Bioorganic & Medicinal Chemistry Letters 25 (2015) 5477–5480

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Lipophilic vancomycin aglycon dimer with high activity against vancomycin-resistant bacteria

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ARTICLE INFO

Article history:
Received 17 September 2015
Accepted 27 October 2015
Available online 27 October 2015

Keywords:
Antibiotic resistance
Vancomycin
Vancomycin-resistant bacteria
Cell wall biosynthesis inhibition
Antibacterial activity

ABSTRACT

Antibiotic-resistant superbugs such as vancomycin-resistant Enterococci (VRE) and Staphylococci have become a major global health hazard. To address this issue, we synthesized vancomycin aglycon dimers to systematically probe the impact of a linker on biological activity. A dimer having a pendant lipophilic moiety in the linker showed ~300-fold more activity than vancomycin against VRE. The high activity of the compound is attributed to its enhanced binding affinity to target peptides which resulted in improved peptidoglycan (cell wall) biosynthesis inhibition. Therefore, our studies suggest that these compounds, prepared by using facile synthetic methodology, can be used to combat vancomycin-resistant bacterial infections.

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Vancomycin, a glycopeptide antibiotic, has long been considered as 'antibiotic of last resort' for the treatment of Gram-positive bacterial infections especially those caused by multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).[1] The extensive use of vancomycin for MRSA infections resulted in emergence of vancomycin resistance in MRSA (vancomycin-intermediate-resistant *S. aureus*, VISA and vancomycin-resistant *S. aureus*, VRSA).[2] In addition to this, the emergence and spread of vancomycin-resistant Enterococci (VRE) is a growing concern worldwide.[3,4] The perennial persistence of vancomycin resistance calls for urgent measures to develop more potent analogs to tackle vancomycin-resistant bacteria.

Vancomycin inhibits bacterial cell wall biosynthesis by binding to the peptidoglycan peptide terminus D-Ala-D-Ala found in cell wall precursors, sequesters the substrate from transpeptidase and inhibits cell wall cross-linking.[1] Conversely, bacteria discerning this vancomycin stress, have altered their cell wall precursors from D-Ala-D-Ala to D-Ala-D-Lac (depsipeptide).[5,6] This alteration is induced by five *van* genes and is ample to lessen the binding efficiency of vancomycin to its target by 1000-fold and as a result, its antibacterial activity reduced by >1000-fold (VanA).[7] Significant approaches have been intended for the development of next-generation glycopeptide antibiotics that address the specter of widespread vancomycin resistance.[8–17] Among numerous semi-synthetic glycopeptides, very few of them such as telavancin, dalbavancin and oritavancin were FDA approved for the treatment of skin infections caused by MRSA.[18] However, both dalbavancin and telavancin are moderately active against more virulent VanA phenotypes of vancomycin-resistant bacteria.[19] In our own efforts, membrane active vancomycin analogs[13,20] and vancomycin–sugar conjugates[12,21] have been developed which showed potent antibacterial activity against more virulent drug-resistant bacteria.

Glycopeptide antibiotics such as vancomycin are known to self-associate into homodimers via hydrogen bonding and hydrophobic interactions in both solution and solid states.[22] This noncovalent dimerization is highly favorable and cooperative with the binding of cell wall precursors which could lead to enhancement in antibacterial activity.[23] This observation prompted the scientific community to study the effects of covalent dimerization on antibacterial properties of glycopeptides.[24–27] Also, it has been shown that the overall binding affinity of a covalent dimer is more than its corresponding monomer towards the bacterial ligands. Here, we report the synthesis of a series of bis-(vancomycin aglycon)carboxamides with variable linkers that differ in lipophilicity and cationic charge. The dimer having a pendant hydrophobic moiety in the linker showed high antibacterial activity against VRE (VanA phenotype). Compared to vancomycin, this dimer caused more accumulation of cell wall (peptidoglycan) precursor indicating the inhibition of cell wall biosynthesis in a greater extent. Unlike vancomycin, the compound was found to be highly bactericidal with improved ex vivo antibacterial activity in whole blood.

In the synthetic strategy employed for preparing the vancomycin aglycon dimers, bis(vancomycin aglycon)carboxamides, polyamines were used as linkers with variable hydrophobicity and positive charge. The linkers bearing a primary amine group

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http://dx.doi.org/10.1016/j.bmcl.2015.10.083
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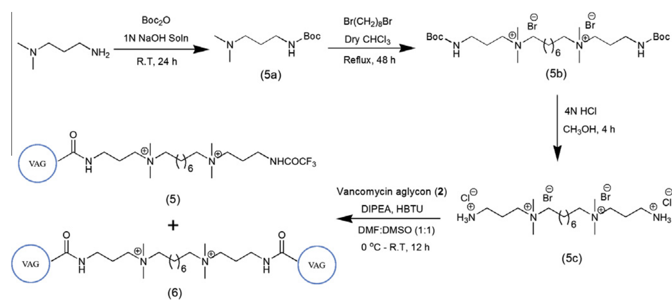
on either side were coupled to the carboxyl group of vancomycin aglycon via amide bond formation by using *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) as a coupling reagent. These vancomycin aglycon dimers were purified by reverse-phase HPLC (High-performance liquid chromatography) using 0.1% trifluoroacetic acid (TFA) in water/acetonitrile solvent system to more than 95% purity in 40–45% yield and characterized by ^{1}H NMR spectroscopy and high resolution mass spectrometry (HR-MS). Also, the corresponding monomeric adducts were obtained in 25% yield as trifluoroacetamides probably due to the possible reactivity of the second free amine group of the monomers with TFA of solvents during purification.

Firstly, we synthesized vancomycin aglycon (**2**) from vancomycin (**1**), which was deglycosylated by using trifluoroacetic acid.[28] Then, the carboxylic group of vancomycin aglycon (**2**) was coupled to the amine group of 1,8-diaminooctane (which does not have permanent positive charge) to give monomer **3** and its dimer **4** (Scheme 1).

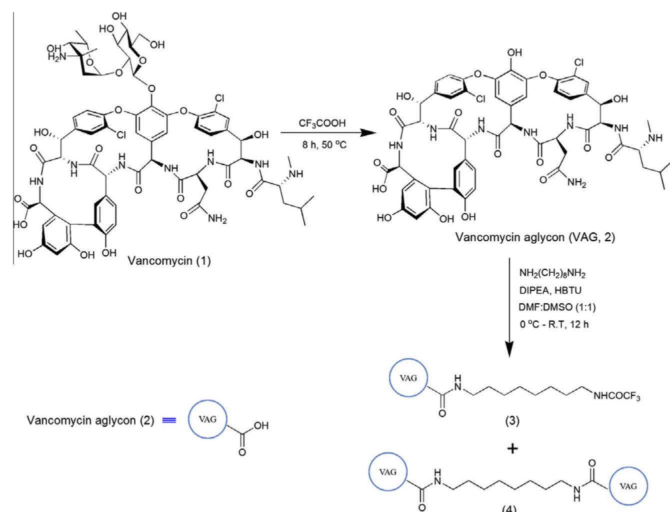
Next, we sought to incorporate permanent positively charged centres (quaternary ammonium centres) and lipophilicity in the linker due to the anticipated favorable electrostatic interactions between the more negatively charged bacterial cell membrane and the cationic centres of molecule, which might lead to favorable biological properties. Utilizing this concept, we synthesized N^1,N^8-bis(3-aminopropyl)-N^1,N^1,N^8,N^8-tetramethyloctane-1,8-diaminium bromide (**5c**) and coupled to vancomycin aglycon (Scheme 2). Here, to synthesize compound **5c**, *N*,*N*-dimethyl-1,3-propanediamine was protected using di-*t*-butylpyrocarbonate to give *t*-butyl (3-(dimethylamino)propyl)carbamate (**5a**). Then, the tertiary amine group of compound **5a** was quaternized with 1,8-dibromooctane to give *N*-Boc protected quaternized compound (**5b**). Now, compound **5b** was subjected to deprotection in presence of acid to give *N*-Boc free compound **5c**. Then, the compound **5c** was coupled to the carboxylic group of vancomycin aglycon (**2**) to yield monomer **5** and dimer **6** (Scheme 2).

Next, we synthesized dimer **8** using *N*′-(3-aminopropyl)-*N*′-octylpropane-1,3-diamine (**7b**) as a linker comprising a pendant lipophilic moiety and tertiary amine (which becomes cationic under physiological conditions). It has been shown in the literature that inclusion of a pendant hydrophobicity to the periphery of glycopeptides leads to enhanced dimerization and stronger association with bacterial membrane. This further leads to enhanced inhibition of cell wall biosynthesis compared to parent glycopeptide thereby accounting for high antibacterial activity.[29] In order to have this beneficial effect along with increased binding affinity, dimer **8** has been synthesized. Initially, octyl amine was subjected to Michael addition with acrylnitrile to give an adduct (**7a**). Then, the nitrile group of the adduct was reduced by lithium aluminium hydride (LAH) to primary amine and then finally coupled to vancomycin aglycon to yield monomer **7** and dimer **8** (Scheme 3).

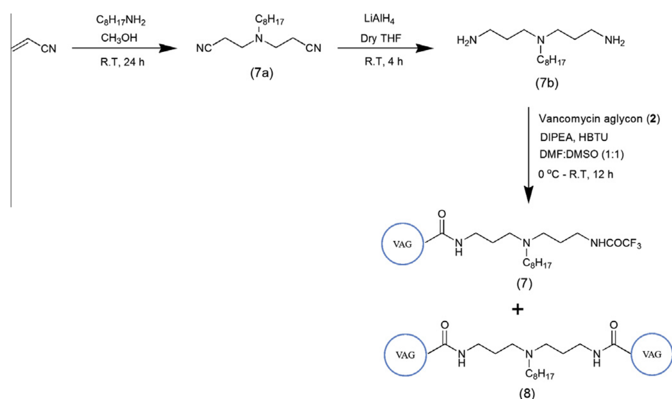
The antibacterial activities (minimum inhibitory concentration, MIC) of all the compounds were determined against vancomycin-sensitive strains of Staphylococci (MSSA and MRSA) and Enterococci (VSE), as well as against vancomycin-resistant strains of Staphylococci (VISA) and Enterococci (VRE) and the results are summarized in Table 1. The activity of vancomycin aglycon was found to be similar to vancomycin against sensitive bacteria with the MIC of ~1 μM. Against VISA, vancomycin aglycon showed ~3-fold more activity than vancomycin whereas in case of VRE it was found to be ineffective. Then, we evaluated the activities of vancomycin aglycon derivatives having various linkers (monomers **3**, **5** and **7**) and their MICs were found to be ~1 μM against vancomycin-sensitive bacteria. In case of VISA, compounds **3**, **5** and **7** showed potent activity with MICs ranging from 0.5 to 0.8 μM. When checked against VRE, monomers **3** and **5** showed moderate activity with the MICs of 30 and 48 μM, respectively, whereas monomer **7** exhibited good activity with the MIC of 6.5 μM which is ~115-fold more active than vancomycin. Next, we have evaluated the antibacterial activity of vancomycin aglycon dimers (**4**, **6** and **8**) and the results are compared with their corresponding monomers (**3**, **5** and **7**). The dimer **4** comprising 1,8-diaminooctylene in the linker showed ~65-fold increase in activity against VISA (MIC = 0.2 μM) and ~30-fold increase in case of VRE (MIC = 25 μM) compared to vancomycin. Further, dimer **4** exhibited ~3-fold more activity than its corresponding monomer **3** against both VISA and



Scheme 2. Synthesis of monomer (**5**) and dimer (**6**).



Scheme 1. Synthesis of vancomycin aglycon (**2**), monomer (**3**) and dimer (**4**). Reagents: CF_3COOH, trifluoroacetic acid; DIPEA, *N*,*N*-diisopropylethylamine; HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate.



Scheme 3. Synthesis of monomer (**7**) and dimer (**8**).

Table 1
In vitro antibacterial activity of the compounds

Compound	Minimum inhibitory concentration (μM)				
	MSSA[a]	MRSA[b]	VISA[c]	VSE[d]	VREm[e]
Vancomycin	0.6	0.7	13.0	0.6	750
2 (VAG)	1.0	1.0	4.0	1.8	>100
3 (monomer)	1.0	2.0	0.6	0.6	30.5
4 (dimer)	0.8	0.6	0.2	0.6	25.8
5 (monomer)	1.0	1.0	0.5	0.2	>100
6 (dimer)	0.2	0.3	0.1	0.05	48.0
7 (monomer)	0.8	1.0	0.8	0.4	6.5
8 (dimer)	0.6	0.6	0.6	0.5	2.5

[a] MSSA (methicillin-sensitive *S. aureus*).
[b] MRSA (methicillin-resistant *S. aureus*).
[c] VSE (vancomycin-sensitive *E. faecium*).
[d] VISA (vancomycin-intermediate-resistant *S. aureus*).
[e] VREm (vancomycin-resistant *E. faecium*, VanA).

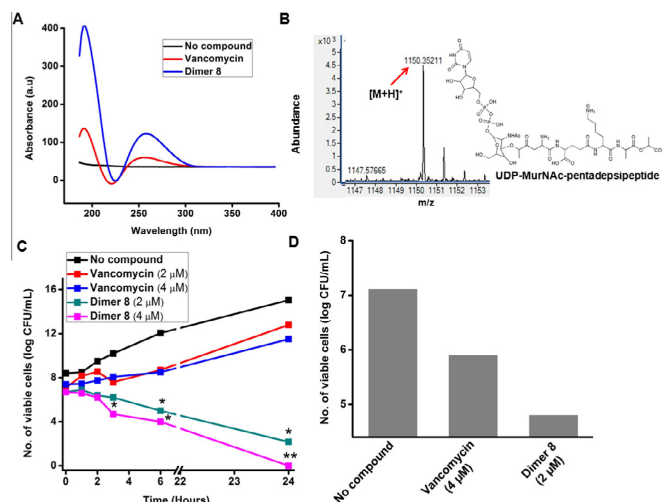


Figure 1. Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentadepsipeptide after treatment of VRE with vancomycin and dimer **8** at 5 μM (A and B). (A) Identification of intracellular UDP-MurNAc-pentadepsipeptide by monitoring absorbance at 260 nm wavelength (B) UDP-MurNAc-pentadepsipeptide was identified by mass spectrometry as indicated by the peak at *m/z* 1150.35; (C) Bactericidal properties of dimer **8** and vancomycin against MRSA in media. Single stars correspond to reduction of $3\log_{10}$CFU/mL and double stars correspond to <50 CFU/mL. (D) Antibacterial activity of vancomycin and dimer **8** after 3 h incubation in 90% human whole blood against MRSA.

MRSA whereas it showed slightly better activity against VRE. Dimer **6** (having two permanent positively charged centres connected together by an octylene linker) was found to be ~2-fold, ~12-fold and ~130-fold more active than vancomycin against MRSA, VSE and VISA, respectively, with the MICs ranging from 0.05 to 0.3 μM. Against VRE, dimer **6** exhibited an MIC of 48 μM which is ~15-fold more active than vancomycin. Further, dimer **6** was ~4-fold more active than its corresponding monomer **5** against MRSA, VISA and VSE whereas it showed slightly lower activity against VRE. Dimer **8** comprising a pendant octyl chain in the linker exhibited an activity of 2.5 μM against VRE which is ~300-fold higher than vancomycin. Further, dimer **8** was ~3-fold more active than its corresponding monomer **7** against VRE whereas against rest of the bacteria dimer **8** showed an MIC of ~0.6 μM which is similar to monomer **7**. Among compounds **3**–**8**, dimer **8** showed the best activity against VRE and dimer **6** demonstrated the best activity against VISA and VSE.

To substantiate these findings, the binding constants of the best active dimer, compound **8** and vancomycin were evaluated using UV-difference spectroscopy against both sensitive and resistant model ligands: *N,N*′-diacetyl-Lys-D-Ala-D-Ala and *N,N*′-diacetyl-Lys-D-Ala-D-Lac, respectively (Supplementary Figs. S1 and S2). The binding affinity of the dimer **8** was found to be similar to vancomycin against *N,N*′-diacetyl-Lys-D-Ala-D-Ala ($\sim 1 \times 10^5\ M^{-1}$). When evaluated against *N,N*′-diacetyl-Lys-D-Ala-D-Lac, this dimer ($K_a = 5.7 \times 10^3\ M^{-1}$) exhibited the binding affinity of 10-fold more than vancomycin ($K_a = 5 \times 10^2\ M^{-1}$). This observation indicates that compound **8** binds more effectively with bacterial ligands compared to vancomycin and leads to improved inhibition of cell wall biosynthesis.

In order to investigate the effect of enhanced binding affinity on peptidoglycan biosynthesis, we determined the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentadepsipeptide (UDP-MurNAc-pp) after treating bacteria (VRE) with the compound **8** and vancomycin at 5 μM. In case of compound **8**, a more intense peak was observed at 260 nm compared to vancomycin, which corresponds to more accumulation of UDP-MurNAc-pp and confirmed by high resolution mass spectrometry (*m/z* = 1150.94 (calcd), 1150.35 (obsd) for $[M+H]^+$) (Fig. 1A and B). This suggests that dimer **8** causes more accumulation of cell wall (peptidoglycan) precursor than vancomycin indicating a higher inhibition of peptidoglycan biosynthesis. This might be due to increased association of the dimer **8** with bacterial ligands and bacterial membranes because of the presence of pendant lipophilicity which presumably serves to anchor the drug thereby allowing it to stay for a longer time at cell wall region and inhibiting the cell wall biosynthesis in a greater extent, which leads to improvement in antibacterial activity against VRE. Similar observation was reported for semi-synthetic lipoglycopeptides such as oritavancin and telavancin wherein they show significantly better inhibition of cell wall biosynthesis compared to parent drug due to the installed additional lipophilicity in the molecule.[18,29]

In order to study the bactericidal activity of the optimum compound, we carried out in vitro time-kill assay with the dimer **8** and vancomycin against MRSA (starting bacterial concentration of $\sim 8\log_{10}$CFU/mL), at two different concentrations (2 and 4 μM). Our results demonstrated a rapid bactericidal activity with dimer **8**, which increased with increasing concentration. Further, dimer **8** caused $\sim 3\log_{10}$CFU/mL reduction in bacterial concentration within 3 h of incubation at 4 μM (Fig. 1C). Also, it displayed complete bactericidal activity in 24 h at 4 μM. On the other hand, vancomycin showed bacteriostatic effect in the initial 6 h and did not show any effect on bacterial growth irrespective of the concentration beyond that time (Fig. 1C). The potent bactericidal activity of compound **8** can be attributed to its improved cell wall biosynthesis inhibition.

Next, an ex vivo assay was developed to provide a relevant challenge to the antimicrobial agent in complex biomatrices such as whole blood. The assay design includes simultaneous introduction of antimicrobial agent and bacterial cells into biomatrices. Here, we performed ex vivo whole blood assay with dimer **8** and vancomycin against MRSA. In whole blood, an inoculum of $\sim 5.0\log_{10}$CFU/mL yielded an end point in MRSA viability of $\sim 7.0\log_{10}$CFU/mL (Fig. 1D) after a 3 h incubation at 37 °C in growth control (no compound). In whole blood containing the dimer at a concentration of 2 μM, $4.5\log_{10}$CFU/mL viable bacterial cells were detectable after a 3 h incubation, which indicates a reduction of $\sim 2.5\log_{10}$CFU/mL, in comparison to vancomycin which showed only $1.0\log_{10}$CFU/mL reduction at 4 μM. These results indicate that the dimer **8** can potentially maintain antibacterial activity in vivo with nominal loss due to non-specific interactions with tissue components.

To summarize, bis(vancomycin aglycon)carboxamides with variable linkers have been developed that differ in permanent positive charge and lipophilicity. The dimer comprising a pendant lipophilic moiety in the linker exhibited 300-fold more activity

than vancomycin against VRE. The high activity of the dimer is credited to its greater cell wall biosynthesis inhibition compared to parent drug. Also, this dimer was found to be bactericidal at low concentrations and retained its activity in the complex media such as whole blood. The results furnished in this report emphasize the potential of this class of compounds as future antibiotics.

Acknowledgments

We thank Prof. C. N. R. Rao (JNCASR) for his constant support and encouragement. J. H. acknowledges Department of Science and Technology (DST), Govt. of India for Ramanujan Fellowship [SR/S2/RJN-43/2009]. V. Y. thanks CSIR for research fellowship.

Supplementary data

Supplementary data (synthetic protocols, assay details, supplementary figures and compound characterization data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.083.

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