Cooperativity between Non-polar and Ionic Forces in the Binding of Bacterial Cell Wall Analogues by Vancomycin in Aqueous Solution

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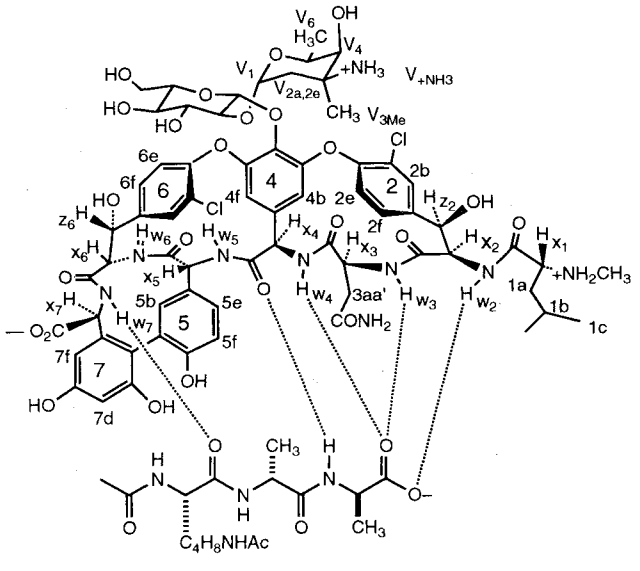
The clinically important glycopeptide antibiotic vancomycin binds to bacterial cell wall peptides of Gram-positive bacteria which terminate in -Lys-D-Ala-D-Ala, thereby inhibiting cell wall synthesis resulting in cell death. We have removed the *N*-terminal leucine residue of vancomycin by an Edman degradation and acylated the exposed amino group of residue 2 with *N*-Me-Gly, *N*-Me-D-Ala, acetyl, butyl, and isohexyl groups to generate novel vancomycin analogues. The binding of vancomycin and these vancomycin analogues to the bacterial cell wall analogue di-*N*-Ac-L-Lys-D-Ala-D-Ala (DALAA) was studied by NMR techniques and UV spectroscopy. The effects that these structural modifications of the carboxylate binding pocket of vancomycin have on the antibiotic-DALAA recognition process show that a cooperative effect between non-polar and ionic forces appears to be partly responsible for the highly efficient sequestering of the DALAA *C*-terminal carboxylate from aqueous solution.

The recent discovery of bacterial strains resistant to vancomycin, which is still the last line of defence in combating the outbreaks of multiply-resistant staphylococci and enterococci in hospitals and clinics worldwide, has greatly increased the interest in the vancomycin family of antibiotics. We wish to elucidate the structural and thermodynamic factors which are responsible for the binding of vancomycin to bacterial cell wall, which ultimately results in the antibacterial activity. A complete understanding of these factors may lead to the design and synthesis of new, more potent vancomycin analogues, or analogues which kill vancomycin-resistant bacteria.

The vancomycin family of antibiotics recognise the *C*-terminal portion of bacterial cell wall precursor peptides ending in the sequence -Lys-D-Ala-D-Ala.[1] These antibiotics share a structural motif which is responsible for binding to the *C*-terminal carboxylate anion of the cell-wall peptide. This part of the cell wall binding pocket is essentially composed of a hydrophobic-walled cavity into which three of the backbone amide N–H bonds converge. In vancomycin (**1**) the hydrophobic walls of the cavity are formed by the side chain of residue 1 (*N*-Me-D-leucine) and the non-polar portions of residues 2 and 3, while the amide N–H groups of these three residues form hydrogen bonds with the carboxylate of the cell wall peptide (Fig. 1).[2]

Previous NMR studies have identified interactions between the *N*-terminal amino acid of the antibiotic and cell wall which are thought to be favourable for binding. The isobutyl side chain of the *N*-Me-D-leucine residue of vancomycin was shown to fold in, and to bury part of a bound cell wall analogue in DMSO-d_6 solution.[3~5] NOESY experiments showed also that the *N*-terminal cationic amine plays a role in stabilizing the peptide-antibiotic complex. The $-^{+}NH_2CH_3$ of residue 1 is oriented such that the hydrophobic methyl group, and not the δ^+ N–H protons, is adjacent to the peptide carboxylate anion. This should thereby enhance the

Fig. 1. The complex of vancomycin (**1**) with the cell wall analogue DALAA.



hydrophobic surroundings of the carboxylate binding pocket;[3~5] probably more significantly, the cationic charge has maximum exposure to the solvent.

Due to the proximity of the amine to the carboxylate in the complex, some form of electrostatic stabilization also seems likely. This conclusion is supported by work of FEENEY 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 becomes less favorable by 5.9 kJ mol^{-1} (a factor of 11 in binding constant).[6] We wished to investigate the roles of electrostatic stabilisation and hydrocarbon packing in the binding of cell wall analogues. We report here the synthesis of analogues of vancomycin in which the *N*-terminal residue of vancomycin has been modified to afford new vancomycin derivatives (Fig. 2), and discuss the corresponding binding affinities of these compounds for the cell wall analogue di-*N*-Ac-Lys-D-Ala-D-Ala (DALAA) in aqueous solution.

Results and Discussion

Previously, the Edman degradation of vancomycin **1**, which selectively removes the *N*-terminal *N*-Me-leucine residue to provide the vancomycin hexapeptide, has been reported.[7] We now show that the *N*-terminal amino group of vancomycin hexapeptide can be selectively acylated with acid anhydrides, or coupled with *N*-*t*-Boc-*N*-Me-Gly or *N*-*t*-Boc-*N*-Me-D-Ala to afford the vancomycin analogues **2**~**6** (Fig. 2). After purification by reverse-phase HPLC, these compounds were characterised by electrospray mass spectrometry and 2D NMR. In addition, the complexes of these analogues with DALAA were studied by 2D NMR.

The structural studies confirm that these analogues bind to the tripeptide in a manner similar to that for vancomycin itself, *i.e.*, the NOE enhancement data are consistent with the binding picture illustrated in Fig. 1.[3~5] Some of the NOE enhancements obtained in NOESY experiments carried out on the **4**-DALAA complex are shown in Table 1, along with those for the corresponding vancomycin-DALAA complex for comparison. The binding constants for the 1 : 1 complexes of **1**~**6** with DALAA in pH 7 aqueous solution were measured by UV spectroscopy as described previously[8] and are shown in Table 2.

The first three entries of Table 2 allow an analysis of the effect of the stepwise removal of the isobutyl sidechain of vancomycin, while retaining the terminal $-^{+}NH_2Me$ group. Removal of three of the four carbon atoms (and associated hydrogen atoms) of the sidechain reduces binding by a factor of 19 (**1** *vs.* **2**). Burying this part of the hydrocarbon surface area of the C_4 sidechain within the complex, thereby removing it from water (and thus exercising the hydrophobic effect) is expected from modelling studies to bury *ca.* 28 Å^2 of hydrocarbon from

Fig. 2. Synthesis of vancomycin analogues **2**~**6**.

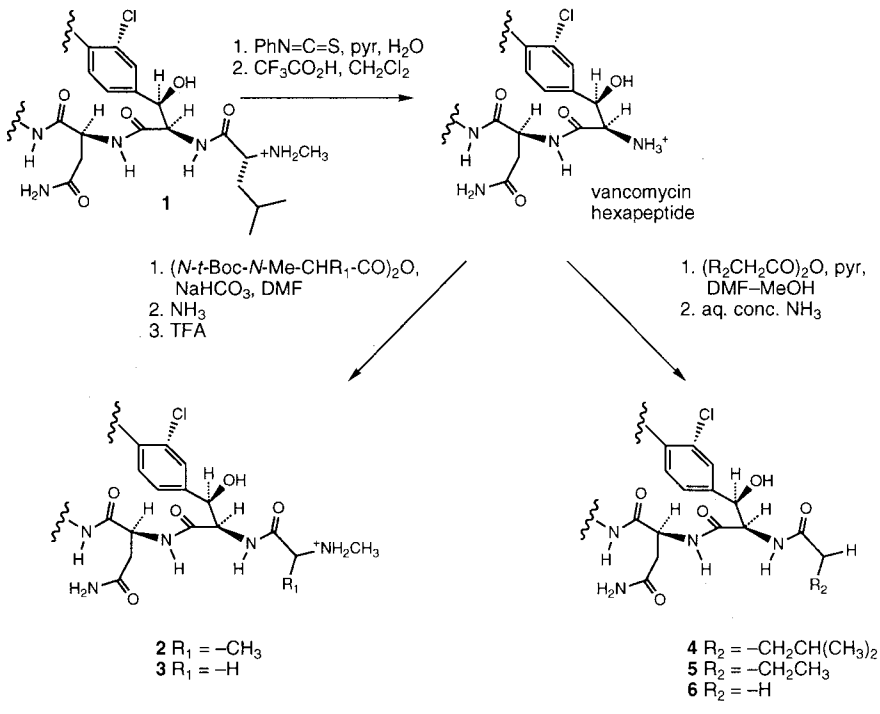


Table 1. Observed NOE enhancements for vancomycin- and **4**-DALAA complexes from NOESY experiments in 9:1 D_2O-H_2O, 283 K[a].

Proton	**1**-DALAA		**4**-DALAA	
	δ (ppm)	NOE	δ (ppm)	NOE
x_1	4.20	1a, 1b, 1c, NMe, 2f, w_2	3.75	1a, 1b, 1c, 2f, w_2
1a	1.63	1b, 1c, x_1	1.6	1b, 1c, x_1
1b	1.75	1a, 1c, NMe, x_1, 2f, Ala_C α-C-H_{bound}	1.7	1a, 1c, x_1
1c	0.9, 0.68 free and bound	1a, 1b, 3a,a', NMe, Ala_C α-C- H_{bound}, x_1, 2f, Ala_C Me_{bound}	0.8	1a, 1b, 2f, 3a,a', x_1
w_2	11.69	x_1, w_3, w_4	10.3	x_1, w_3, w_4
2e	7.35	2f, 4b, Ala_C Me_{bound}	7.38	2f, Ala_C Me_{bound}, Ala_C Me_{free}
2f	7.70	1b, 1c, 2e, x_1, NMe, Ala_C α-C-H_{bound}, Ala_C Me_{free},Ala_C Me_{bound}	7.74	1c, 2e, x_1, Ala_C Me_{bound}
3a,a'	2.60	1b, 1c, w_3, w_4	2.75	1c, w_3, w_4, 5b, 5e
Ala_C Me_{bound}	0.36	1c, 2e, 2f, NMe, x_1, Ala_C Me_{free}	0.70	V6, Ala_C Me_{free}, 2e, Ala_C α-C-H_{bound}
NMe	2.70	1b, 1c, 2f, x_1, Ala_C Me_{bound}, Ala_C α-C-H_{bound}	—	—

[a] Concentration of antibiotic *ca.* 10 mM; with twofold excess of DALAA.

Table 2. UV association constants[a] for antibiotic-DALAA complexes at 298 K[b].

Compound	R_1	R_2	K_{assoc} (M^{-1})	$-\Delta G$ (kJ mol^{-1})
1	$-^+NH_2Me$	$-CH_2CH(CH_3)_2$	1.6×10^6	35
2	$-^+NH_2Me$	$-CH_3$	8.5×10^4	28
3	$-^+NH_2Me$	$-H$	7.7×10^4	28
4	$-H$	$-CH_2CH(CH_3)_2$	7.4×10^4	28
5	$-H$	$-CH_2CH_3$	5.4×10^4	27
6	$-H$	$-H$	4.3×10^4	26

[a] Titrations were performed in triplicate; uncertainties in K_{assoc} are estimated to be 20%.
[b] Concentration of antibiotic *ca.* 0.05 mM, pH 7, 0.05 M KH_2PO_4 buffer.

water exposure. Therefore the potential increase in binding constant due to this effect in isolation (taking the hydrophobic effect as lying in the range 0.20~0.23 kJ mol^{-1} $Å^{-2}$ at 298 K[9,10]) is a factor of 10~13, which is reasonably close to the factor of 19 above.

The effect of removing all 4 carbon atoms of the sidechain is a similar reduction in binding, in this case by a factor of *ca.* 20 (**1** *vs.* **3**, Table 2). We conclude that the enhancement of binding upon introduction of the D-Ala methyl group into the antibiotic (**3** *vs.* **2**) is small or negligible.

We now consider the relative binding affinities of vancomycin with those of compounds in which the $-^+NH_2Me$ group has been removed, and the isobutyl sidechain is progressively removed (Table 2, rows 1 and 4 to 6). The loss in binding affinity associated with removal of the $-^+NH_2Me$ group from vancomycin is about a factor of 20 to 40 in binding constant, and is found to lie in this range irrespective of whether the leucine side chain is retained (**1** *vs.* **4**), partially removed (**1** *vs.* **5**), or totally removed (**1** *vs.* **6**). Thus, in the absence of the $-^+NH_2Me$ group, the $-CH_2CH(CH_3)_2$ sidechain promotes binding by only a factor of *ca.* 2 (although it is able to promote binding by a factor of *ca.* 20 in the presence of the $-^+NH_2Me$ group—see above).

A consistent picture therefore emerges from these data: the main promotion of binding by a hydrocarbon group and a $-^+NH_2Me$ group requires the presence of both. A number of factors may be involved in this cooperativity. First, electrostatic binding energy between the carboxylate anion of the bacterial cell-wall analogue and the $-^+NH_2Me$ group of the antibiotic may only promote binding significantly when the antibiotic-bound carboxylate anion is more effectively sequestered from water by the presence of the $-CH_2CH(CH_3)_2$ sidechain. This consideration is justified by the fact that electrostatic interactions are known to be strengthened in a less polar environment.[11] Second, the hydrophobic

effect may be more efficiently exercised and strengthened when the charged $-^{+}NH_2Me$ group is in the immediate proximity of the hydrophobic interaction. An analogous strengthening has previously been proposed to account for the fact that the hydrophobic interaction (between the 6-methyl group of the amino-sugar vancosamine and the methyl group of the *C*-terminal alanine in di-*N*-Ac-L-Lys-D-Ala-D-Ala) promotes binding by a factor of 5 greater when the amino group of the sugar is charged ($-NH_3^+$) relative to when it is acetylated ($-NHCOCH_3$).[12] This effect may have the same physical basis as the lower solubility of an organic compound in a brine solution relative to the solubility in water (the well known "salting out" effect).

Comparisons between pairs of compounds other than those so far directly made support the general tenor of the above conclusions. Thus, introduction of the $-^{+}NH_2Me$ group in the absence of the $-CH_2CH(CH_3)_2$ sidechain only slightly changes binding (*cf.* data for **3** and **6**, in Table 1), whereas the same introduction in the presence of the $-CH_2CH(CH_3)_2$ sidechain promotes binding by a factor of *ca.* 20 (*cf.* data for **1** and **4** in Table 2). The general conclusion is therefore clear—binding is cooperatively promoted by the $-^{+}NH_2Me$ and $-CH_2CH(CH_3)_2$ groups possibly through at least two effects:

(i) the strengthening of the $-CO_2^-\cdots{}^{+}NH_2Me$ interaction in the presence of the $-CH_2CH(CH_3)_2$ sidechain.

(ii) the strengthening of the hydrophobic effect in the presence of the positive charge of the $-^{+}NH_2Me$ group.

The weaker binding seen in the complexes corresponding to rows 2~6 (Table 2), relative to the corresponding vancomycin complex, is reflected in the difference in the chemical shift of the N–H w_2 proton of the antibiotics ($\Delta\delta$), in the free and complexed state. For example, in free vancomycin at pH 4.5 and 1 mM concentration, the w_2 resonance (see Fig. 1) occurs at 8.79 ppm. Addition of excess DALAA tripeptide (to ensure that formation of the complex is >95%) to the vancomycin solution causes the w_2 resonance to shift downfield to 11.69 ppm. This rather large $\Delta\delta$ value of 2.90 ppm for vancomycin reflects the strong intermolecular hydrogen bond formed between the w_2 and the DALAA carboxylate in the complex. For comparison, the $\Delta\delta$ values for the **2**-, **3**-, **4**-, **5**-, and **6**-DALAA complexes are 1.90, 2.06, 2.05, 1.96, and 2.09 ppm respectively (available from the data presented in Table 3). We take the extent of the downfield shift of the w_2 resonance as a measure of the strength of the electrostatic interaction of the carboxylate anion of the bacterial cell wall analogue with the w_2 amide N–H. Therefore, these data show that the strength of this electrostatic interaction is not significantly changed by the addition of the $-CH_2CH(CH_3)_2$ sidechain in the absence of the $-^{+}NH_2Me$ group (*cf.* $\Delta\delta$ values of 2.09 and 2.05 ppm); nor by the addition of the $-^{+}NH_2Me$ group in the absence of the $-CH_2CH(CH_3)_2$ sidechain (*cf.* $\Delta\delta$ values of 2.09 and 2.06 ppm). However, the addition of the same hydrocarbon sidechain in the presence of the $-^{+}NH_2Me$ group strengthens this electrostatic interaction (*cf.* Δd values of 2.06 and 2.90 ppm). These experiments seem to provide strong evidence for the cooperative effects of $-^{+}NH_2Me$ and $-CH_2CH(CH_3)_2$ groups in promoting an electrostatic interaction adjacent to both groups.

Table 3. Chemical shifts of the w_2 resonance for the complexes of **1**~**6** with DALAA.

Complex	δ (w_2)
1-DALAA	11.69
2-DALAA	10.69
3-DALAA	10.85
4-DALAA	10.84
5-DALAA	10.75
6-DALAA	10.88

Experimental

General Procedures

Vancomycin was obtained as the hydrochloride salt as a gift from Eli Lilly and Company (Indianapolis) and was used without further purification. Samples of other compounds for NMR spectroscopy were typically purified by preparative HPLC prior to use. Samples were dissolved in DMSO-d_6, D_2O, D_2O/H_2O, or deuterated phosphate buffer. Deuterated phosphate buffer (pD 7.0) was prepared by dissolving KD_2PO_4 (50 mM) and NaOD (29 mM) in D_2O. Successive lyophilisation of KH_2PO_4 from D_2O was used to prepare the deuterated salt, while NaOD was purchased as a 40% (w/w) solution in D_2O. All pH and pD sample readings were measured with a Corning pH meter 125 equipped with a Russell combination glass electrode. The pD readings quoted throughout are pH meter readings and no corrections have been made for isotope effects. The pH or pD of NMR samples was adjusted using solutions of DCl and NaOD.

NMR spectra were obtained using Bruker WM250, AM400, AM500, or AMX500 spectrometers. Chemical shifts were referenced to internal TSP (δ 0.0 ppm) or dioxane (δ 3.74 ppm). One dimensional ^1H spectra were

recorded with quadrature detection as 8 K or 16 K data points after a $\pi/2$ pulse. Recycle delays of between 1.5 and 2.0 seconds were employed. Carrier frequencies and spectral widths were adjusted to allow *ca.* 1 ppm either side of the observable resonances. In those cases where the residual HOD signal was intense, selective suppression was achieved by presaturation of the signal during the relaxation delay. All two dimensional experiments were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in f_1. The spectral widths used were the same as for the one dimensional experiments. Typically, either 32 or 64 transients of 2 K data points each were recorded in the f_2 dimension and 512 t_1 increments were recorded then zero-filled to 1 K data points. In cases where the residual water signal in deuterated solvent was intense, suppression was achieved by presaturation during the recycle delay. Prior to Fourier transformation, the spectra from double quantum filtered COSY experiments were subjected to a phase-shifted sine bell weighting function in each dimension. The mixing time (τ_m) for phase-sensitive NOESY spectra was set to 400 ms, with a 20 ms z-filter to reduce zero-quantum scalar coupling correlation by random variation of t_m. Data sets were subjected to a Lorentz-Gaussian weighting function in both dimensions prior to Fourier transformation.

UV spectra were recorded on a UVIKON 940 dual beam spectrophotometer at 303.2 ± 0.1 K. Glycopeptide and ligand solutions were buffered with aqueous KH_2PO_4/NaOH (50 mM/29 mM) at pH 7.0. The initial concentration of glycopeptide used was 0.05 mM. Four wavelengths were monitored during ligand binding titrations. These were chosen on the basis of a difference spectrum, determined with antibiotic in the reference cell, and antibiotic-ligand complex in the sample cell. This showed the wavelengths at which the UV spectra of antibiotic and antibiotic-ligand complex were most different. Two wavelengths at the apex of a peak due to a difference between antibiotic and complex (typically 293 and 294 nm) and two wavelengths at the bottom of a valley due to a difference between antibiotic and complex (typically 283 and 284 nm) were chosen. Binding constants were determined by measuring the increase in the difference in absorption between the peak and the valley, with antibiotic solution in both the reference and sample beams. Ligand dissolved in the antibiotic solution was titrated into the cell in the sample beam (the ligand concentration used was in the range 5 ~ 30 mM, depending on the magnitude of the binding constant to be measured). The solutions were stirred (20 seconds), left to settle (3 minutes) and the absorbances were then measured. The values for the absorbances at 293 and 294 nm were added together, and from this the sum of the absorbances at 283 and 284 nm pairs of wavelengths was subtracted. The resulting data was fitted to the theoretical equation for 1 : 1 binding using least-squares methods.

Electrospray mass spectra were recorded on a VG Bio-Q ESMS machine, using H_2O-CH_3OH-CH_3CO_2H (50 : 50 : 1) as the carrier solvent. Myoglobin was used to calibrate the spectrometer.

High pressure liquid chromatography was performed using a Shimadzu Series LC-10A system under the control of Shimadzu CLASS LC-10 software. The solvents used were distilled, deionised (Millipore Milli-Q Reagent Grade Water System) water and HPLC grade methanol and acetonitrile. All solvents were filtered (Millipore, 0.22 mm) and degassed immediately prior to use. Analytical HPLC was carried out using a 3.9×300 mm Waters μ-Bondapak C-18 column. A gradient of aqueous acetonitrile with trifluoroacetic acid (0.1%) as modifier was used at a flow rate of 1 ml min^{-1}. The gradient used was 0 ~ 30% for **3** or 0 ~ 70% MeCN (over 15 minutes) for **4** ~ **6**, then isocratic MeCN (30 or 70%, 15 minutes). The wavelengths monitored were 254 and 280 nm.

N-Me-D-Ala-vancomycin **2**

N-*t*-Boc-*N*-Me-D-Ala (56.9 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (1 ml) and cooled to 0°C. To this was added DCC (28.9 mg, 0.14 mmol) in CH_2Cl_2 (1 ml) and the solution was stirred with cooling for 30 minutes and at room temperature for 30 minutes. After this time, a white precipitate which had formed was removed by filtration and discarded. A white solid was obtained from the supernatant after removal of the solvent under reduced pressure, and this was dissolved in DMF (2 ml) at 0°C. The solution was added to a mixture of vancomycin hexapeptide (100 mg, 0.07 mmol) and sodium hydrogen carbonate (25 mg, 0.3 mmol) in DMF (2 ml), which had been cooled in an ice bath. The mixture was stirred at 0°C for 2 hours, and then a few drops of conc ammonia solution were added, and the stirring was continued for 10 minutes. The mixture was evaporated under vacuum to dryness. A 1 : 1 solution of TFA in CH_2Cl_2 (2 ml) was added and then removed under reduced pressure after 5 minutes. The resulting material was purified by HPLC, to give **2** (64 mg, 60% yield). ESMS: *m/z* 704.4 (calcd for $(MH_2)^{2+}$ $C_{63}H_{70}Cl_2N_9O_{24}$, 704.1).

N-*t*-Boc-*N*-Me-Gly-vancomycin

N-*t*-Boc-*N*-Me-Gly (143 mg, 0.76 mmol) was dissolved in dichloromethane (3 ml). To this was added DCC (78 mg, 0.38 mmol) as a solution in dichloromethane (3 ml). After 1 hour at room temperature, the mixture was filtered to remove a white precipitate, which was discarded. A white solid was obtained from the supernatant after removal of the solvent under reduced pressure, and this was dissolved in DMF (5 ml). Vancomycin hexapeptide (57 mg, 0.04 mmol) and $NaHCO_3$ (anhydrous, 30 mg, 0.36 mmol) were added to this, and the reaction monitored by HPLC. After 2 hours, a single peak with longer retention time than the starting material was seen, and reaction was judged to be complete. The solvent was removed under reduced

pressure, and aqueous ammonia (pH 11.0, 10 ml) was added to the resultant white solid. After reaction at 311 K for 1 hour, the solvent was removed under reduced pressure to give a pale brown solid (188 mg). Preparative HPLC of a portion of this gave *N-t*-Boc-*N*-Me-Gly-vancomycin (64% yield overall). ESMS: *m/z* 1493.8 (calcd for $(MH)^+$ $C_{67}H_{75}Cl_2N_9O_{25}$, 1493.3).

N-Me-Gly-vancomycin **3**

N-t-Boc-*N*-Me-Gly-vancomycin (64 mg) was dissolved in aqueous trifluoroacetic acid (10%, 50 ml), and stirred at room temperature. The reaction was monitored by HPLC, and after 3 hours was judged to be complete. The solvent was removed under reduced pressure. Preparative HPLC of the resulting white solid gave **3** (42 mg, 66%). ESMS: *m/z* 697.3 (calcd for $(MH_2)^{2+}$ $C_{62}H_{67}Cl_2N_9O_{24}$, 696.6).

Preparation of Acylated Vancomycin Derivatives **4**~**6**, General Procedure

To a solution of vancomycin hexapeptide (100 mg, 0.067 mmol) in 2 ml of 20% DMF/MeOH containing pyridine (5 μl, 0.13 mmol) was added the neat anhydride (total amount added *ca.* 10 equiv, 0.61 mmol) in about five portions spaced over 3 days. The reaction was monitored periodically by reverse-phase HPLC using a 4.9 × 300 mm C_{18} column employing a gradient elution of 0~70% CH_3CN/H_2O containing 0.1% TFA as modifier over a 20 minutes period. Anhydride was added until the reaction was judged complete. The acylated products had slightly longer retention times than did the starting material. The reaction mixture was then treated with distilled H_2O (3 ml) and extracted with diethyl ether (2 ml × 3). The aqueous layer was evaporated to dryness under vacuum. The resulting white solid was purified by column chromatography over reverse phase silica gel. The crude product was dissolved in 1~2 ml of H_2O-0.1% TFA and applied to a 15 cm bed of Whatman ODS reverse phase silica. A gradient elution was employed starting with 100 ml of H_2O-0.1% TFA, then 100 ml of 3% CH_3CN-H_2O-0.1% TFA and gradually up to 25% CH_3CN-H_2O-0.1% TFA maintaining a flow rate of 5 cm of solvent per minute. The fractions (20 ml) were analysed by HPLC and the appropriate fractions containing the product were pooled and evaporated to provide the acylated hexapeptides **4**~**6** in 60~70% yield. HPLC analysis indicated that the products were at least 98% pure. Electrospray mass spectrometry gave the expected molecular ion; **4** ESMS: *m/z* 1421.4 (calcd for $(MH_1)^{1+}$ $C_{65}H_{73}Cl_2N_8O_{24}$, 1421.2), **5** ESMS: *m/z* 1392.1 (calcd for $(MH_1)^{1+}$ $C_{63}H_{68}Cl_2N_8O_{24}$, 1392.2, **6** ESMS: *m/z* 1365.3 (calcd for $(MH_1)^{1+}$ $C_{61}H_{64}Cl_2N_8O_{24}$, 1365.2). COSY and NOESY experiments on the antibiotic derivatives were performed in DMSO-d_6, D_2O, and 9:1 H_2O-D_2O solutions and allowed nearly complete assignment of the proton spin systems (data not shown).

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