Pergamon

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1683–1686

BIOORGANIC &
MEDICINAL
CHEMISTRY
LETTERS

Identification of Potent and Broad-Spectrum Antibiotics from SAR Studies of a Synthetic Vancomycin Analogue

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Received 7 January 2003; revised 7 March 2003; accepted 7 March 2003

Abstract—Dimeric vancomycin analogues based on a lead compound identified from a library of synthetic analogues of vancomycin have up to 60-fold greater activity than vancomycin against vancomycin-resistant *Enterococcus faecium* (VRE, VanA phenotype). Simplified analogues have also been prepared and found to maintain activity against VRE and have broad-spectrum antibiotic activity.

The glycopeptide antibiotic vancomycin is used clinically as a last line of defense for the treatment of serious infections caused by Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* strains and multiply resistant *Streptococcus pneumoniae* strains. Consequently, the emergence of bacterial resistance to vancomycin poses a serious threat towards the effective treatment of bacterial infections.[1] Vancomycin is believed to exert its main antibacterial effect through inhibition of cell wall cross-linking by binding to the D-Ala-D-Ala terminus of the mucopeptide precursors through a network of five hydrogen bonds.[2] The most common form of antibiotic resistance results from the biosynthesis of an altered bacterial cell wall precursor ending in D-Ala-D-lactate. Replacement of the D-alanine residue with D-lactate eliminates a key amide N–H hydrogen bond interaction with vancomycin and introduces an unfavorable electrostatic interaction (Fig. 1) resulting in a > 1000-fold drop in binding affinity.[3]

We have previously described a strategy for the design and synthesis of vancomycin analogues targeting resistant bacteria.[4] Herein, we report the preparation of a library of approximately 27,000 synthetic analogues of vancomycin. Direct screening of the library in solution for antibacterial activity has resulted in the identification of synthetic analogues with up to 4-fold improved MIC values relative to vancomycin against vancomycin-resistant *Enterococcus faecium* (VRE, VanA phenotype). We further report the development of dimers of the most potent synthetic analogue that are up to 60 times more potent than vancomycin towards VRE, as well as greatly simplified analogues that maintain activity against VRE and have broad-spectrum antibiotic activity.

Similar to our previous efforts,[4] we designed the synthetic receptors **1** (Scheme 1) such that they incorporated the right-hand carboxylate binding pocket of

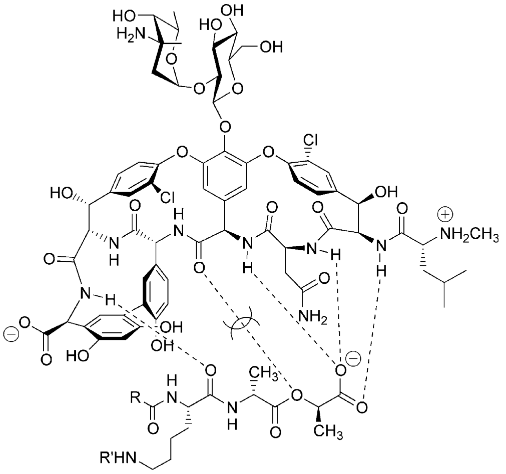

Figure 1. The molecular basis for vancomycin resistance.

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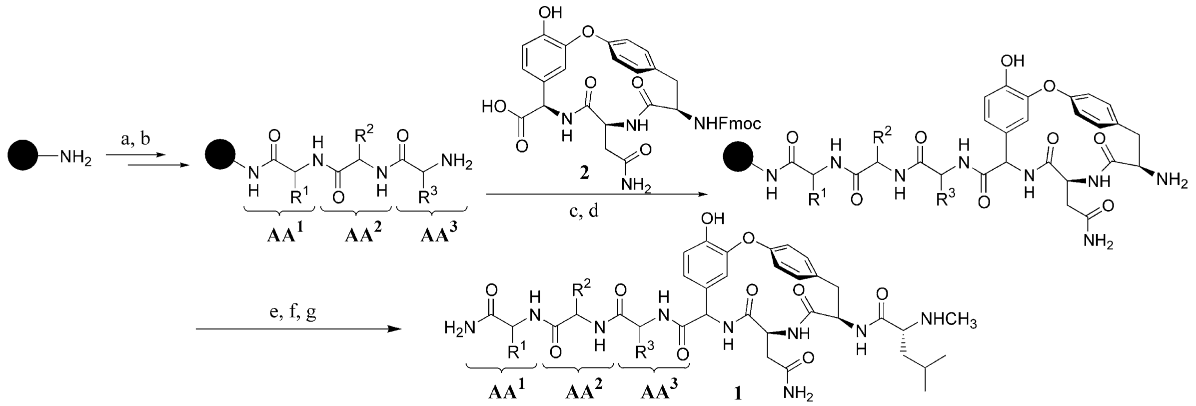
doi:10.1016/S0960-894X(03)00243-9



Scheme 1. Reagents and conditions: (a) Fmoc amino acid (5 equiv), PyBOP (5 equiv), HOAt (5 equiv), $i\mathrm{Pr_2NEt}$ (10 equiv), NMP, rt, 6 h; (b) 20% piperidine/DMF (v/v), rt, 2 h; (c) **2** (3 equiv), PyBOP (6 equiv), HOAt (6 equiv), $i\mathrm{Pr_2NEt}$ (6 equiv), NMP, rt, 6 h; (d) 20% piperidine/DMF (v/v), rt, 2 h; (e) Fmoc-*N*-Me-D-Leu (5 equiv), PyBOP (5 equiv), HOAt (5 equiv), $i\mathrm{Pr_2NEt}$ (10 equiv), NMP, rt, 6 h; (f) 20% piperidine/DMF (v/v), rt, 2 h; (g) TFA/$\mathrm{H_2O}$/i-$\mathrm{Pr_3SiH}$ (95:2.5:2.5), rt, 30 min.

vancomycin, since it retains productive binding interactions with D-Ala-D-lactate. In contrast, the left-hand side of vancomycin, where unproductive interactions with D-Ala-D-lactate occur, was replaced with a tripeptide segment for introducing diversity. To facilitate library synthesis, the vancomycin E-ring chlorine[5] and the sugar residues[6] were not included in the receptor design since these groups make relatively modest contributions to the binding interactions of vancomycin.

To prepare the library of vancomycin analogues the three amino acid inputs, $\mathrm{AA^1}$ to $\mathrm{AA^3}$, were loaded onto Rink substituted ArgoGel-$\mathrm{NH_2}$ resin using standard solid-phase peptide synthesis procedures (Scheme 1). Sequential coupling of the *N*-Fmoc-protected peptide macrocycle **2**[7] and *N*-Fmoc-*N*-Me-D-Leu were followed by side-chain deprotection and cleavage from support.

For the library synthesis, 30 amino acids were introduced at each of the three amino acid diversity inputs ($\mathrm{AA^1}$–$\mathrm{AA^3}$). The amino acid inputs included the D- and L-enantiomers of many of the proteinogenic amino acids as well as a number of constrained amino acids.[8] The library was prepared with 96-well Robbins® blocks using a combination of split synthesis and parallel synthesis methods to provide 90 pools. Each pool contained roughly 300 compounds composed of a mixture of the 30 amino acids at $\mathrm{AA^1}$, a defined amino acid at $\mathrm{AA^2}$, and a pool of 10 amino acids at $\mathrm{AA^3}$.

The library was first screened against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VanA phenotype).[9] While no activity was observed against MRSA, 13 out of 90 pools showed $\geq$50% inhibition against vancomycin-resistant *E. faecium* at 600 μg/mL. Only the aromatic amino acids, L-Phe, L-Tpi, D- and L-Trp, and D- and L-*N*-Me-Phe were present at the $\mathrm{AA^2}$ position of the active pools. Based upon these initial screening results, 130 pools were next screened with each pool composed of a mixture of the 30 amino acids at $\mathrm{AA^1}$, one of the selected amino acids listed above at $\mathrm{AA^2}$, and a defined amino acid at $\mathrm{AA^3}$. Ten out of the 130 pools showed MIC values $\leq$600 μg/mL (Table 1).

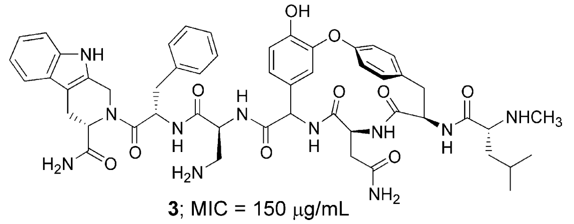
As a final deconvolution step the 180 compounds represented by entries 1–6 in Table 1 were prepared and screened as discrete compounds with 33 compounds showing $\leq$600 μg/mL MIC values.[9] Library screening clearly selected for aromatic amino acids as well as constrained amino acid derivatives as verified by resynthesis, purification and reassay of several of the most potent library members (data not shown). The most potent compound **3**, which incorporated L-Tpi, L-Phe and L-Dapa at $\mathrm{AA^1}$, $\mathrm{AA^2}$ and $\mathrm{AA^3}$, respectively, had an $\mathrm{IC_{50}}$ value of 150 μg/mL and was 4-fold more potent than vancomycin.

In an effort to improve potency and to help elucidate the mechanism of action of the synthetic vancomycin analogues, we next prepared a series of covalently linked dimers of **3**. Several research groups have described vancomycin dimers with up to 100-fold greater activities than vancomycin against VRE.[10] The enhanced potency of vancomycin dimers against resistant bacteria has been primarily attributed to avidity for D-Ala-D-lactate.[11]

Table 1. Most active pools against vancomycin-resistant *E. faecium* (VanA) after the second deconvolution step[a,b]

Pool	$\mathrm{AA^2}$	$\mathrm{AA^3}$	MIC (μg/mL)
Vancomycin	—	—	600
1	L-Phe	L-Tpi	300
2	*N*-Me-D-Phe	*N*-Me-L-Phe	300
3	D-Trp	*N*-Me-L-Phe	300
4	L-Phe	L-Dapa	600
5	*N*-Me-D-Phe	L-Pro	600
6	L-Tpi	D-Lys	600
7	*N*-Me-L-Phe	Disc	600
8	*N*-Me-D-Phe	D-Pro	600
9	L-Trp	L-Dapa	600
10	L-Tpi	D-His	600

[a] Mixture of 30 amino acids are present at $\mathrm{AA^1}$.
[b] See ref 8 for abbreviations of non-proteinogenic amino acids.



3; MIC = 150 μg/mL

The synthesis of the vancomycin analogue dimers **4–6** is outlined in Scheme 2. Symmetrical diazido-alcohols were loaded onto DHP resin[12] followed by reduction of the azide moieties. Sequential coupling of the *N*-Fmoc protected amino acids and macrocycle **2**[7] were followed by side-chain deprotection and cleavage from support.

Purified dimers **4–6** were tested for their activity against vancomycin-resistant *E. faecium* (VanA phenotype).[9] A significant increase in activity was found for all of the dimeric analogues over both the monomeric analogue **3** and vancomycin (Table 2). Furthermore, the activity of the dimers increased with decreasing tether length. The analogue with the shortest tether, compound **4**, was approximately 60-fold more active than vancomycin.

In order to gain insight into the mechanism of antibacterial activity of dimers **4–6**, the truncated compounds **7** and **8** (Table 3) were prepared by a similar reaction sequence as outlined in Scheme 2. Presumably, if the vancomycin analogues **4–6** exhibit antibacterial activity via binding to D-Ala-D-lactate, **7** and **8** would have little or no activity against VRE (VanA phenotype). To our surprise, the truncated dimers **7** and **8** were nearly as potent as compounds **4–6** against VRE (Table 3). Significantly, the corresponding monomeric peptides **9** and **10** showed a considerable reduction in activity.

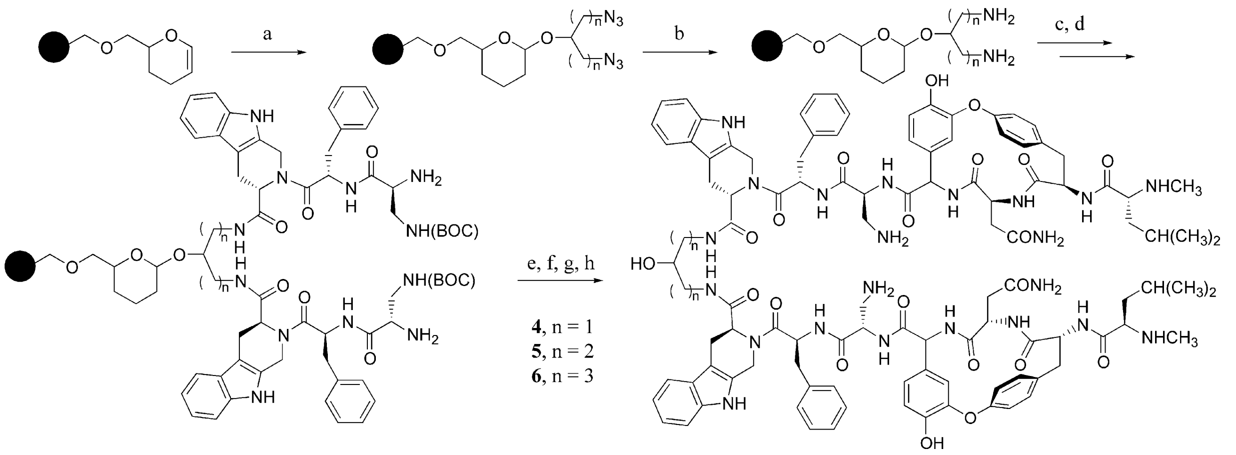
Table 2. Susceptibility of *E. faecium* (VanA) towards compounds **4–6**

Compd	MIC (μg/mL)
Vancomycin	600
3	150
4	9
5	18
6	38

Compounds **7** and **8** were comparably active against vancomycin-susceptible *E. faecium* as they were against the resistant strain. Furthermore, no potentiation of antibacterial activity against VRE was observed in the presence of 20 μg/mL of vancomycin. These results further suggest that **7** and **8** do not inhibit bacterial growth through binding D-Ala-D-lactate.

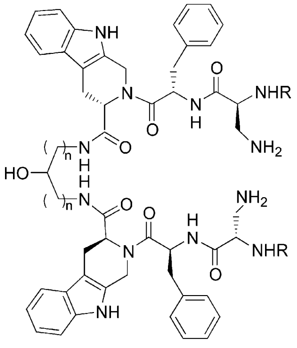
Since **7** and **8** appear to inhibit growth of VRE by a mechanism other than binding D-Ala-D-lactate, the susceptibility of a broad range of bacterial strains towards **7** was determined (Table 4).[9] Compound **7** exhibits broad-spectrum antibiotic activity, though in most cases it is more potent towards Gram-positive (*Staphylococcus*, *Enterococcus*, *Streptococcus*) than Gram-negative organisms (*Escherichia*, *Klebsiella*, *Pseudomonas*, *Haemophilus*, *Moraxella*). This may be attributed to the outer membrane of the Gram-negative bacteria serving as an effective permeability barrier. *Escherichia coli* and *Haemophilus influenzae* efflux pump mutants were more susceptible to **7** than the non-mutated strains, although the increase in susceptibility is not significant. Notably, **7** shows good efficacy against strains of methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and penicillin-intermediate *Streptococcus pneumoniae*. In preliminary investigations compound **7** does not appear to be cytotoxic towards K562 human leukemia and P388 murine leukemia cells lines at 100 μg/mL.

In conclusion, dimeric vancomycin analogues based on a lead compound from a library of ~27,000 synthetic analogues of vancomycin were synthesized and found to be up to 60-fold more active than vancomycin against vancomycin-resistant *E. faecium* (VRE, VanA phenotype). In addition, greatly simplified analogues of these

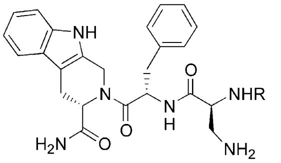


Scheme 2. Reagents and conditions: (a) diazido alcohol (2 equiv), *i*PrOH (2 equiv), PPTS (2 equiv), 1,2-dichloroethane, 70 °C, 12 h; (b) $SnCl_2$ (4 equiv), PhSH (16 equiv), NEt_3 (20 equiv), THF, rt, 4 h; (c) Fmoc amino acid (5 equiv), PyBOP (5 equiv), HOAt (5 equiv), *i*Pr_2NEt (10 equiv), CH_2Cl_2/DMF (9:1), rt, 6 h; then repeat; (d) 20% piperidine/DMF (v/v), rt, 2 h; (e) **2** (1.5 equiv), PyBOP (3 equiv), HOAt (3 equiv), *i*Pr_2NEt (6 equiv), CH_2Cl_2/DMF (9:1), rt, 6 h; then repeat; (f) 20% piperidine/DMF (v/v), rt, 2 h; (g) BOC-*N*-Me-D-Leu (5 equiv), PyBOP (5 equiv), HOAt (5 equiv), *i*Pr_2NEt (10 equiv), CH_2Cl_2/DMF (9:1), rt, 6 h; then repeat; (h) TFA/DMS/H_2O (90:5:5), rt, 30 min.

Table 3. Susceptibility of *E. faecium* (VanA) towards compounds **7**–**10**



7, R = *N*-Me-D-Leu
8, R = Ac



9, R = *N*-Me-D-Leu
10, R = Ac

Compd	MIC (μg/mL)
7	18
8	18
9	>1200
10	1200

Table 4. Susceptibility of bacterial strains towards compound **7**

Organism	MIC (μg/mL)
Staphylococcus aureus	16[a]
S. aureus (MRSA)	32
Staphyloccus epidermidis	24 (±11)[a]
S. epidermidis (MRSE)	8
Enterococcus faecium	4
Enterococcus faecalis	8
Streptococcus pneumoniae	16
S. pneumoniae (Penicillin-intermediate)	8
Streptococcus pyogenes	2
Escherichia coli	32[a]
E. coli (efflux mutant)	16
Klebsiella pneumoniae	>128
Pseudomonas aeruginosa	128
Haemophilus influenzae	96 (±45)[a]
H. influenzae (efflux mutant)	16
Moraxella catarrhalis	8

[a] Values are means of 2–3 experiments; standard deviation is given in parentheses.

dimers were prepared that maintain potency against VRE and have broad-spectrum antibiotic activity. The data suggest these compounds inhibit VRE by a mechanism other than binding to D-Ala-D-lactate. These results also raise the possibility that the principal antibacterial activity of vancomycin dimers against resistant bacteria may be independent of binding to D-Ala-D-lactate.[13]

Acknowledgements

This Research was supported by NIH grant GMRO153696. K.A.A. thanks Pharmacia for a graduate fellowship. J.A.O. thanks the LEO Foundation and the Knud Jøjgaard Foundation for financial support. M.W. thanks the Japan Society for the Promotion of Science for a postdoctoral fellowship (JSPS 01798).

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7. The synthesis of the macrocycle **2** is described in the Supporting Information of ref 4.
8. The following amino acid inputs were used: D- and L-isomers of Ala, Asn, Glu, His, Lys, *N*-Me-Phe, Phe, Pro, Thr, tetrahydroisoquinoline-3-carboxylic acid (Tic), Trp, Tyr, Val; the L-isomer of diaminopropionic acid (Dapa) and 1,2,3,4-tetrahydronorharman-3-carboxylic acid (Tpi); and racemic 1,3-dihydroisoindole carboxylic acid (Disc) and 1-aminocyclohexane-carboxylic acid (Achc).
9. Compounds were tested according to NCCLS protocols. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard*, 5th ed.; NCCLS Document M7-A5; National Committee for Clinical Laboratory Standards: Wayne, PA, 2000.
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