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Solid-phase synthesis and antibacterial evaluations of *N*-demethylvancomycin derivatives

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Abstract—Twenty-five *N*-demethylvancomycin derivatives were synthesized on solid-support and their structures were determined by LC–MS/MS. Biological evaluation of these compounds indicated that bulky hydrophobic substituent on vancosamine of *N*-demethylvancomycin can increase antibacterial activity against vancomycin-resistant *Enterococcus faecalis*.

Vancomycin, a potent glycopeptide antibiotic, is the last resort against Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus*.[1] Vancomycin exerts its antibacterial action by binding to cell wall peptide precursor terminating in -Lys-D-Ala-D-Ala, thereby inhibiting cell wall growth and peptidoglycan crosslinking which results in bacterial lysis.[2] NMR studies of vancomycin complexed with -D-Ala-D-Ala showed that (i) there are a total of five hydrogen bonds between the amides in the antibiotics and the amides and carboxylate anion of -D-Ala-D-Ala,[3] (ii) hydrophobic interactions affect the hydrogen bonds strengths,[4] and (iii) there is electrostatic interaction between the carboxylate anion of -D-Ala-D-Ala and the amino terminus of antibiotic.[5] Unfortunately, vancomycin-resistant *Enterococci* has emerged and poses a serious threat to public health. In vancomycin-resistant *Enterococci*, the D-Ala-D-Ala peptide terminus is replaced by D-Ala-D-Lac. This replacement of an amide linkage with an ester linkage leads to the loss of one hydrogen-bond interaction between vancomycin and -D-Ala-D-Lac, resulting in a 1000-fold decrease in affinity and biological activity.[6] Recent studies, however, indicate that carbohydrate derivatives of vancomycin inhibit the transglycosylation step of peptidoglycan biosynthesis without binding to -D-Ala-D-Ala[7] through hydrophobic substitutions on the vancosamine nitrogen atom. Some of these compounds have excellent activity against vancomycin-resistant *Enterococci*.[8]

N-Demethylvancomycin, an analogue of vancomycin that has been used clinically in China since 1967, differs from vancomycin at the *N*-terminus, where the *N*-methyl-leucine is replaced by leucine.[9] This free primary amine at the *N*-terminus allows one to add another point of diversity for building vancomycin libraries. However, there have been only a few reports on vancomycin derivatization at this site.[10] Both solution-phase and solid-phase methods have been used in the past to modify vancomycin.[11] We herein report a new solid-phase synthesis strategy for *N*-demethylvancomycin derivatization, which involves chemoselective protection of amino groups by Fmoc at the *N*-terminus. Twenty-five *N*-demethylvancomycin derivatives (Table 1) were successfully synthesized on solid-support and subsequently cleaved, released and analyzed by LC–MS/MS. Their antibacterial activities were evaluated to determine the contribution of hydrophobic substituents to antibacterial activity.

Both vancomycin and *N*-demethylvancomycin consist of a tricyclic central core of heptapeptide with five

Keywords: Solid-phase synthesis; Demethylvancomycin derivatives.
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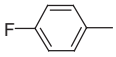
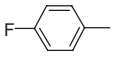
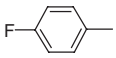
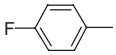
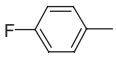
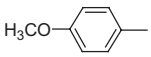
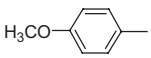
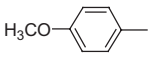
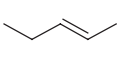
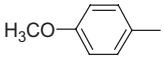
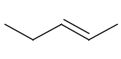
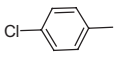
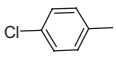
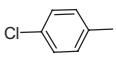
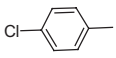
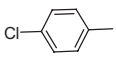
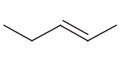
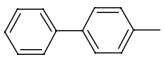
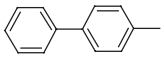
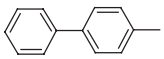
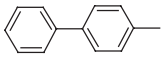
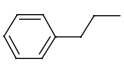
Table 1. Compounds and their antibacterial activity (MIC μg/mL) against vancomycin-susceptible and vancomycin-resistant bacteria

Compound	R^1	R^2	Purity (%)	1	2	3	4
1		**(A)** 	97.5	>10	>10	>10	>10
2	H	**(A)** 	96.3	>10	>10	10	10
3		**(A)** 	99.1	>10	>10	>10	>10
4		**(A)** 	98.2	>10	>10	>10	>10
5		H	98.0	2.5	>10	2.5	2.5
6		**(B)** 	79.7	>10	>10	>10	>10
7		**(B)** 	93.6	>10	>10	>10	10
8		**(A)** 	98.9	>10	>10	>10	>10
9		**(B)** 	96.7	>10	>10	10	>10
10		**(A)** 	75.4	>10	>10	10	10
11		**(A)** 	97.7	>10	>10	>10	>10
12		**(A)** 	95.9	>10	>10	10	10
13		**(B)** 	96.9	>10	>10	>10	>10
14		H	83.1	10	>10	5	5
15		**(B)** 	70.3	>10	>10	>10	>10
16		**(A)** 	98.3	5	10	5	2.5
17		**(B)** 	94.3	>10	10	5	5
18		H	99.6	5	>10	5	5
19		**(A)** 	96.2	10	>10	5	5
20		**(B)** 	89.8	>10	>10	>10	>10
21		**(A)** 	97.5	>10	>10	>10	>10
22	H	Fmoc	89.3	5	>10	1.25	5
23	H	CH_3, Fmoc	93.9	10	>10	10	10
24	Fmoc	CH_3	94.2	2.5	5	2.5	2.5
25	Fmoc	CH_3, Fmoc	99.1	10	5	>10	5
Demethylvancomycin	H	H	95.1	2.5	>10	<0.6	<0.6
Vancomycin	H	CH_3	89.2	2.5	>10	0.62	1.25

(**A**) monoalkylated product; (**B**) dialkylated product. 1: *E. faecalis* (ATCC 29212); **2**: vancomycin-resistant *E. faecalis* (ATCC 51299); **3**: *S. aureus* (ATCC 29213); **4**: Methicillin resistant *S. aureus*.

α-aromatic amino acids and two aliphatic amino acids. One of the aromatic amino acids carries α-L-vancosaminyl-β-D-glucose, a disaccharide. In addition, the phenol groups that emerged from the side chain of three residues are crosslinked to form a highly constrained molecule.[9] Confronted with such complex structure, appropriate choice of protecting group[12] and linkage to solid support[13] would be critical for regioselective

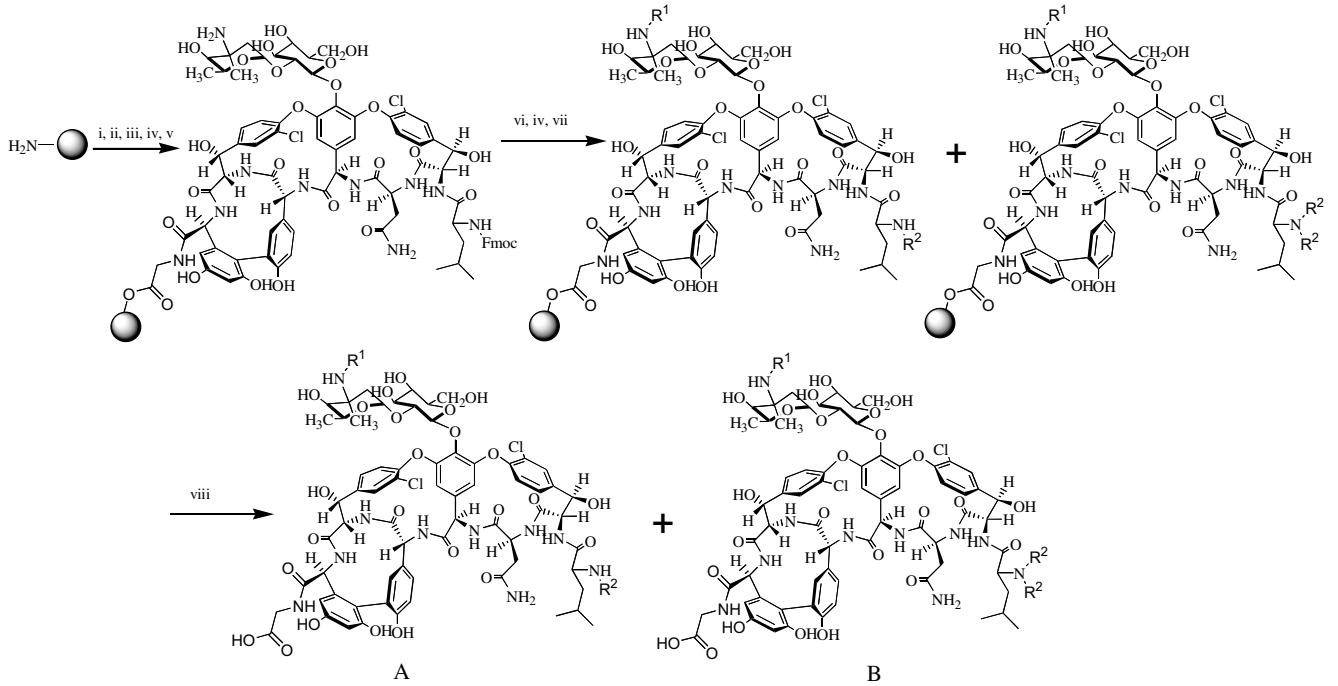
modification of the two amino groups on *N*-demethylvancomycin.

Our strategy is to first derivatize the *N*-terminal free amino group of the molecule with *N*-fluorenylmethoxycarbonyl (Fmoc), and then couple Fmoc-*N*-demethylvancomycin onto solid support through its C-terminus, followed by selective modification of the remaining amino group of the vancosamine.

To regioselectively derivate the *N*-terminus of vancomycin with 9-fluorenylmethyl-*N*-succinimidyl carbonate (Fmoc–OSu), the pH is critical. The vancosamine amino group, being more basic than the amino group at the *N*-terminus, could be fully protected by cation formation at pH 6.4–6.8. Under this condition, Fmoc protection occurred predominantly at the *N*-terminus, and reverse phase HPLC analysis showed 74% regioselective conversion **23** (Table 1). However, when pH was over 7, a mixture of **23**, **24**, and **25** (Table 1) was obtained. *N*-Demethylvancomycin was pH independent with completely regioselective conversion to produce **22** (Table 1) because of little steric hindrance at *N*-terminus. MS of compound **22** (*m*/*z* 1656.5) and **23** (*m*/*z* 1670.4) revealed the presence of Fmoc group. Additionally, MS/MS analysis, showing elimination of vancosamine fragments (143.3 Da) and disaccharide fragments (305.2 Da), indicated that *N*-terminus of vancomycin and *N*-demethylvancomycin were protected. However, compound **24** and **25** lost vancosamine and disaccharide containing Fmoc group fragments (365.4 Da and 527.9 Da, respectively), which demonstrated the amino groups of vancosamine were protected. Furthermore, NMR data of compound **22**, **23**, **24**, and **25** also showed the presence of Fmoc group.

Tenta Gel S NH_2 resin with medium loading capacity (0.27 mmol/g) and a long hydrophilic PEG spacer was chosen as solid support. A base-labile HMBA linker was incorporated into the Tenta Gel beads prior to the synthesis of *N*-demethylvancomycin derivatives because deglucosylation of vancomycin occurs under strong acid cleavage condition, such as TFA.[14] To introduce the third potential diversity, an amino acid was assembled onto resin before the Fmoc-*N*-demethylvancomycin was attached onto solid support as outline in Scheme 1. The esterification of Fmoc-glycine onto resin was performed by a standard Fmoc method.[15] Subsequent reaction with 15% Ac_2O end-capped any unreacted hydroxyl groups. Following Fmoc deprotection, Tenta Gel S NH_2 resin was allowed to react with *N*-Fmoc-*N*-demethylvancomycin in the presence of HBTU, HOBt, and DIEPA in DMF for 24 h.

The subsequent reductive alkylation of amino group of vancosamine with sodium cyanoborohydride ($NaBH_3CN$) has proved to be problematic because of steric hindrance.[16] However, the majority of reactions could be driven to completion by using a high molar excess of aldehyde (25 equiv) and $NaBH_3CN$ (40 equiv) in 1% AcOH/DMF (v:v), an elevated temperature of 40 °C, and longer reaction time (18 h). Following Fmoc deprotection, unhindered amino group of *N*-terminus was smoothly reductive alkylated with excess aldehyde (5 equiv) and $NaBH_3CN$ (15 equiv) to give monoalkylated products (**A**) for 8 h at room temperature.

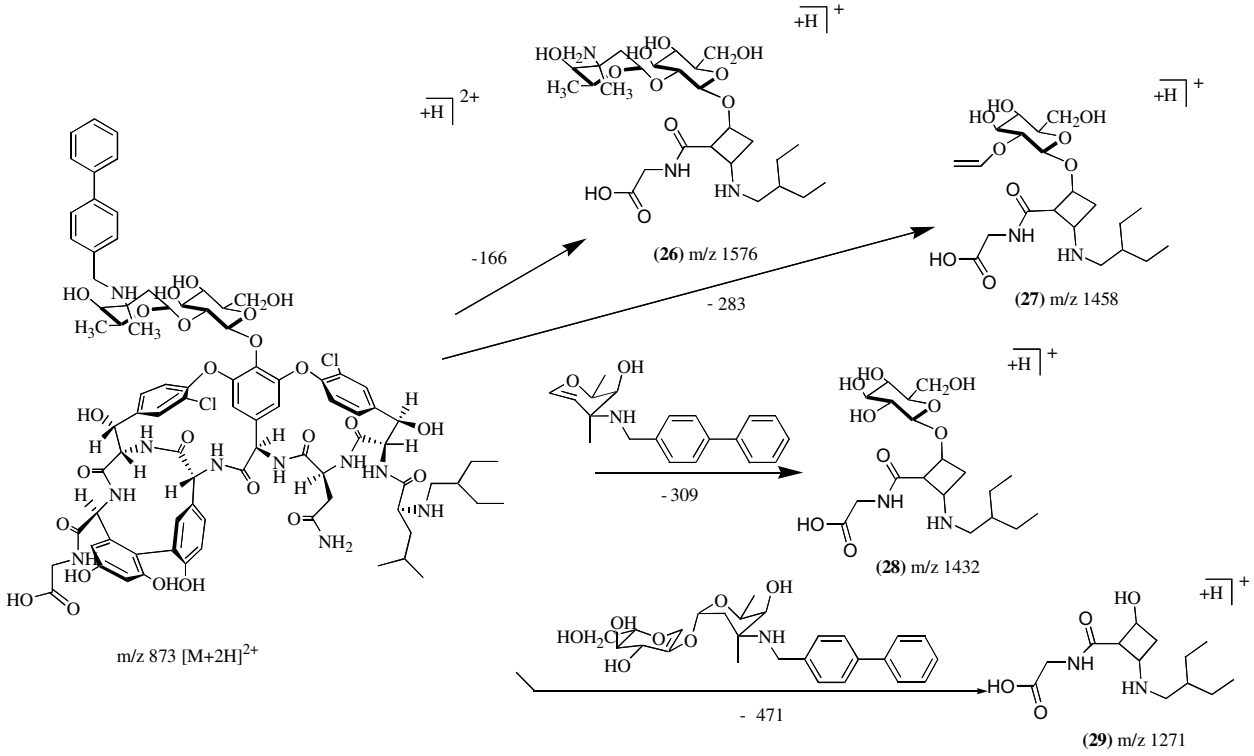


Scheme 1. Solid phase synthesis of *N*-demethylvancomycin derivatives. Reagents and conditions: (i) HMBA (2.5 equiv), HOBt (2.5 equiv), DIC (2.5 equiv), 1 h; (ii) Fmoc-Gly (5 equiv), HOBt (5 equiv), DIC (5 equiv), DMAP (0.1 equiv), 12 h; (iii) 15% Ac_2O/DMF, 50 min; (iv) 20% piperidine/DMF, 20 min; (v) *N*-Fmoc-demethylvancomycin (2.5 equiv), HBTU (2.5 equiv), HOBt (2.5 equiv), DIPEA (5 equiv), rt, 24 h; (vi) aldehyde (**R**^{1} in Table 1), (25 equiv), $NaCNBH_3$ (40 equiv), 1% AcOH/DMF, 6 h at 40 °C, 18 h at rt; (vii) aldehyde (R^2 in Table 1), (5 equiv), $NaCNBH_3$ (15 equiv), 1% AcOH/DMF, rt, 8h; (viii) 1.0 M NaOH:dioxane (v:v = 1:3), 15 min.

However, small aliphatic aldehydes, such as butyraldehyde, *trans*-2-pentenal and 3-phenylpropionaldehyde, commonly generated dialkylated products (**B**). The products were cleaved from the resins by treatment with 1.0 M NaOH in dioxane (1:3) for 15 min. The final products were obtained after desalting and purification with reverse phase column chromatography.

Sensitive and rapid analytical MS techniques can provide a detailed structural characterization of a small quantitative sample.[17] The structures of new *N*-demethylvancomycin derivatives (Table 1) were demonstrated by MS/MS analysis. For example, the proposed fragmentation pathway of typical compound **16** was illustrated in Scheme 2. The full scan MS spectrum revealed a doubly charged ion $[M+2H]^{2+}$ of the expected molecular weight. In the full scan MS/MS spectrum, losing biphenyl group (166 Da) yielded fragment **26** (*m*/*z* 1576). The fragment **27** (*m*/*z* 1458), which was produced by vancosamine ring cleavage and elimination of fragment moiety (283 Da) containing biphenyl substituent, indicated that the amino group of vancosamine was selectively alkylated by biphenyl aldehyde. This was further confirmed by generation of **28** (*m*/*z* 1432) through losing biphenylated vancosamine (309 Da) and **29** (*m*/*z* 1271) through elimination of biphenylated disaccharide (471 Da). On the other hand, the major fragments **26**, **27**, **28**, and **29**, which all contained 2-ethylbutyl group, also demonstrated that the *N*-terminus of demethylvancomycin was selectively 2-ethylbutylated by 2-ethylbutylaldehyde. Therefore, this fragment pattern clearly outlines the chemical alkylation's pathway of demethylvancomycin on Tenta Gel resin. The structures of compound **16** were also confirmed by NMR spectrum.

The antibacterial activity (MIC, μg/mL) of *N*-demethylvancomycin, vancomycin, and the 25 *N*-demethylvancomycin derivatives against *Enterococcus faecalis* (ATCC 29212), vancomycin-resistant *E. faecalis* (ATCC 51299), *S. aureus* (ATCC 29213), and methicillin resistant *S. aureus* (ATCC 43300) are shown in Table 1. As expected, *N*-demethylvancomycin exhibited more antibacterial activity against both *S. aureus* and methicillin resistant *S. aureus* than vancomycin. However, both of them were ineffective against low-level vancomycin-resistant *E. faecalis* (ATCC 29212) with minimum inhibitory concentration (MIC) of over 10 μg/mL. Many of the 25 *N*-demethylvancomycin derivatives tested showed a lost of antibacterial activity to the four bacterial strains. However, substituting the R^1 position with a more bulky aliphatic group (compounds **16** and **17**) restored the activity against vancomycin-resistant *E. faecalis* with a MIC of 10 μg/mL. Interestingly, compound **24**, with a Fmoc group at the R^1 position and a methyl group at the R^2 position, showed improved activity against vancomycin-resistant *E. faecalis* (ATCC 51299) with a MIC of 5 μg/mL, while retained its activity against the other three bacterial strains. Compound **25** also has a Fmoc group at R^1 position, but a methyl and Fmoc-group at the R^2 position. Like compound **24**, it was also active in the vancomycin-resistant *E. faecalis*. However, activity against the other bacterial strains was decreased.



Scheme 2. Fragmentation pathways of compound **16** (cyclobutyl in fragments **26**, **27**, **28**, and **29** represent the core structure of *N*-demethylvancomycin).

In conclusion, we have developed a solid-phase synthesis method with Fmoc protection strategy to prepare *N*-demethylvancomycin derivatives. This method allows us to efficiently and regioselectively reductive alkylate *N*-demethylvancomycin at both the vancosamine and *N*-terminal sites. Derivatization of the vancosamine amino group of *N*-demethylvancomycin with bulky hydrophobic structure leads to the restoration of antibacterial activity on vancomycin-resistant *E. faecalis*. The synthetic route outlined in this report is relatively simple and can be readily applied to large-scale combinatorial synthesis, including substituting the glycine with many other natural and unnatural amino acids.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bmcl.2005.02.086.

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