Antibiotic Activities and Affinities for Bacterial Cell Wall Analogue of *N*-Demethylvancomycin and Its Derivatives

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N-Demethylvancomycin, which has been clinically used in China, is one member of vancomycin group (glycopeptide) antibiotics. It differs from vancomycin only in that methyl group on the amino group of the *N*-terminal residue of vancomycin has been replaced by H. By reductive alkylation of *N*-demethylvancomycin, we synthesized *N*-alkyl and *N*,*N'*-dialkyl *N*-demethylvancomycins, which closely correlated with vancomycin in structure. The association constants of the complexes of *N*-demethylvancomycin and its analogues with di-*N*-Ac-L-Lys-D-Ala-D-Ala and the antibiotic activity against *Staphylococcus aureus* of the glycopeptides were determined. Results showed that *N*-demethylvancomycin has higher affinity for bacterial cell wall analogue di-*N*-Ac-L-Lys-D-Ala-D-Ala and more potent antibiotic activity against *Staphylococcus aureus* than vancomycin. Both *N*-alkylation and *N*,*N'*-dialkylation of *N*-demethylvancomycin reduced the affinity and antibiotic activity. The longer the alkyl groups, the less potent antibiotic activities and lower affinities have the glycopeptides. The antibiotic activities against *Staphylococcus aureus* of *N*-demethylvancomycin and its analogues roughly parallel their affinities for di-*N*-Ac-L-Lys-D-Ala-D-Ala.

The first member of the glycopeptide class of antibiotics to be reported is vancomycin, which was isolated from *Amycolatopsis orientalis* (*Nocardia orientalis*) in 1956.[1] Since then a great number of antibiotics of this group have been isolated and characterized. This group of antibiotics, which is active against Gram-positive bacteria, share a very similar extended heptapeptide backbone and vary mainly in the number, type, and placement of sugar substituents attached to the peptide nucleus.[2~5] The mode of action of the group antibiotics is based on their ability to bind to the cell-wall peptidoglycan of Gram-positive bacteria terminating in tripeptide -L-Lys-D-Ala-D-Ala. Cell wall mimics such as di-*N*-Ac-L-Lys-D-Ala-D-Ala (DALAA) have been used extensively to characterize the molecular nature of the interaction with the antibiotics.[6~8] These antibiotics exhibit in their bound state a carboxylate binding pocket, which is essentially composed of a hydrophobic-walled cavity. In vancomycin the hydrophobic walls of the cavity are formed by the side chain of residue 1 (*N*-Me-D-leucine) and non-polar portions of residues 2 and 3, while the N–H groups of these three residues form hydrogen bonds with the carboxylate of the cell wall peptide.[9] NOESY experiments showed that the *N*-terminal cationic amine plays a role in stabilizing the peptide-antibiotic complex. The $-^{+}NH_2CH_3$ of residue 1 is orientated such that the hydrophobic *N*-methyl group, and not the δ^{+} N–H protons, is adjacent to the peptide carboxylate anion. In this way, the hydrophobic pocket around the peptide carboxylate anion has another boundary.[8,10,11]

The vancomycin group antibiotics have received considerable attention both for the growing importance in the treatment of methicillin-resistant *Staphylococci* and multiply resistant strains of *Streptococcus pneumoniae*[12] and because their complexes with small peptides provide an excellent system for studying substrate-receptor interactions.[13~15] Of the group antibiotics, vancomycin and teicoplanin have been clinically used in America and Europe. *N*-Demethylvancomycin, another member of the vancomycin group, was first isolated from Van-23, a strain of *Amycolatopsis orientalis* from soil sample collected in Guizhou Province, China, in 1959[16] and has

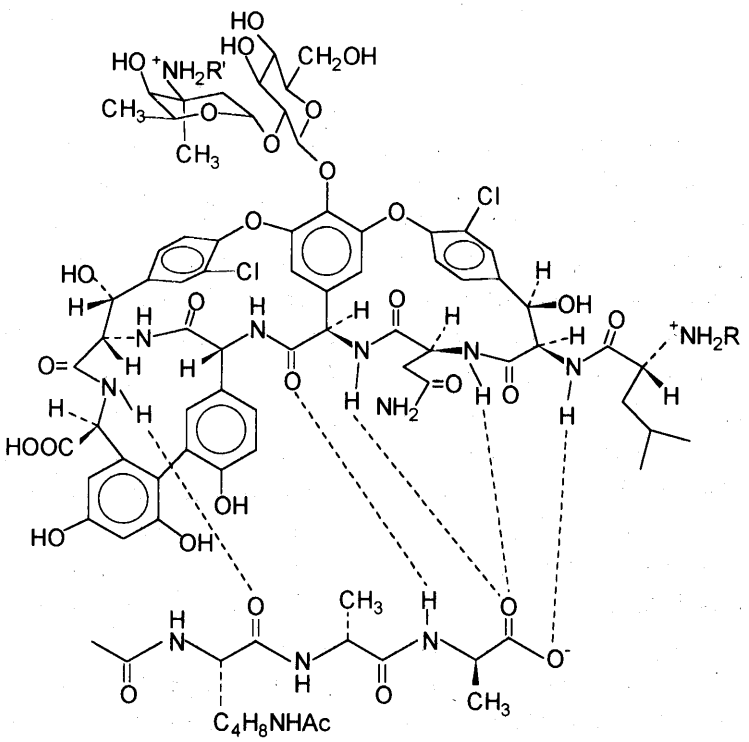
been clinically used in China since 1967. The structure of *N*-demethylvancomycin was determined by BOECK *et al.*[17] and LIN *et al.*[18]

Recently resistance to vancomycin, which is still considered as the last line of defense against multiply resistant bacteria, has been reported.[19~21] Therefore, interest in this group of antibiotics as well as elucidation of the structure-function relationship, which may lead to the design and synthesis of new, more potent vancomycin analogues, or analogues killing vancomycin-resistant bacteria, has greatly increased. In this paper, we study on antibiotic activities and combination with bacterial cell wall analogue DALAA of *N*-demethylvancomycin and its analogues, *N*-alkyl *N*-demethylvancomycins (including vancomycin).

Results and Discussion

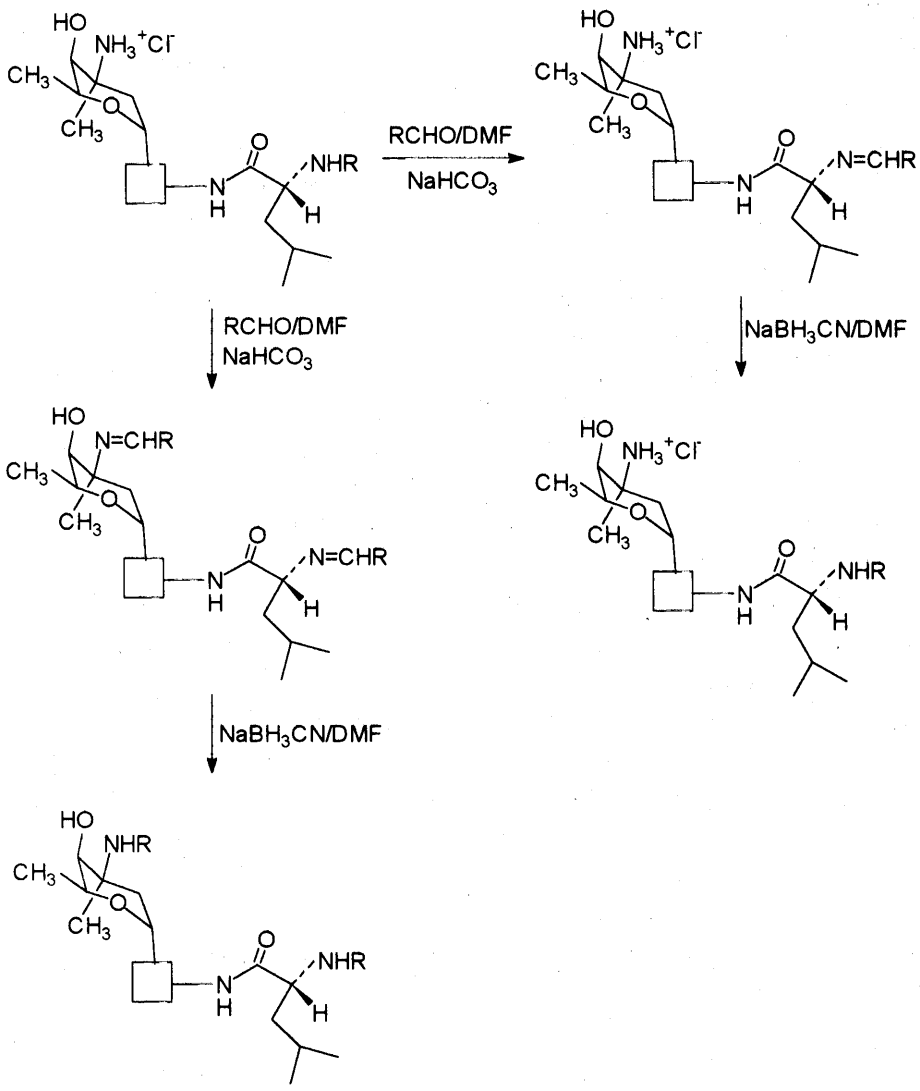
N-Demethylvancomycin, which has been clinically used in China for 30 years, is one member of vancomycin group antibiotics. It differs from vancomycin only in that methyl group on the amino group of the *N*-terminal residue of vancomycin has been replaced by H, as shown in Figure 1. *N*-Demethylvancomycin may be the closest analogue of vancomycin in the natural occurring members in this group. It is easy to prepare *N*-alkyl *N*-demethylvancomycins, which are closely correlated with vancomycin in structure, by reductive alkylation of *N*-demethylvancomycin with aldehydes, as shown in Scheme 1. The appropriate aldehyde first reacted with *N*-demethylvancomycin to form Schiff base. Then the Schiff base formed *in situ* was reduced by sodium cyanoborohydride to give the *N*-alkyl *N*-demethylvancomycin. The products were purified by semi-prepared reversed-phased HPLC. The structures of the synthetic *N*-alkyl *N*-demethylvancomycins were confirmed through mass spectra of them and their aglycones. *N*-demethylvancomycin has two amino groups: the amino group of vancosamine (amino sugar) and the amino group of the peptide *N*-terminal. Electrospray mass spectrum of aglyco-*N*-heptyl *N*-demethylvancomycin (NHDV) shows that molecular weight is 1129, indicating the alkylation is occurred on the *N*-terminal amino group. The result shows that, in the most cases studied in the reaction condition, the product of alkylation on the *N*-terminal amino group is the major product, indicating the *N*-terminal amino group is more reactive than the vancosamine amino group. Similar reactivity orders were found for vancomycin[8] and vancomycin hexapeptide.[22] The reason for these may be the vancos-

Fig. 1. Structures of *N*-demethylvancomycin, vancomycin and complex with cell wall analogue DALAA.



N-Demethylvancomycin: R=H, R′=H; vancomycin: R=CH_3, R=H

Scheme 1.



amine amino group is more hindered than the *N*-terminal amino group in the tertiary structure of the glycopeptides. Table 1 shows the structures of synthetic *N*-alkyl *N*-demethylvancomycins. When butyraldehyde was used as the alkylation reagent, besides *N*-butyl *N*-demethylvancomycin, *N*,*N*′-dibuyl *N*-demethylvancomycin, which was confirmed by mass spectra of it and its aglycon, was also obtained. When *n*-nonyl aldehyde was used as alkylation reagent, not *N*-nonyl *N*-demethylvancomycin but *N*,*N*′-dinonyl *N*-demethylvancomycin was obtained, as shown in Table 1.

Affinity constants for the binding of *N*-demethylvancomycin and its analogues with DALAA were measured by UV titration method, as tabulated in Table 2. It can be seen that *N*-demethylvancomycin has higher binding constant than vancomycin and other *N*-alkyl and *N*,*N*′-dialkyl *N*-demethylvancomycins. The longer the *N*-alkyl chains, the weaker are the binding of *N*-alkyl *N*-demethylvancomycins with DALAA. NOE experiment showed[8] that in vancomycin-DALAA complex, the cationic *N*-terminal amine is orientated such that the hydrophobic *N*-methyl group, and not the δ^+ N–H protons, is adjacent to the DALAA carboxylate anion. Our results show that removal of the *N*-terminal methyl group from vancomycin or the *N*-alkyl groups from the *N*-alkyl *N*-demethylvancomycins increases their affinities for DALAA, and increasing the length of the *N*-alkyl groups are unfavorable for the binding. Therefore, the orienting *N*-methyl group in vancomycin seems to have a negative contributor to the binding; and thus the orientation of δ^+ N–H protons appears to be favored for the binding. As shown in Figure 1, due to proximity of the amine to the carboxylate in the complex, some form of electrostatic stabilization seems likely. This

Table 1. Structures of *N*-demethylvancomycin and its analogues.

Glycopeptide	R[a]	R'[a]	Molecular weight	
			Calculated	Found
N-Demethylvancomycin	H	H		
Vancomycin	CH_3	H		
NBDV	$CH_3CH_2CH_2CH_2$	H	1490	1490[b]
DBDV	$CH_3CH_2CH_2CH_2$	$CH_3CH_2CH_2CH_2$	1546	1547[b]
IBDV	$(CH_3)_2CHCH_2$	H	1490	1490[b]
NPDV	$CH_3CH_2CH_2CH_2CH_2$	H	1504	1504[b]
IPDV	$(CH_3)_2CHCH_2CH_2$	H	1504	1504[c]
NHDV	$CH_3(CH_2)_5CH_2$	H	1532	1533[c]
DNDV	$CH_3(CH_2)_7CH_2$	$CH_3(CH_2)_7CH_2$	1686	1685[b]
BZDV	$C_6H_5CH_2$	H	1524	1524[c]
MBZDV	*m*-$CH_3C_6H_4CH_2$	H	1538	1539[b]

[a] See Figure 1.
[b] Determined by FAB-MS.
[c] Determined by ES-MS.

Table 2. Association constants and antibiotic activities for *N*-demethylvancomycin and its analogues.

Glycopeptide	K_a (M^{-1})	MIC against *S. aureus* CMCC(B) 26003 (μg/ml)
N-Demethylvancomycin	5.8×10^6	1.4
Vancomycin	1.5×10^6	2.3
NBDV	5.3×10^5	2.7
DBDV	4.0×10^5	6.3
IBDV	4.7×10^5	3.1
NPDV	4.3×10^5	6.3
IPDV	4.5×10^5	3.1
NHDV	5.0×10^5	3.1
DNDV	4.2×10^5[a]	4.5
BZDV	3.7×10^5	12.5
MBZDV	3.8×10^5	12.5

[a] Experiment carried out in 30% methanol at pH 6.2.

conclusion is supported by work of FEENY and co-workers who showed that upon deprotonation of the vancomycin *N*-terminal amine in aqueous solution, complex formation with *N*-Ac-D-Ala-D-Ala became less favorable by 5.9 kJ mol^{-1} (a factor of 11 in binding constants).[23] Both acylation[8] and removal[22] of $-^+NH_2Me$ of *N*-terminal residue of vancomycin also reduce the binding to DALAA by a factor of 20. Therefore, some distance between the amine and the carboxylate seems to be necessary for tight binding. Similar phenomena were observed for epiristocetin[24] and epiavoparcin[25] which show diminished binding even though models suggest that the *N*-terminal amino group in the epi compounds is in the better position for electrostatic interaction with the carboxylate anion than in the natural isomer. *N'*-Alkylation of *N*-alkyl *N*-demethylvancomycin also reduced the affinity for DALAA.

Table 2 also gives the antibioic activities of *N*-demethylvancomycin and its analogues against *Staphylococcus aureus* CMCC(B) 26003. The decreases in antibiotic activities parallel the decreases in the binding constants for these glycopeptides. Among the glycopeptide antibiotics studied, *N*-demethylvancomycin is the most potent antibiotic. The antibiotic activity of *N*-demethylvancomycin against *Staphylococcus aureus* CMCC(B) 26003 is slightly greater than that of vancomycin. The unpublished results obtained in the North China Pharmaceutical Corporation showed that the antibiotic activity of *N*-demethylvancomycin against all other strains of *Staphylococcus aureus* tested as well as some other Gram-positive bacteria are greater than that of vancomycin by a factor of 1.1 ~ 1.3.[26]

Conclusion

1. *N*-Demethylvancomycin has more potent antibiotic activity against *Staphylococcus aureus* and higher affinity for bacteria cell wall analogue DALAA than vancomycin.

2. *N*-Alkylation of *N*-demethylvancomycin decreases antibiotic activity and affinity for DALAA. Further *N'*-alkylation of *N*-alkyl *N*-demethylvancomycin also

decreases the antibiotic activity and the affinity. The longer the alkyl groups, the less potent antibiotic activities and lower affinities have the glycopeptides.

3. The antibiotic activities against *Staphylococcus aureus* of *N*-demethylvancomycin and its analogues roughly parallel their affinities for DALAA.

Experimental

Materials

Vancomycin hydrochloride was obtained from Eli Lilly and Company. *N*-Demethylvancomycin hydrochloride was obtained from North China Pharmaceutical Corporation. Dicyclohexylcarbodiimide (DCC) was obtained from Shanghai Sheshan Chemical Plant. 001×7 Strongly acidic cation exchange resin was obtained from Nankai Group. N^{α}, N^{ε}-Dibenzyloxycarbonyl lysine (di-*N*-*Z*-L-Lys) was prepared as described by BERGMANN *et al.*[27] D-Ala-OCH_3·HCl was prepared referring to the method of BRENNER & HUBER.[28] Pd/C was synthesized according to the method reported in literature.[29]

Synthesis of *N*-Alkyl *N*-Demethylvancomycins

General procedure for the preparation of *N*-alkyl *N*-demethylvancomycins: *N*-demethylvancomycin hydrochloride was first neutralized with an equimolar amount of triethylamine, and then was reacted with an equimolar amount of the desired aldehyde at room temperature for 2 days or at 50°C for 12 hours to form Schiff base in DMF. The Schiff base was then reduced with sodium cyanoborohydride to form *N*-alkyl *N*-demethylvancomycin. The crude product formed was purified by semipreparative reversed phase HPLC (column: μ-Bondpak C_{18}, i.d. 7.8×300 mm, Waters; eluent: methanol-water containing 0.1% of trifluoroacetic acid, gradient elution). The purity of the product was checked by analytical reversed phase HPLC (column: Novo-Pak C_{18}, i.d. 3.9×150 mm, Waters, eluent: methanol-water containing 0.1% of trifluoroacetic acid, gradient elution). Synthesis of *N*-heptyl *N*-demethylvancomycin as an example is given below.

0.1 g of *N*-demethylvancomycin hydrochloride (0.068 mmol) and 9.5 μl of triethylamine (6.9 mg, 0.068 mmol) were dissolved in 2 ml of DMF. To this solution 9.2 μl of heptaldehyde (7.8 mg, 0.068 mmol) was added and the mixture was stirred at 50°C for 12 hours. Then 6 mg of sodium cyanoborohydride was added to the solution and the mixture was stirred at room temperature for 2 days. After removing the solvent under reduced pressure the crude product was purified by HPLC on a μ-Bondpak C_{18} column (i.d. 7.8×300 mm) using a gradient of aqueous methanol containing 0.1% of triflouroacetic acid as eluent to give *N*-heptyl *N*-demethylvancomycin trifluoroacetate (41 mg, 37% yield).

Preparation of aglyco-*N*-alkyl *N*-demethylvancomycins: 10 mg of *N*-alkyl *N*-demethylvancomycin was dissolved in 1 ml of 1 M HCl and the mixture was heated in a boiling water bath for 5 minutes. The solution was cooled and then centrifuged to give the correspondent aglyco-*N*-alkyl *N*-demethylvancomycin as white precipitate.

The molecular weight was measured by the fast atom bombardment mass spectrometry (FAB-MS) on a Zabspec machine or electrospray mass spectrometry (ES-MS) on a VG Bio-Q ESEM machine.

Synthesis of Di-*N*-*Z*-L-Lys-D-Ala

Di-*N*-*Z*-L-Lys (1.0 g, 2.4 mmol), D-Ala-OCH_3·HCl (0.38 g, 2.7 mmol) and triethylamine (0.3 g, 3.0 mmol) were dissolved in dichloromethane (10 ml). With ice bath cooling DCC (0.5 g, 2.4 mmol) was added to the solution and the solution was stirred for 2 hours with cooling and 4 hours at room temperature. After this time a white precipitate formed was removed by filtration and discarded. Then a part of the solvent of the filtrate was evaporated under reduced pressure and a small amount of precipitate formed was removed by filtration again. The filtrate was evaporated to dryness under reduced pressure. The solid obtained was dissolved in chloroform (10 ml) and the solution was washed with aqueous citric acid (10%), water and aqueous $NaHCO_3$ (5%), respectively. The organic phase was dried with anhydrous Na_2SO_4 and the dried solution was evaporated to dryness. The solid residue obtained was dissolved in THF (10 ml). Under ice bath cooling aqueous 2 M LiOH (2.0 ml) was added to the solution. The solution was stirred at room temperature. The reaction was monitored by TLC, and after about 20 hours was judged to be complete. The mixture was evaporated to dryness and the solid residue was washed several times with chloroform. The solid residue was dissolved in DMF (20 ml) and the solution was acidified to pH 3 with 1 M HCl. The mixture was evaporated to dryness. The solid was washed with water and was used in the following reaction as Di-*N*-*Z*-L-Lys-D-Ala without further purification.

Synthesis of DALAA. Di-*N*-*Z*-L-Lys-D-Ala obtained above, D-Ala-OCH_3·HCl (0.38 g, 2.7 mmol) and triethylamine (0.3 g, 3.0 mmol) were dissolved in DMF (10 ml). To this was added DCC (0.5 g, 2.4 mmol) with ice bath cooling. The mixture was stirred for 2 hours

with cooling and 1 day at room temperature. Then the solvent was removed under reduced pressure. The solid residue was dissolved in chloroform and the insoluble residue was removed by filtration. The filtrate was washed repeatedly with each of the followings: aqueous citric acid (10%), water and aqueous $NaHCO_3$ (5%), and then the solvent was removed by evaporation under reduced pressure. The solid obtained was dissolved in DMF (10 ml) and to this was added Pd/C (10%, 40 mg). The mixture was treated with H_2. After the completion of the reaction had been confirmed by TLC, the mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The solid was dissolved in DMF and excess acetic anhydride was added. The mixture was stirred for 4 hours at room temperature and 2 hours at 50°C. The reaction was monitored by ninhydrin test. After the reaction was complete, the solvent was removed under reduced pressure. The solid obtained was dissolved in THF and to this was added excess aqueous 2 M LiOH. The solution was stirred for 2 days at room temperature and then evaporated to dryness. The crude product was dissolved in water and loaded on 001 × 7 strongly acidic cation exchange resin (H form) column and eluted with water. The eluent was evaperated to dryness, 508 mg of product was obtained, yield 56.8%. TLC determination showed that the product was pure. Amino acid analysis showed that peptide is composed of Lys and Ala with a ratio of 1 : 2, indicating the product is DALAA.

Determination of Association Constant

The determination of association constants for binding of *N*-demethylvancomycin and its derivatives with DALAA by UV spectrophotometry was carried out on a Shimadzu UV-2101PC dual beam spectrophotometer at 25±1°C. Both glycopeptide and DALAA solutions were buffered with KH_2PO_4 (0.05 M)/NaOH, pH 7.0. Solutions containing glycopeptide (*ca.* 0.08 mg/ml) were placed in the sample and reference cells. The sample cell was titrated with the ligand solution (*ca.* 1 mM, 5~600 μl) which contained the same concentration of the glycopeptide as the glycopeptide solution. The differences in absorbence were measured. The association constants were determined by means of a nonlinear least-squares program based on the assumption of 1 : 1 binding.

Antibiotic Assays

Minimal inhibitory concentrations (MIC's) of the glycopeptides for *Staphylococcus aureus* CMCC (B) 26003 were determined by the microdilution method in an agar dilution assay. MIC values were defined as the lowest concentration of antibiotic that completely inhibits growth of the organism.

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