Subtle differences in molecular recognition between modified glycopeptide antibiotics and bacterial receptor peptides identified by electrospray ionization mass spectrometry

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In determining structure–activity relationships, it is advantageous if binding constants for a variety of ligands to a given target molecule can be directly obtained from a single aqueous solution containing a mixture of ligands and the target molecule. In this paper further evidence is provided showing that electrospray ionization mass spectrometry (ESI-MS) can be used in the rapid quantitative analysis of mixtures of vancomycin-group antibiotics and their bacterial cell-wall receptors allowing the identification of even subtle differences in binding constants. Differences in affinities are quantified for a mixture of vancomycin antibiotics (vancomycin, dechlorovancomycin and *N*-demethylvancomycin) and for a mixture of ristocetin A and its pseudoaglycone. Binding constants determined by ESI-MS were found to be in close agreement with those determined by more direct methods in aqueous solution.

Introduction

In recent years, there has been much interest in the potential of electrospray ionization mass spectrometry (ESI-MS) for the measurement of association constants for non-covalent interactions that are of importance in biological systems (*e.g.*, protein–ligand interactions).[1–3] When a solution containing a mixture of A and B, corresponding to the molecular association A+B→A·B, is introduced into a mass spectrometer using electrospray ionization the spectrum may show ion signals not only due to A and B but also to A·B. The crucial question is whether the relative abundance of these three ion signals can be used to derive an association constant which is a reflection of the binding occurring in solution. There has already been considerable debate as to which conditions (if any) should be applied for ESI-MS that will allow the determination of binding constants that are reliable. For example, it has been shown that in the binding of acyl-coenzyme A (acyl CoA) compounds to acyl CoA binding protein (ACBP), ESI-MS spectra showed similar proportions of acyl CoA–ACBP complexes due to acyl CoA ligands containing C_8, C_{12}, and C_{16} acyl chains; this is despite the measurement of solution binding affinities which increase from $5 \pm 2.5 \times 10^6$ M^{-1} to $>10^{10}$ M^{-1} as the acyl chain is increased from C_8 to C_{16}.[4] In contrast, in the binding of acyl CoAs to several mutant ACBPs (in which tyrosine residues of the wild type, which are crucial for high affinity binding of acyl CoAs, are mutated to other residues), the ESI-MS data clearly reflected the reduced stability of the mutant complexes and, in two out of three cases, with a reasonably good quantitative reflection of the binding affinities determined in solution.[4]

There is a variety of data in the literature, which variously indicate the promise of ESI-MS to evaluate solution binding constants,[5–15] or the caution with which the data should be treated.[16–18] In the present paper, we further illustrate that for the here studied antibiotic–receptor interactions, ESI-MS data can, when determined under appropriate experimental conditions, as previously discussed in detail,[19] predict solution binding constants where the effects are both subtle and unexpected from previous studies.

The clinically important group of glycopeptide antibiotics, such as vancomycin (Fig. 1A), act by binding to bacterial cell-wall precursors terminating in the sequence –Lys-D-Ala-D-Ala. This interaction inhibits cross-linking of the growing cell wall, leading to cell death.[20,21] Binding constants of these antibiotics with a variety of peptide cell-wall precursor analogs (*e.g.*, *N*,*N*′-diAc-Lys-D-Ala-D-Ala, *N*-Ac-D-Ala-D-Ala, see Fig. 1B) have previously been determined using various, quite laborious, methods including ^{1}H NMR spectroscopy, UV difference spectrophotometry, microcalorimetry, and capillary electrophoresis.[22]

Results and discussion

Previously, we and others have demonstrated that given a careful choice of solution and interface conditions, ESI mass spectra can be obtained which reflect the structure-specific interactions of vancomycin and *N*-Ac-D-Ala-D-Ala in aqueous solution.[19] In the present work, we show that for a number of mixtures of glycopeptide antibiotics and bacterial cell-wall precursor analogs, determination of binding constants by ESI mass spectrometry is sufficiently reliable to predict subtle and unexpected effects.

The interaction of vancomycin with its bacterial receptor is based on a complex combination of different interactions including hydrogen bonds and hydrophobic interactions. Therefore, minor changes in the chemical structure of the antibiotic

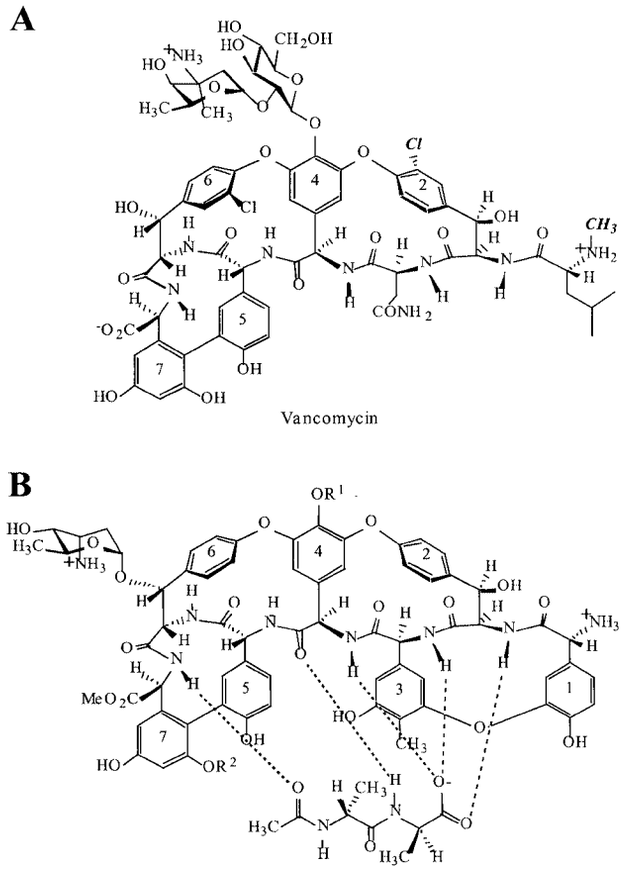

Fig. 1 A) Structure of the glycopeptide antibiotics vancomycin, dechloro- and *N*-demethyl-vancomycin. In dechloro- and demethylvancomycin a single Cl and a single CH_3 group (given in bold italics) have been replaced by a H. B) Exploded view of the binding of *N*-Ac-D-Ala-D-Ala to ristocetin A and ristocetin-Ψ. Dashed lines indicate hydrogen bonds. In ristocetin A R^1 = tetrasaccharide and R^2 = mannose, in ristocetin-Ψ $R^1 = R^2 = H$.

or its receptor may have considerable effect on the molecular recognition between the antibiotic and its receptor. To investigate whether ESI-MS can be used to monitor the effect of minor modifications on the antibiotics' affinity towards the receptor we investigated a mixture of the antibiotics vancomycin, dechlorovancomycin and *N*-demethylvancomycin. Their chemical structures are given in Fig. 1. *N*-Demethylvancomycin (also known as A51568A), isolated from *Amycolatopsis orientalis*, has been clinically used in China since 1967, and differs from vancomycin only in that the methyl group on the amino group of the N-terminal residue of vancomycin has been replaced by H (see Fig. 1). Some of the effects of these particular modifications have been reported previously,[23,24] but never in a single measurement from one solution. The upper spectrum in Fig. 2 displays the positive ion ESI mass spectrum of an equimolar mixture (15 μM) of vancomycin, dechlorovancomycin and *N*-demethylvancomycin, the lower spectrum was obtained after mixing in 25 μM of the receptor mimicking peptide *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala. Running the mass spectrometer in the positive ion mode the "free" antibiotics are (almost exclusively) detected as doubly protonated ions at mass to charge ratios (*m*/*z*) of approximately 706, 717 and 724 for dechlorovancomycin, *N*-demethylvancomycin and vancomycin, respectively. The signals appearing at *m*/*z* = 892, 903 and 910 after mixing in *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala originate from the doubly protonated ions of the noncovalent complexes of the latter three antibiotics with the receptor mimicking peptide. Assuming that these spectra reflect directly the affinity of the antibiotics towards the *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala receptor it may be extracted from the spectra shown in Fig. 2 that the order of affinity for the receptor is *N*-demethylvancomycin > vancomycin

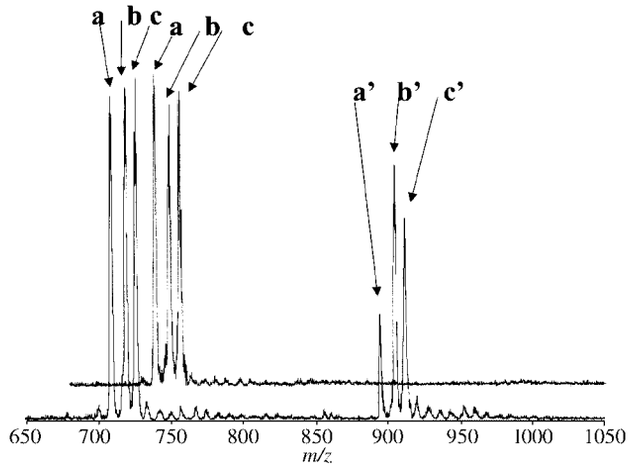

Fig. 2 Nanoflow ESI mass spectrum of an equimolar mixture (15 μM) of vancomycin, dechloro- and *N*-demethyl-vancomycin with (lower spectrum) and without (upper spectrum) the peptide (25 μM) *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala. The ion signals appearing at **a**, **b** and **c**, originate from doubly protonated ions of dechlorovancomycin, *N*-demethylvancomycin and vancomycin, respectively. The ion signals appearing at **a**′, **b**′ and **c**′, originate from doubly protonated ions of the noncovalent complexes of *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala with dechlorovancomycin, *N*-demethylvancomycin and vancomycin, respectively. The minor signals observed at slightly higher *m*/*z* values originate predominantly from alkali metal (*e.g.* Na^+) cationised ions.

> dechlorovancomycin. As described previously,[19] if the ionization probabilities of the antibiotics and their noncovalent complexes are identical, the ion intensities (*i.e.*, the integrals of the signals) in these mass spectra can be used to calculate the solution phase equilibrium concentrations of the antibiotics, the antibiotic–peptide complexes and therefore also the free peptides. In short, the equilibrium concentrations of the antibiotics ([A1], [A2] and [A3]) can be derived from eqn. (1)

$$[A1] = [A1]_t\,(A1/(A1 + A1L)) \quad (1)$$

(square brackets denote concentrations), where A1 and A1L are the peak intensities of one antibiotic and its complex with the ligand, respectively. $[A1]_t$ is the total concentration of the antibiotic. The concentration of the complex between the antibiotic and the ligand is given by eqn. (2). The concentration of

$$[A1L] = [A1]_t - [A1] \quad (2)$$

the other antibiotics ([A2] and [A3]) and their complexes with the ligands ([A2L] and [A3L]) can be derived in an analogous manner. For the concentration of unbound ligand we have eqn. (3), where $[L]_t$ is the total concentration of the ligand. The

$$[L] = [L]_t - [A1L] - [A2L] - [A3L] \quad (3)$$

binding constant of the antibiotic to the ligand can now be calculated from eqn. (4). An analogous equation leads to the

$$K_{A1L} = [A1L]/([A1]\,[L]) \quad (4)$$

binding constants for the other antibiotics. Table 1 summarizes the binding constants determined by ESI-MS and for comparison literature values determined by more direct methods in solution (UV difference spectrophotometry). The binding constant of dechlorovancomycin ($K = 3.5 \times 10^5$ M^{-1}) towards *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala is approximately half that of vancomycin ($K = 7.3 \times 10^5$ M^{-1}), in agreement with literature data. The dechlorinated derivative has also about 70% reduced activity towards *B. subtilis*,[23] when compared to vancomycin. The present data do indicate that demethylation increases the affinity towards *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala slightly, but

Table 1 Binding constants for the tested vancomycin-type antibiotics with the cell-wall precursor analog *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala

Antibiotic	$K_{ass}/10^5$ M^{-1}	
	ESI-MS	UV difference
Vancomycin	7.3	15[23,24] 4.1,[28] 3.0[29]
N-Demethylvancomycin	9.0	58[24]
Dechlorovancomycin	3.5	5.9[23]

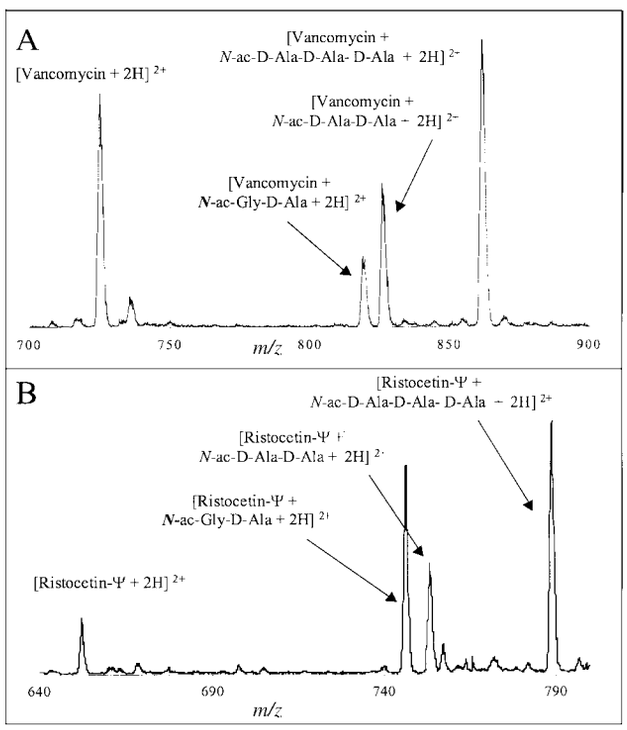

Fig. 3 A) ESI mass spectrum of an equimolar mixture (50 μM) of vancomycin with the three peptides *N*-Ac-D-Ala-D-Ala-D-Ala, *N*-Ac-D-Ala-D-Ala and *N*-Ac-Gly-D-Ala. B) As in Fig. 3A for ristocetin-Ψ.

significantly by a factor of approximately 25%. It has been previously shown that *N*-demethylvancomycin has a higher affinity for the bacterial cell-wall analogue *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala by UV difference spectrophotometry.[24] *N*-Demethylvancomycin was observed also to have more potent antibiotic activity against *Staphylococcus aureus* and other Gram-positive bacteria than vancomycin, typically by a factor 1.1–1.3,[24] again in good agreement with the present findings. In the literature a widespread range of association constants can be found for almost each of these association constants, *e.g.* Table 1, with absolute values differing by more than 100%. These large differences are probably due to the huge effect several experimental parameters, such as temperature, pH and solvent, have on the values of the absolute association constants. These parameters have to be carefully controlled during the different experiments. Using the present method, based on ESI-MS, the association constants of the different antibiotics are at least studied under exactly the same conditions from a single mixture. Therefore, we believe the present method is uniquely appropriate for the determination of the subtle effect chemical modifications may have on binding constants.

To evaluate the usage of this method for mixtures of antibiotics and bacterial receptor precursor peptides we further analyzed first a mixture of precursor peptides with one antibiotic. In Fig. 3A the positive ion ESI mass spectrum of an equimolar mixture (50 μM) of vancomycin with the three peptides *N*-Ac-D-Ala-D-Ala-D-Ala, *N*-Ac-D-Ala-D-Ala and

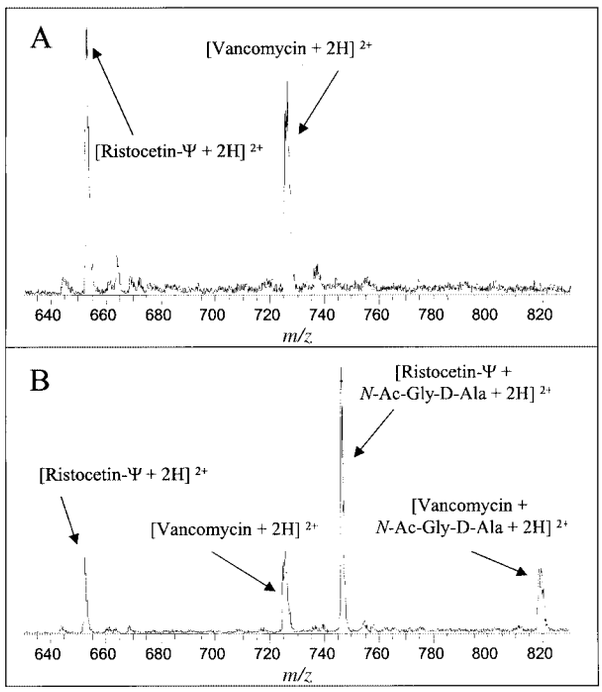

Fig. 4 A) ESI mass spectrum of an equimolar mixture (12.5 μM) of vancomycin and ristocetin-Ψ. B) ESI mass spectrum of an equimolar mixture (12.5 μM) of vancomycin and ristocetin-Ψ containing *N*-Ac-Gly-D-Ala (100 μM). Note that the signals for vancomycin and its complexes are broader due to the chlorine isotopes. Therefore, the integrated intensities of the signals in the spectra are quite different from their heights.

N-Ac-Gly-D-Ala is shown. In Fig. 3B is shown the ESI mass spectrum of a similar mixture of ristocetin pseudoaglycone (ristocetin-Ψ) with the same three peptides. Again these spectra show predominantly the doubly protonated ions of the antibiotic and its complexes with the peptides. In "reversed" experiments a mixture of antibiotics was analysed with a single receptor peptide. Fig. 4A displays the ESI mass spectrum of vancomycin (12.5 μM) and ristocetin-Ψ (12.5 μM). The spectrum in Fig. 4B was obtained from a solution containing these two antibiotics together with the ligand *N*-Ac-Gly-D-Ala (100 μM). Table 2 summarizes the binding constants determined from the spectra as shown in Fig. 3 and Fig. 4 by ESI-MS and, for comparison, binding data directly obtained in solution using UV difference spectrophotometry.

A key new observation in the present work was that association of ristocetin-Ψ to a cell-wall precursor terminating in –Gly-D-Ala was found by ESI mass spectrometry to be stronger than that occurring to one terminating in –D-Ala-D-Ala (see Fig. 3B and Table 2). This observation was contrary to the result expected on the basis of analogous binding to other glycopeptide antibiotics, such as ristocetin A and vancomycin (see Fig. 3A and Table 2). Subsequent UV difference spectrophotometry titration measurements confirmed, however, that the relative order of the binding constants determined by ESI-MS results were correct. In the complex between ristocetin A and the cell-wall precursor analog *N*-Ac-D-Ala-D-Ala, the methyl group of the N-terminal alanine residue of the ligand is situated near rings 5 and 7 of ristocetin A, forming hydrophobic interactions with this part of the antibiotic (Fig. 1). Removal of the *N*-terminal alanine methyl group (*N*-Ac-D-Ala-D-Ala→*N*-Ac-Gly-D-Ala) only results in a slight reduction of the binding energy (see Table 1), despite the loss of the hydrophobic contribution of the Ala methyl group.

It was proposed earlier[25] that the removal of the Ala methyl group enables the *m*-dihydroxylated benzene ring of residue 7 of the antibiotic to approach more closely to the π-face of the acetyl group (*i.e.*, the π orbitals of its carbonyl carbon and the

Table 2 Binding constants for several antibiotics with cell-wall precursor analogs *N*-Ac-D-Ala-D-Ala, *N*-Ac-Gly-D-Ala and *N*-Ac-D-Ala-D-Ala-D-Ala determined by several experimental methods. Numbers in bold were determined in the present work

		$K_{ass}/10^4$ M^{-1}		
Antibiotic	Ligand	ESI-MS	UV	Calorimetry
Ristocetin A	*N*-Ac-D-Ala-D-Ala	10.5[19]	11.2[30]	11.0[32]
			26.0[31]	
	N-Ac-Gly-D-Ala	3.8[19]	4.4[30]	9.5[32]
	N-Ac-D-Ala-D-Ala-D-Ala	14.5[19]	**16.5**	
Ristocetin-Ψ	*N*-Ac-D-Ala-D-Ala	**3.6**	**2.3**	
			3.3[33]	
	N-Ac-Gly-D-Ala	**7.6**[a]	**4.5**	
		4.6[b]		
	N-Ac-D-Ala-D-Ala-D-Ala	**11.5**	**8.2**	
Vancomycin	*N*-Ac-D-Ala-D-Ala	1.9[19]	2.0[34]	3.3[32]
			3.3[31]	
	N-Ac-Gly-D-Ala	1.3[a,19] **1.0**[b]	1.1[34]	
	N-Ac-D-Ala-D-Ala-D-Ala	5.1[19] **5.2**	5.0[34]	

[a] Determined from experiments as shown in Fig. 3. [b] Determined from experiments as shown in Fig. 4.

amide NH which is attached to this carbonyl group) of *N*-Ac-Gly-D-Ala. Thus, increased π-stacking interactions may partially offset the loss of hydrophobic interactions. We now propose that the surprising result that *N*-Ac-Gly-D-Ala binds more strongly to ristocetin-Ψ than *N*-Ac-D-Ala-D-Ala, may be explained by the lack of the mannose of residue 7 of ristocetin-Ψ. This mannose moiety can prevent the *N*-acetyl group of the ligand from forming optimal π-stacking interactions with ring 7 even after removal of the methyl group of the *N*-terminal Ala residue of the ligand. Therefore, in the ristocetin-Ψ–*N*-Ac-Gly-D-Ala complex (which lacks the mannose of ring 7), optimized π-stacking interactions may not only offset, but actually overcome the loss of hydrophobic interactions caused by removal of the Ala methyl group.

It is important to attempt to understand why specific solution interactions appear to have been quantitated in the present work, whereas in numerous other studies nonspecific interactions have clearly also been observed. For example, why are interactions of porcine elastase with both L- and D-enantiomers of an inhibitor of elastase observed in earlier reported ESI spectra,[16] whereas only the anticipated binding of *N*,*N*′-diAc-L-Lys-D-Ala-D-Ala (and not of *N*,*N*′-diAc-L-Lys-L-Ala-L-Ala) to vancomycin are observed in another study?[19] We are as yet unable to give a completely satisfactory answer to this question, but note that in obtaining the encouraging quantitations of the present, and earlier,[19] work it appears to have been important to (i) use nanoflow electrospray ionization, (ii) use a cone voltage just sufficient to desolvate the ions, but insufficient to cause collision-induced decomposition of the complex, (iii) use an (ammonium acetate) buffer solution. Although it is unlikely that these conditions will prevent the formation of spurious adducts in all cases, the present work does establish that they are sufficient in this particular case.

Conclusion

There are two crucial points with regard to the results. First, under carefully chosen conditions, the ESI-MS data are of sufficient reliability and precision to predict subtle variations in solution binding constants. Some of the present findings were *a priori* not anticipated, but were indeed confirmed from subsequent determinations of solution binding using more traditional, but also more laborious, methods. Second, and most importantly, that relative affinities of individual components in mixtures could be obtained rapidly from a single experiment. The results emphasize the potential of the method (for carefully chosen systems, and where the receptor–ligand complexes can be obtained as ions in the gas phase) in rapid screening as employed in the pharmaceutical industry.

Experimental

Antibiotics and peptides

Vancomycin hydrochloride and the vancomycin derivatives were kindly provided by Torben Koch from Dumex-Alpharma (Copenhagen, Denmark). Ristocetin A sulfate was kindly donated by Alpharma (Oslo, Norway) and Abbott Laboratories (Chicago, USA) and used without further purification. Ristocetin-Ψ was obtained by acid methanolysis from ristocetin A, as described previously.[26] Peptides were purchased from Sigma with free N-termini and acetylated with acetic anhydride.

Electrospray ionization mass spectrometry

ESI mass spectra were recorded on a Finnigan TSQ700 triple quadrupole mass spectrometer and/or a Thermoquest LC-Q ion trap. All samples were introduced using a nanoflow electrospray source (Protana, Odense, DK). Solutions were made up in aqueous 5 mM ammonium acetate, pH = 5.1, 298 K. The experimental procedures have been reported in more detail previously.[19] The procedure used to determine binding affinities directly from the observed signal intensities (integrated) in competition experiments as displayed in Figs. 2, 3 and 4 has also been described in detail earlier.[19] The experiments were typically repeated at different concentrations and the reported binding constants were averaged over at least 3 different measurements. The deviations found in the calculated binding constants in different experiments were less than 20%. As indicated before, the present method relies on the assumption that the ion intensities reflect directly the concentrations in solution of the antibiotic and its complexes with the peptides. That this assumption is valid, in the case of the present work can be, and was, confirmed by varying the initial concentrations of the compounds in the mixture.

UV difference spectrophotometry

UV difference spectrophotometry was carried out on a UVIKON 940 dual beam spectrophotometer. Both the reference and sample cells contained 50 μM antibiotic in 0.1 M phosphate buffer (pH 4.5, 300 K). Aliquots of a ligand solution containing 50 μM antibiotic were added to the sample cell. The solution was stirred after each addition, and the absorbance at both *ca.* 245 nm and *ca.* 285 nm was measured repeatedly until stable. The data at *ca.* 245 nm were subtracted from those at *ca.* 285 nm and analyzed as previously described.[27] Measurements were carried out in triplicate. The errors in the binding constants were estimated to be approximately ±15 000 M^{-1} in the case of ristocetin A and ristocetin-Ψ and ±5000 M^{-1} in the case of vancomycin.

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