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Vancomycin in vacuo

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Abstract

In this paper, we give an overview of mass spectrometry (MS)-based efforts over the last decade to characterize in detail the non-covalent interaction of vancomycin-group antibiotics with ligands that mimic the natural cell-wall receptor. We describe first direct methods to probe the strength of such interactions in solution by monitoring the ions of the free and complexed vancomycin by electrospray ionization-mass spectrometry (ESI-MS). We show that these methods can be used to monitor subtle differences in non-covalent interactions.

Additionally, we describe recent work on gas-phase studies on non-covalent complexes of vancomycin, including gas-phase hydrogen/deuterium (H/D) exchange, collisionally-induced dissociation and electron capture-induced dissociation (ECD).

Keywords: Vancomycin; Non-covalent interactions; Binding constants

1. Introduction

Glycopeptide antibiotics are complex molecules characterized by a macrocyclic peptide backbone with sugar moieties attached at various sites [1,2]. A wide array of glycopeptide antibiotics exists in nature. These glycopeptide antibiotics differ primarily in the amino acids and sugar moieties present. The precise chemical nature of the amino acids and number of sugar moieties is known to influence the efficacy of the antibiotics. Vancomycin (see Fig. 1A), the prototypical glycopeptide antibiotic, is of great clinical importance as it is currently the drug of last resort particularly against methicillin-resistant *Staphylococcus aureus* (MRSA).

From a vast amount of research, to which Dudley Williams made an essential contribution, it is by now well established that the antibiotic activity of vancomycin is first and foremost a direct result of non-covalent interactions between the drug and the bacterial cell-wall peptidoglycan precursor UDP-*N*-acetylmuramylpentapeptide. These interactions originate from a mixture of hydrogen bonds, electrostatic and hydrophobic interactions [3]. More specifically, vancomycin targets the D-alanyl-D-alanine terminus of the pentapeptide. Schematically, the interactions between ristocetin, a family member of the glycopeptide antibiotic family, and the D-alanyl-D-alanine peptide are shown in Fig. 1B, whereby some of the hydrogen bond interactions are indicated. The binding of vancomycin to the bacterial cell-wall peptidoglycan precursors inhibits transpeptidation which in turn results in bacterial cell death [4–7]. Interestingly, thus the primary recognition site of vancomycin is the "unnatural" –D-Ala-D-Ala sequence, whereas vancomycin does not show any significant interaction with the stereoisomeric –L-Ala-L-Ala moiety.

Over the years the vancomycin/D-Ala-D-Ala interaction has become a model system for investigating the nuances of receptor–ligand biomolecular recognition processes. Moreover, the vancomycin/D-Ala-D-Ala interaction has proven to be an ideal system to use during the development of novel analytical techniques for probing biomolecular interactions. In most of these experiments solely the D-Ala-D-Ala or L-Lys –D-Ala-D-Ala peptides feature as a mimic for the natural cell-wall peptidoglycan precursor UDP-*N*-acetylmuramylpentapeptide receptor. Although the

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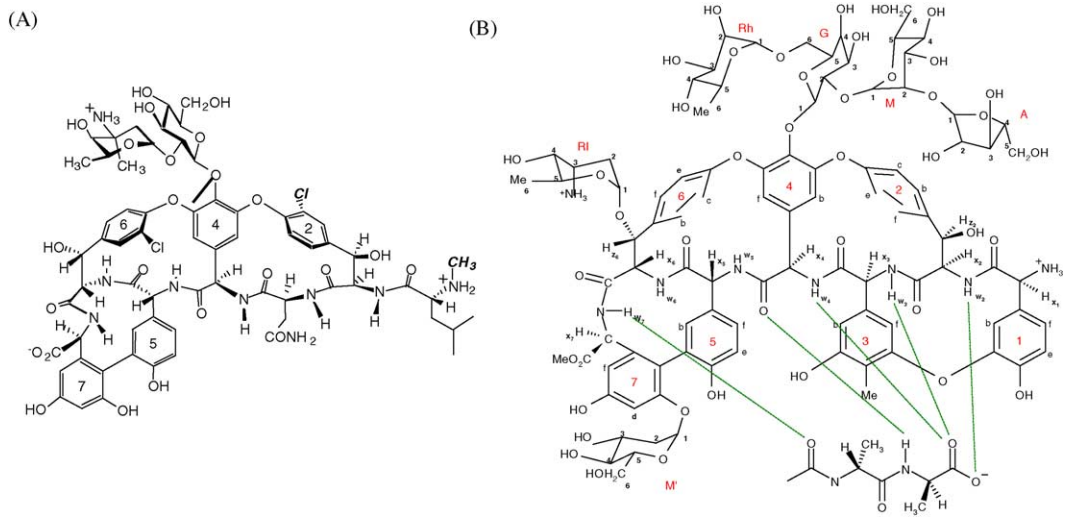

Fig. 1. Chemical structures of the antibiotics (A) vancomycin and (B) ristocetin. In B also the chemical structure of the acetylated –D-Ala-D-Ala peptide is shown, whereby some of the most relevant hydrogen bonds between the peptide and the antibiotic are indicated.

primary mode of action of vancomycin is well understood [1,2], important questions remain to be answered before a complete understanding of the subtleties of receptor–ligand interactions related to the antibacterial properties of the vancomycin system is achieved. The investigation of these molecular subtleties is important because bacterial resistance, also against vancomycin, is growing [8–10]. Derivatives of vancomycin are one means of developing new weapons against resistant bacteria.

The strength and structural features of the molecular interactions of glycopeptide antibiotics with cell-wall precursor analogues are generally studied by a variety of analytical techniques such as UV difference spectroscopy [5,6], nuclear magnetic resonance (NMR) [11,12], surface plasmon resonance (SPR) [12], affinity capillary electrophoresis (ACE) [13–16] and X-ray crystallography [17]. In recent years, bioaffinity mass spectrometry (MS) [18] has emerged as a relatively new technique to probe molecular interactions. The aim of the present paper is to review the development and applications of these bioaffinity mass spectrometry methods, with an emphasis on the application in the area of the glycopeptide antibiotics.

1.1. How Dudley Williams got us started

One of the earliest attempts to probe the interactions of vancomycin with bacterial cell-wall peptide analogues by mass spectrometry was, not surprisingly, performed in the group of Dudley Williams by amongst others Carol Robinson (maiden-name Bradley) and Gustav Bojesen [19]. Very soon after the introduction of fast atom bombardment [20], when molecules such as vancomycin were still considered large biomacromolecules, Williams et al. [19] recorded in 1981 FAB–MS spectra of vancomycin and its aglycon. Additionally, they probed the interaction of these antibiotics with peptides terminating in –D-Ala-D-Ala. Excitingly, the positive ion FAB mass spectrum of an equimolar mixture of aglucovancomycin and acetyl-D-Ala-D-Ala showed $[\text{aglucovancomycin}+H]^+$ and $[\text{aglucovancomycin}+\text{peptide}+H]^+$ ions in a ratio of 2: l; whereas the corresponding ratio for an equimolar mixture of aglucovancomycin with the non-binding acetyl-L-Ala-L-Ala peptide were 8: l, suggesting stronger binding of the antibiotic ions to the D-form of the peptide. Being careful experimentalists, the experiments were repeated with: (i) a matrix containing aglucovancomycin/CH_3CO-D-Ala-D-Ala/CD_3CO-L-Ala-L-Ala in equimolar ratios. In these experiments, the antibiotics showed adduct ions of very similar abundances with both CH_3CO-D-Ala-D-Ala and CD_3CO-L-Ala-L-Ala, and it was concluded that FAB–MS was not an appropriate method to probe the selective binding of the stereochemically different peptides.

Approximately 15 years later in 1996, Williams gave an RSC lecture in the Chemistry Department of Warwick University that aroused much enthusiasm. For one of us (AJRH), it became really the starting point to endeavor into the “vancomycin-world”. Of course at that time electrospray ionization-mass spectrometry (ESI-MS) had been introduced, and already developed into a method that allowed the analysis of minute amounts of sample, leaving not only the analytes intact, but also being able to preserve weaker non-covalent complexes through the ionization process making them amendable for mass spectrometric analysis [21,22]. Particularly this latter aspect of electrospray ionization intrigued us and the well-studied vancomycin system seemed to be an ideal model system to explore the possibilities and limitations for using ESI-MS to probe non-covalent interactions qualitatively and quantitatively. Shortly, hereafter TJDJ arrived in Warwick for a “summer-studentship” as part of his Ph.D. program, under supervision of Peter Roepstorff and Gustav Bojesen (the latter co-author on the aforementioned FAB–MS paper). To both our surprise, it

turned out we were working on the similar subject, i.e., probing the interactions of vancomycin-group antibiotics with bacterial cell-wall peptide analogues by electrospray ionization mass spectrometry, for TJDJ a logical subject because of the link with Bojesen. From that time on, we started to collaborate on this subject and set out to carefully optimize and validate the non-covalent bio-affinity mass spectrometry methods.

2. Solution-phase studies of vancomycin–peptide complexes

2.1. Determination of solution binding constants of glycopeptide antibiotics with bacterial cell-wall peptide analogues by electrospray ionization mass spectrometry

Traditionally, the interactions of vancomycin with bacterial cell-wall peptide analogues have been quantitatively probed, as most biological interactions, by titration experiments. In these experiments, often one of the interaction partners concentration is kept constant, whereby the second entity is titrated in. The interaction between the two partners changes a physiochemical property of the system, which may then be concentration dependent monitored. A titration curve can be made, from which by using appropriate fitting procedures dissociation constants can be derived. Many different methods to probe interactions by this titration methodology exist, such as by probing differences in UV absorbance, circular dichroism absorbance, NMR chemical shifts, migration time as in affinity capillary electrophoresis, heat released as in microcalorimetry, and indeed all these methods have been used in probing the interactions of vancomycin with bacterial cell-wall peptide analogues [5,6,11–16,23]. The disadvantage of such methods is that they are often quite elaborate, and care must be taken to perform the series of titration experiments without changing any environmental factors, such as solution pH and temperature. Additionally, these methods whereby often just a single physicochemical parameter is monitored, are less sensitive to probe more complex interactions, leading for instance to cooperativity [24–29], multi-site binding or formation of multimer non-covalent complexes. Still, prior to our studies, also the initial non-covalent electrospray ionization mass spectrometry studies on the interactions of vancomycin with bacterial cell-wall peptide analogues [14,30] were performed in such a titration mode. In experiments by Lim et al. [30], the intensity of the ion signals of the vancomycin and the non-covalent complex between vancomycin and the diacetyl-L-Lys-D-Ala-D-Ala peptide were monitored as a function of the variable diacetyl-L-Lys-D-Ala-D-Ala concentration. Using Scatchard plots, they were able to determine dissociation constants that were in reasonable agreement with literature data, although their samples were dissolved in non-physiological water/acetonitrile mixtures.

As shown first by us, one of the real advantages of electrospray ionization mass spectrometry to probe non-covalent interactions is that in principle several dissociation constants can be measured in one single experiment [31]. When a solution containing A and B, which do interact, is electrosprayed under gentle desolvation and ionization conditions the spectrum shows ions of not only A and B, but also of AB. The crucial question is always whether the intensities of these ions can be related to the abundance of the corresponding species in solution. If that is the case ESI-MS can be used to determine the concentrations of these species in solution, and from these dissociation constants can be derived. However, the electrospray process relies on a phase-transfer from solution to the vacuum, and the ionization cross-section or response factor of species of different physico-chemical nature may be quite different [32]. In case of the measurements on the vancomycin–peptide ligand system, it may be evident that the nature and size of the relatively small di- or tri-peptides is quite different from that of the much larger vancomycin. Of course not only the size but also the hydrophobicity and polarity of the species play a large role in the ionization cross-sections. Besides the ionization, cross-section of the species other factors may influence the dependence of the magnitude of the ion signal measured, such as the mass to charge ratio of the ions, which may influence their transfer and detection efficiency inside the mass spectrometer. Therefore, a priori the assumption that ion abundances may be used to determine solution phase concentrations in a single experiment is far from self-evident. However, in case of the vancomycin–peptide ligand system, the assumption was only made that the response factor of the vancomcyin ion (Mw = 1447 Da), would be similar to that of the non-covalent vancomycin–peptide ligand complex (Mw ∼ 1700–1900 Da). In the initial experiments [31] with this direct method, it was shown that using this assumption solution-phase equilibrium concentrations of various complexes could be determined solely from the ratio of the ion peak intensity of the given complex relative to the summed peak intensities of all the complexes and the free antibiotic. As an example is shown in Fig. 2A part of the ESI mass spectrum of an equimolar mixture (50 μM) of vancomycin and the three peptide ligands, acetyl-D-alanyl-D-alanine-D-alanine, acetyl-D-alanyl-D-alanine, and acetyl-glycyl-D-alanine, in aqueous 5 mM ammonium acetate (pH 5.1). In this solution of vancomycin and the three peptide ligands, L1, L2, and L3, the equilibrium concentration of species V_i (V_i refers to all forms of the antibiotic) is given by,

$$[V_i] = \frac{V_i[V]_0}{V + VL1 + VL2 + VL3}$$

where V, VL1, VL2, and VL3 are the peak intensities of the antibiotic and its three complexes with the peptide ligands, respectively, the square brackets denote concentrations, and $[V]_0$ is the initial concentration of the antibiotic (or initial concentration of each of the peptides, since the mixtures are

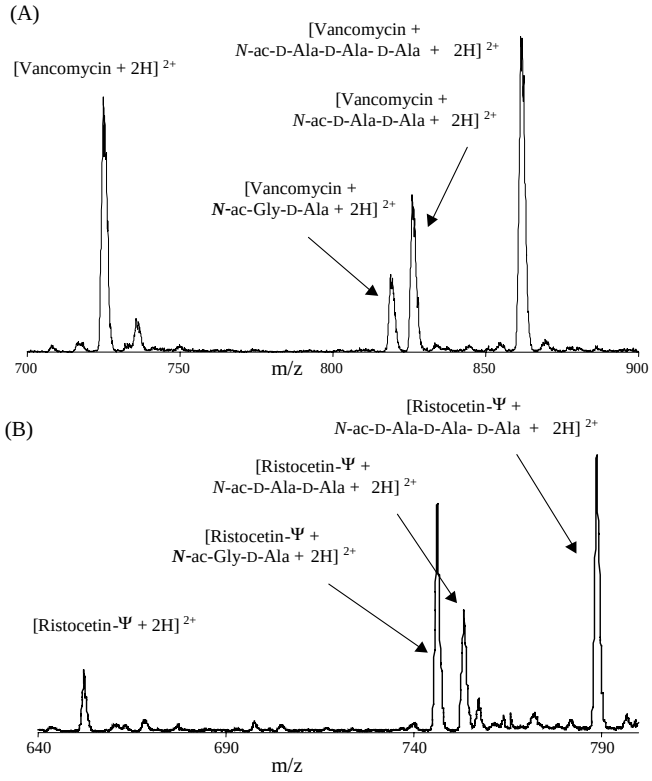

Fig. 2. ESI-MS spectra of an equimolar (50 μM) mixture of: (A) vancomycin with the three peptides Ac-Gly-D-Ala, Ac-D-Ala-D-Ala and Ac-D-Ala-D-Ala-D-Ala and (B) the aglucon, ristocetin-Y with the three peptides Ac-Gly-D-Ala, Ac-D-Ala-D-Ala and Ac-D-Ala-D-Ala-D-Ala. Note the reversed order of intensity of the non-covalent complexes with Ac-Gly-D-Ala and Ac-D-Ala-D-Ala in spectrums A and B. Reprinted with permission from reference [33].

equimolar). The above equation allows the determination of the solution equilibrium concentrations of the free peptide ligands (without measuring/using the ion signal of these peptides), the free antibiotic, and each of the three complexes. This calculation is simplified by virtue of working with an equimolar mixture, although this is not an absolute requirement. Solution binding constants can then be calculated by inserting the derived equilibrium concentrations into the expression

$$K_{VL1} = \frac{[VL1]}{[V][L1]} = \frac{[VL1]}{[V]([V] + [VL2] + [VL3])}$$

where [L1] is the equilibrium concentration of peptide ligand 1. As shown in Table 1, for a different mixture of three peptides, dissociation constants were determined at different equimolar concentrations of the species, and showed good agreement with each other and with known literature data obtained by alternative solution-based methods. To further validate this novel method, we determined the pH dependent behavior of the dissociation constants, which was consistent with that probed by circular dichroism, the effect of experimental parameters as cone voltage and used a stable isotope labeled –L-alanyl-L-alanine control peptide. From all these measurements it was concluded that this new method based on ESI-MS allowed to directly determine solution association constants for complexes between glycopeptide antibiotics (vancomycin and ristocetin) and several peptide ligands in one single experiment as the measured values were in good agreement with previously reported values obtained by standard titration techniques. An additional advantage of using this direct nano-electrospray bioaffinity method is that approximately only 10 pmol of sample was consumed to probe three dissociation constants in a matter of a few seconds.

In this initial report, we had probed only known dissociation constants. Of course, we were encouraged that our method was able to confirm known data, however, we had not learned anything new about the structure–function relationship of the vancomycin system. The first exciting novel finding we monitored was the remarkable behavior of the aglucon of the glycopeptide antibiotic ristocetin in binding to the two bacterial cell-wall peptide analogues acetyl-Gly-D-Ala and acetyl-D-Ala-D-Ala [33]. As it was believed that the interaction between the antibiotics and the peptide required preferentially a peptide terminating in D-Ala-D-Ala, we were surprised to find that association of the aglucon of ristocetin to the peptide terminating in –Gly-D-Ala seemed to be stronger than that to the peptide terminating in –D-Ala-D-Ala, as revealed by the relatively higher ion signal of the adduct with –Gly-D-Ala, when compared to D-Ala-D-Ala (See Fig. 2B), whereas both ristocetin

Table 1
Dissociation constants, K_d, calculated with equilibrium concentrations (conc.) deduced from relative ion abundances ($[M + 2H]^{2+}$ ions) for competitive binding experiments with vancomycin and three peptide ligands: diacetyl-L-Lys-D-Ala-D-Ala, acetyl-D-Ala-D-Ala, and acetyl-Gly-D-Ala at three different equimolar concentrations

	Equimolar concentrations (μM)								
	12.5			25.0			50.0		
	$(M)^{2+}$	Concentration (M)	K_d (M^{-1})	$(M)^{2+}$	Concentration (M)	K_d (M^{-1})	$(M)^{2+}$	Concentration (M)	K_d (M^{-1})
Vancomycin	32	2.8×10^{-6}		19	3.6×10^{-6}		12	4.7×10^{-6}	
V + KAA	100	8.6×10^{-6}	8.0×10^{5}	100	1.9×10^{-5}	8.6×10^{5}	100	3.9×10^{-5}	7.8×10^{5}
V + AA	7.3	6.3×10^{-7}	1.9×10^{4}	7.4	1.4×10^{-6}	1.6×10^{4}	8.5	3.3×10^{-6}	1.5×10^{4}
V + GA	5.5	4.7×10^{-7}	1.4×10^{4}	5.2	9.9×10^{-7}	1.1×10^{4}	6.8	2.7×10^{-6}	1.2×10^{4}

and vancomycin displayed the expected preference of binding to the –D-Ala-D-Ala terminating peptide (see Fig. 2A). With this data in hand, we approached, and were able to trigger the interest of Dudley Williams, who subsequently not only experimentally confirmed our unexpected data (by performing UV difference spectroscopy titration experiments) but also did come with a proper physicochemical explanation for the observed phenomena based on differences in subtle π-stacking interactions [33] between ristocetin and its aglucon.

The vancomycin molecule is chemically very intriguing, and very difficult to synthesize, although this has been done [34]. The synthesis of vancomycin is difficult largely due to the complex nature of the molecule, being an unusual cyclic peptide, with various sugar moieties attached, and having a wide range of stereo-centers and (e.g., halogen and methyl) substituents. It seems that every group or even atom plays an role in the molecular interactions that lead to bacterial cell-wall peptide analogue recognition [35]. As already mentioned one of the main advantages of the mass spectrometric approach is that several binding interactions may be monitored in a multiplex manner, allowing the analysis of subtle effects, as described already above in the competitive measurements with the –Gly-D-Ala and –D-Ala-D-Ala terminating peptides. Although competition experiments may be done by using more than one peptide ligand, they can also be performed with a series of analogue antibiotics with just one bacterial cell-wall peptide analogue. An example of this approach has been where equimolar ratios of vancomycin, *N*-demethylvancomycin and deschlorovancomycin were mixed with the peptide diacetyl-L-Lys-D-Ala-D-Ala. From the abundance of the non-covalent complexes of the three antibiotics with the peptide, it could be derived that the substitution of just a single chlorine by a hydrogen atom reduces the affinity between the peptide and the anitibiotic by a factor two (which may be explained by an increased flexibility in the peptide backbone) [36], whereas the mutation of the N-terminal methyl group into a hydrogen leads to an increase in affinity of 25%. Interestingly, this *N*-demethylvancomycin is also a natural product, isolated from *Amycolatopsis orientalis*, and has been clinically used in China since the 1960s. These single mutations are not directly involved in the binding pocket, showing the subtle interconnections between different chemical moieties in the antibiotic. Similar observations were made by ESI-MS for the to vancomycin-related avoparcin, a recently banned antibacterial growth-promoting food-additive, whereby subtle differences were detected between the affinities of α- and β-avoparcin for the bacterial cell-wall analogue peptides, whereby the α- and β-form only differ by a Cl to H mutation in the phenyl-ring of the third amino acid [37]. More dramatic changes were observed when vancomycin was allowed to react to formaldehyde or acetealdehyde. These reactions, which may mimic reactions in the human body following alcohol abuse, were shown to lead to a N-terminal

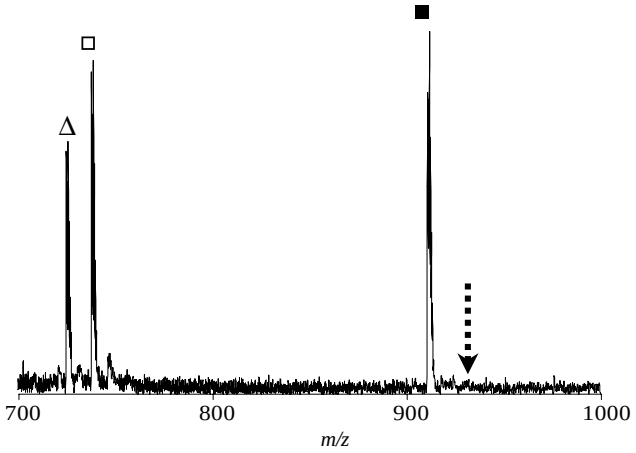

Fig. 3. ESI-MS spectra of a 20 μM solution of vancomycin, partly reacted with acetaldehyde for 10 h at room temperature, to which 25 μM *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala had been added immediately prior to mass analysis. The mass spectrum shows doubly protonated ions originating from vancomycin ((Δ) $m/z = 725$ Th), the M^+ 26 Da adduct of vancomycin ((▭) $m/z = 737$ Th) and of the non-covalent complex of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala ((■) $m/z = 910$ Th). If binding would have occurred a signal would be observed of the non-covalent complex of the M^+ 26 Da adduct of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala at the *m*/*z* value of the dashed arrow. Reprinted with permission from reference [38].

ring closure reaction, effectively blocking the interaction site of the antibiotic, which was verified by competetive bioaffinity mass spectrometry measurements using vancomycin and chemically modified ring-closed vancomycin [38]. These experiments revealed that the modifications resulted in a complete deactivation of the antibiotics towards binding to their natural cell-wall mimicking peptide analogues, as illustrated by the mass spectrum shown in Fig. 3. Additionally, vancomycin is prone to degradation into a product, which has been termed CDP-1, which may be formed via thermal degradation, and is caused by the chemically minor rearrangement of the third residue from aspartic acid into iso-aspartic acid. The bio-affinity method revealed that the CDP-I product does not bind at all to the common cell-wall peptide analogues [39], in agreement with the fact that it is biologically inactive as antibiotic [40].

Of special interest is the role of the sugar moieties in the glycopeptide antibiotics. Although the sugar moieties seem generally to be quite distant from the binding pocket, they do have a significant effect not only on the binding energies towards the cell-wall peptide analogues, but also towards biological activity as measured by minimum inhibitory concentrations (MICs) for bacterial growth. In a comparative study, where MICs and K_ds were determined (the latter by ESI-MS) it was reported that the inhibitory concentration for the aglucon form of vancomycin was four times higher than that of vancomcyin, in agreement with a very significant decrease of the dissociation constant found for the aglucon form [39].

2.2. Dimerization of glycopeptide antibiotics

Formation of dimers is another factor that has been suggested to affect the efficacy of glycopeptide antibiotics. The ability to dimerize varies greatly among glycopeptide antibiotics. For example, eremomycin, an antibiotic structurally related to vancomycin, has a high dimerization constant in the μM range, whereas teicoplanin does not dimerize at all. In some cases, dimerization is cooperative with ligand binding thus enhancing antibacterial activity [12,41], although anti-cooperative effects have been observed as well [25,42]. Specifically, a dimer can bind on a bacterial cell surface by initial attachment (of one-half of the dimer) to a mucopeptide precursor. The binding of a second mucopeptide precursor into the other half of the dimer is then effectively an intramolecular event, and this may be enhanced because of the chelate effect. As vancomycin group antibiotics do often form dimers it may also be apparent that two structurally-related glycopeptide antibiotics might form heterodimers. The analysis of NMR spectra to investigate this phenomenon is a complex task, due to the very large numbers of proton resonances involved, and also other techniques such as UV difference spectrophotometry and microcalorimetry lack the specificity to enable the unambiguous detection of heterodimers. However, since mass spectrometry gives a direct measure of the masses of the species involved, it has an unique potential in an examination of this phenomenon. Using bio-affinity electrospray ionization mass spectrometry, we evaluated several mixtures of glycopeptide antibiotics to investigate whether they would interact and form specific heterodimers [43,44]. Fig. 4 shows the result of one such experiment. Two antibiotics that do not significantly homo-dimerize (vancomycin and ristocetin) were mixed and analyzed by ESI-MS. The resulting spectrum displayed relatively intense ion signals of the hetero-dimer (no-homo-dimer signals). Using a similar approach as described above for the interactions of the peptides which the antibiotics, we were able to determine homo- and hetero-dimerization constants of several glycopeptide antibiotics by ESI-MS. Interestingly, the dimerization constants for the several hetero-dimers measured by ESI-MS were significantly higher than the geometric mean of the equilibrium constants of the homodimers (as clearly evident from the data shown in Fig. 4). Much more laborious and complex experiments by proton NMR, done in collaboration with the group of Dudley Williams, were used to successfully validate the ESI-MS bioaffinity data. In order to find out whether the formation of these relatively strong heterodimers would lead to an improvement in the biological activity of glycopeptide antibiotics, one-to-one mixtures of antibiotics were tested against a vancomycin susceptible and vancomycin-resistant strains of *Enterococcus faecium*, however, no significant increase in activity of the mixture relative to the more active antibiotic (of the

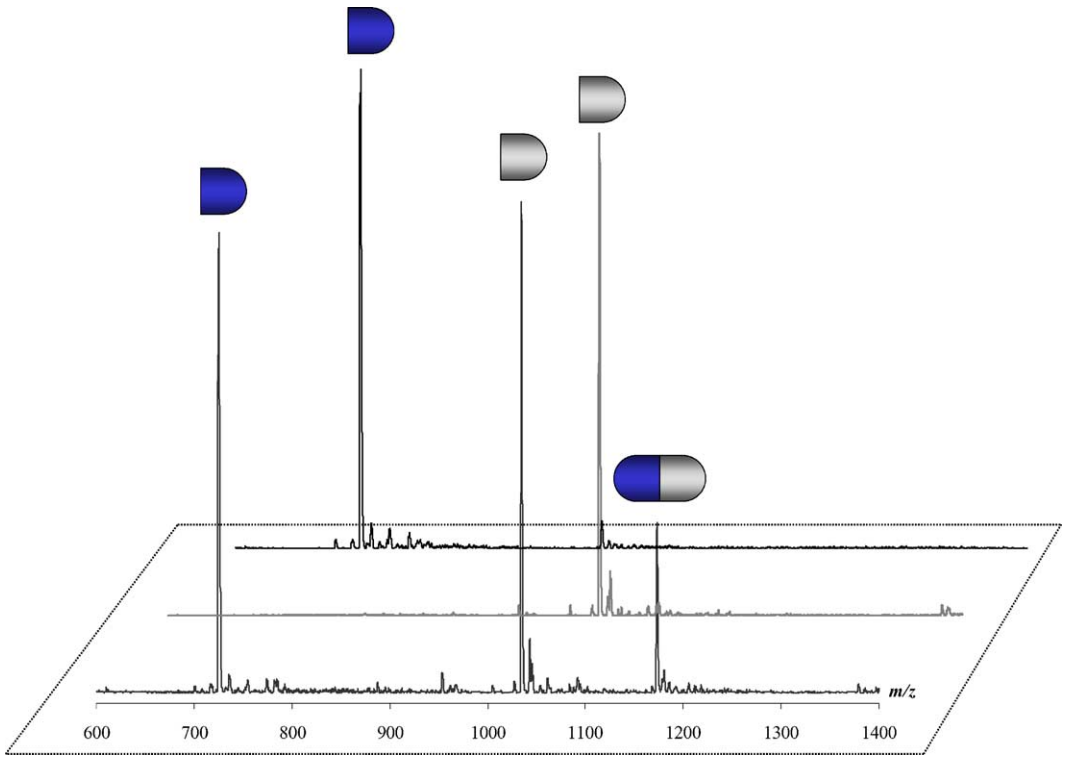

Fig. 4. Electrospray ionization mass spectra obtained from solutions containing from top to bottom vancomycin, ristocetin, and an equimolar mixture of the two (22 μM). The doubly protonated vancomycin and ristocetin can be observed at *m*/*z* 725 and 1034, respectively. The triply charged hetero-dimer ion is observed at *m*/*z* 1172. No significant ion signals of the homo-dimers of vancomycin or ristocetin are detected. Reprinted with permission from reference [44].

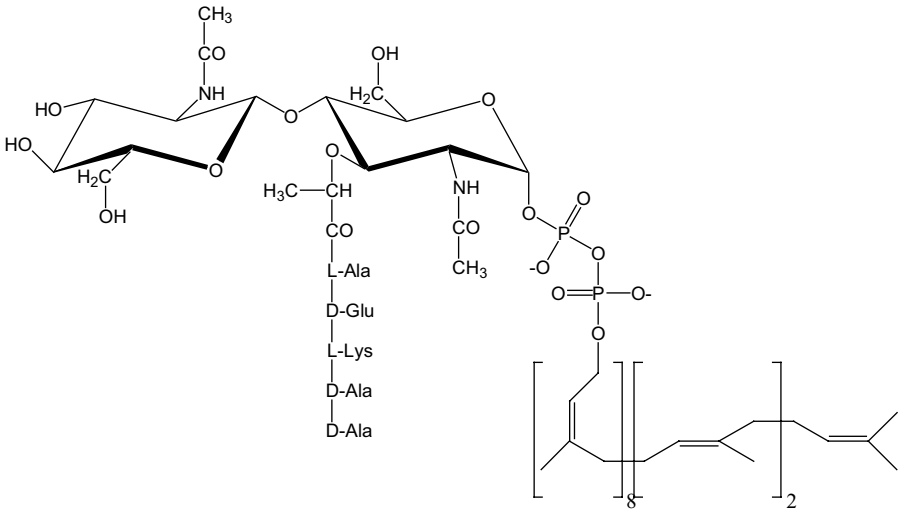

Fig. 5. Chemical structure of Lipid II. The synthesized water-soluble form of Lipid II, used in the bio-affinity measurements, contains 3 isoprene units in the tail as opposed to the 11 in the natural form.

mixed pair) alone could be observed, questioning whether dimerization really plays a role in vivo.

2.3. Interactions between glycopeptide antibiotics and Lipid II

Evidently, rather than using the D-Ala-D-Ala peptide models for the bacterial cell-wall, one would like to study the interactions of glycopeptide antibiotics with intact Lipid II (see Fig. 5). Unfortunately, intact Lipid II is insoluble in water and therefore not amenable to many of the above mentioned approaches to probe molecular interactions, including analysis by bioaffinity ESI-MS. Recently, we were able to synthesize a water-soluble variant of Lipid II [45], which allowed a more comprehensive characterization of the molecular interactions that underlie glycopeptide antibiotic efficacy [39]. Using mass spectrometry, we investigated the non-covalent complexes formed between the glycopeptide antibiotics and the soluble Lipid II, and compared them with those formed with the traditional diacetyl-L-Lys-D-Ala-D-Ala model peptides. In a direct comparison, as illustrated in Fig. 6, of the interactions of vancomycin and ristocetin with the two different ligands, we found that both antibiotics show a very similar affinity towards the diacetyl-L-Lys-D-Ala-D-Ala peptide. However, when the same antibiotics were evaluated against the soluble Lipid II molecule, it was observed that vancomycin binds much stronger than ristocetin, potentially indicating that this new ligand may be a better probe. Additionally, we were able to show that binding to the diacetyl-L-Lys-D-Ala-D-Ala peptide may induce cooperative dimerization of a synthetically produced vancomycin-dimer. However, similar experiments with Lipid II as interacting moiety revealed no further oligomerization, even not at high concentration of Lipid II [39]. These results show that the cooperative effect is ligand dependent. As the Lipid II ligand is a more natural ligand, these results further question whether dimerization may play a role in in vivo efficacy.

3. Gas-phase studies of vancomycin–peptide complexes

It has often been suggested that non-covalent biomolecular complexes generated by electrospray ionization partially retain their solution structure [46–48]. There exist, however, no direct experimental methods for determining three-dimensional (3D) structures of gaseous macromolecules at atomic resolution, so the question as to how closely the gas-phase structure resembles that in solution still remains largely unanswered. The present experimental methods for probing the structures of large biomolecular ions in the gas-phase are based on chemical reactivity, collision cross-section or fragmentation (CID, ECD or BIRD). The chemical reactivity studies are typically ion-molecule reactions such as proton transfer reactions and hydrogen/deuterium (H/D) exchange reactions. Collision cross-sections of protein ions are typically determined with ion mobility techniques. Most of the investigations on the higher order structure of large biomolecular ions in the gas-phase have been concerned with the different charge states of various proteins ions. The results of many experiments have shown that protein ions in many instances maintain a rather compact conformation in the absence of solvent and that protein ions exist in different conformations, which are distinguishable by different levels of hydrogen exchange and by different collision cross-sections [49–51]. Such observations have suggested that at least some aspects of the 3D solution structure of proteins are retained after transfer into the gas-phase. We have used the non-covalent complexes formed between vancomycin antibiotics and peptide ligands as a model system for the investigation of gas-phase structures of non-covalent com-

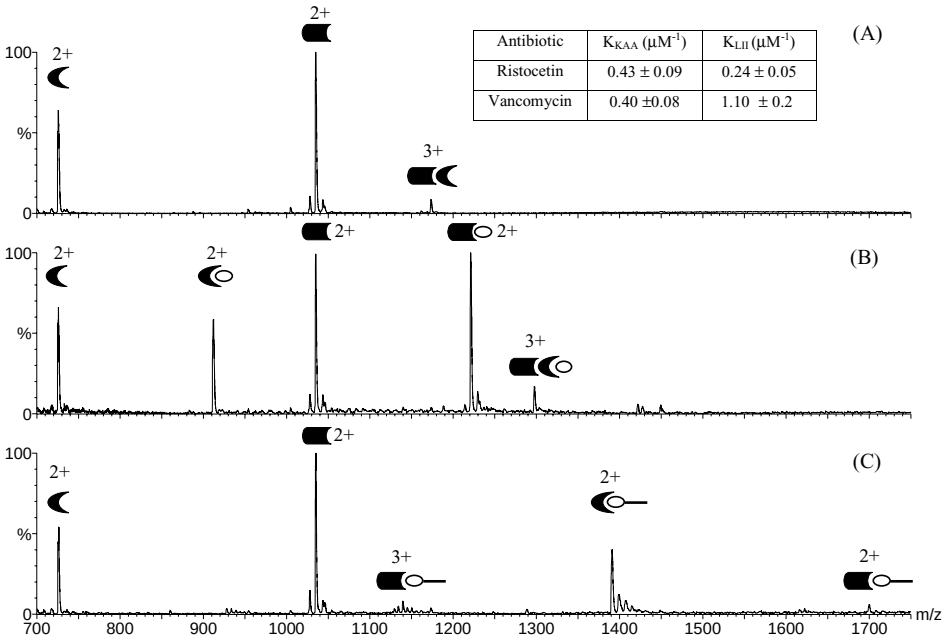

Fig. 6. ESI-MS of: (A) mixture of 10 μM V ( ) and Ris ( ). The doubly protonated V and Ris can be observed at *m*/*z* 725 and 1034, respectively. The triply protonated heterodimer formed between V and Ris ( ) can be found at *m*/*z* 1172. (B) Competition experiment between V and Ris after adding 16 μM KAA ( ). The doubly protonated complex of V with KAA ( ) and Ris with KAA ( ) can be found at *m*/*z* 910 and 1220, respectively. A triply protonated species belonging to the complex of the heterodimer with KAA can also be seen at *m*/*z* 1297 ( ). (C) Competition experiment between V and Ris after adding 8 μM LII ( ). The doubly protonated complex of V with LII ( ) and Ris with LII ( ) can be found at *m*/*z* 1390 and 1700, respectively. The triply protonated species of the complex of Ris with LII is also observed (*m*/*z* 1139). Additionally, the determined dissociation constants for these four different interactions are shown at the top of Fig. 6. Reprinted with permission from reference [39].

plexes. In particular, we have examined the relationship between known solution behavior and gas-phase stability, as measured by the ease of fragmentation of such complexes after collisional activation. Further, we have probed the gas-phase structures of the antibiotic–peptide complexes by using hydrogen/deuterium exchange in vacuo and by measuring destruction cross-sections for specific versus non-specific complexes.

3.1. Collision-induced dissociation of (1:1) antibiotic–peptide complexes

The structural feature of a peptide ligand which is most critical for binding to vancomycin in solution is the stereochemical configuration of the two C-terminal L-Ala residues [6]. If any of these two residues are substituted by L-Ala the binding affinity is dramatically decreased. This stereoselective binding is the result of a high degree of complementarity in the interface between the van der Waals surfaces of the interacting molecules which also provides an optimal geometry for five intermolecular hydrogen bonds (Fig. 1) If the antibiotic–peptide complexes retain their structure from solution to gas-phase then we would expect peptides with the optimal configuration (i.e., –D-Ala-D-Ala) to bind more strongly to vancomycin antibiotics in the gas-phase than peptides with the wrong stereochemistry for binding (e.g., –L-Ala-L-Ala). Such a difference in binding energy would be reflected in their ease of dissociation upon collisional activation in tandem mass spectrometry experiments. In positive ion mode, however, we did not detect any differences in the dissociation thresholds between (1:1) vancomycin complexes with either –D-Ala-D-Ala or –L-Ala-L-Ala peptides [52,53], indicating that the specific interactions known from solution were absent in the cationic gas-phase complexes. In contrast, the dissociation thresholds for the corresponding anionic vancomycin–peptide complexes depended strongly on the stereochemical configuration of the peptides, in a way that paralleled known solution binding affinity (Fig. 7) [52]. This remarkable difference in gas-phase reactivity between cationic and anionic complexes was explained by a protonation of the ligand's carboxylate anion that occurs in the cationic complexes but not in the anionic complexes. In solution, the vancomycin–peptide complex is zwitterionic having two anionic C-termini and two cationic ammonium ions (i.e., net charge is zero). To generate a gaseous doubly protonated ion (with a net charge of +2) two protons must be added to this structure. The most likely protonation sites are the anionic C-termini. Protonation converts them into neutral carboxyl groups and disrupts the three ionic hydrogen bonds that are otherwise formed between the anionic C-terminus of the ligand and amide hydrogens in the back-bone of vancomycin. Thus, the intermolecular interactions in the protonated gaseous complexes are likely very different than those known from solution. In contrast, in the anionic complexes the specific intermolecular hydrogen bonds are preserved upon the phase transition from

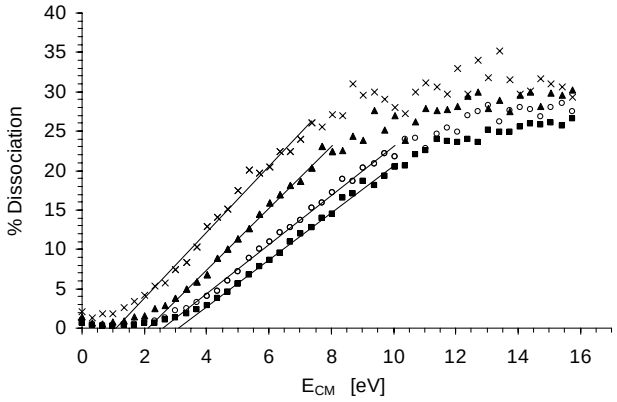

Fig. 7. Dissociation efficiency curves of four vancomycin–tripeptide complexes: [vancomycin+tripeptide–2H]$^{2-}$. The four isomeric complexes are: (closed square) [V+ac$_2$KAA(LDD)–2H]$^{2-}$ (open circle) [V+ac$_2$KAA(DDD)–2H]$^{2-}$ (closed triangle) [V+ac$_2$KAA(LLL)–2H]$^{2-}$ (cross) [V+ac$_2$KAA(DLL)–2H]$^{2-}$. The ions were collided with xenon at single collision conditions, pressure 0.080 mTorr at various collision energies. The *y*-axis represents percent dissociation, which is the relative intensity of the tripeptide ion (*m*/*z* 371) in proportion to the sum of the intensities of the precursor ion (*m*/*z* 908.9) and tripeptide ion.

solution to gas-phase. This was further substantiated when we investigated how a change in the binding pocket of vancomycin affects the gas-phase stability [54]. In CDP-1 (crystalline degradation product 1 of vancomycin), the conformation of the binding pocket has changed so that binding to Ac$_2$-L-Lys-D-Ala-D-Ala in solution is decreased by more than a factor of 1000 [55]. The decreased binding affinity to –D-Ala-D-Ala peptides is thought to originate from an extra methylene group in the peptide backbone, slightly elongating the carboxylate binding pocket [55]. The difference this makes to the shape of the binding site is small, and it is possible to fit the peptide into the binding site, yet the structure does not preserve the right distances for optimal hydrogen-bonding network in solution or in the gas-phase. Thus, we found that the dissociation threshold for the dianionic complex formed between CDP-1 and Ac$_2$-L-Lys-D-Ala-D-Ala was markedly lower than that of the specific vancomycin complex [54] reflecting that Ac$_2$-L-Lys-D-Ala-D-Ala cannot form as strong electrostatic interactions with CDP-1 as with vancomycin.

The gas-phase structure of the complex with the structural specific interactions retained would be expected to be rather compact because of the optimal fit for a –D-Ala-D-Ala peptide in the binding pocket. In contrast, the complex with the non-specifically bound –L-Ala-L-Ala peptide would be expected to be less compact since this stereochemical configuration has the wrong shape for accommodation in the binding pocket. To probe whether such a difference in the compactness exists we measured the destruction cross-section of the specific complex [V+Ac$_2$-L-Lys-D-Ala-D-Ala – 2H]$^{2-}$ and that of the non-specific complex [V+Ac$_2$-D-Lys-L-Ala-L-Ala – 2H]$^{2-}$. The total destruction cross-sections were derived from the exponential dependence of the precursor ion beam intensity I on the thickness (μ) of the target gas, $I(\mu) = I(0)e^{-\sigma\mu}$. The destruction cross-section σ is the sum of all loss processes for the precursor ion including fragmentation and scattering. $I(0)$ the initial intensity of the precursor ion signal (i.e., without collision gas in the collision cell), $I(\mu)$ the intensity of the precursor ion signal after addition of the collision gas, at target thickness μ. Surprisingly, no difference in their destruction cross-section was found [54]. This indicates that despite the dissimilarity in non-covalent bonding interactions between the complexes (as reflected by their different dissociation thresholds) the gas-phase structures appears to have similar compactness. This intriguing finding suggests that although the binding pocket of vancomycin does not support optimal binding to Ac$_2$-D-Lys-L-Ala-L-Ala, the flexibility of this tripeptide allows it to adopt a conformation which still generates a rather compact non-covalent complex. It is thus likely that the same number of hydrogen bonds may exist in both complexes and this leads to compact gas-phase structures in both cases. But the dissociation thresholds will still be different since the energy of a hydrogen bond is critically dependent upon its exact geometry [56]. Such a picture is in accordance with the recent knowledge on the gas-phase structures of peptides, where both experiments and calculations have demonstrated that it is energetically favorable to adopt fairly compact gas-phase structures with extensive hydrogen bonding [50,51]. Moreover, Wu et al. [57] have shown that the non-covalent complexes between a protein and various peptide ligands are likely to form collapsed structures in the gas-phase. This means that the flexible polar regions, which are hydrogen bonded to solvent molecules in solution will fold up in the gas-phase and form hydrogen bonds with other polar regions within the non-covalent complex.

3.2. Collision-induced dissociation of (1:2) antibiotic–peptide clusters

The monomeric vancomycin antibiotics are known to have a single peptide binding site in solution. By using high concentrations of peptide ligands (100 μM) in the electrospray solution we were nevertheless able to generate cluster ions comprised of one antibiotic molecule with two peptide ligands. Such non-specific aggregates are frequently observed in ESI-MS when using high solute concentrations. To probe the molecular recognition in such gaseous cluster ions we used isotopically labelled peptide stereoisomers and examined the fragmentation pathways of such clusters by tandem mass spectrometry. Fig. 8 shows the CID spectrum of the dianionic cluster ion comprised of pseudoaglycoristocetin (Ψ), Ac$_2$-L-Lys-D-Ala-D-Ala and d_6-Ac$_2$-L-Lys-L-Ala-L-Ala. This cluster ion fragment by charge separation yielding two singly charged product ions [d_6-Ac$_2$-L-Lys-L-Ala-L-Ala – H]$^-$ and [Ψ+Ac$_2$-L-Lys-D-Ala-D-Ala – H]$^-$. The exclusive loss of the –L-Ala stereoisomer from the cluster ion strongly indicates that this ligand is very loosely attached

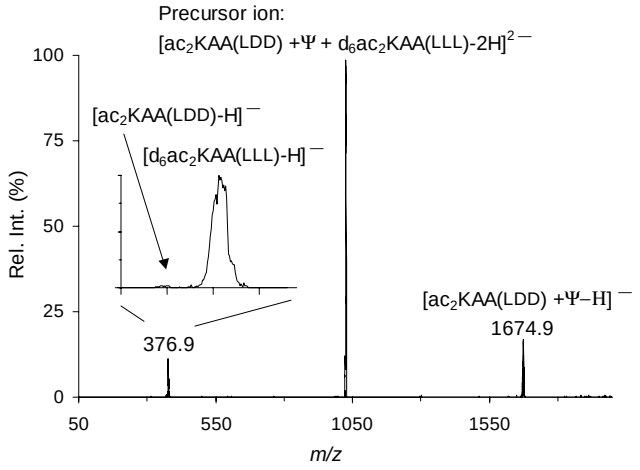

Fig. 8. CID spectrum of the dianionic cluster ion comprised of the pseudoaglycone of ristocetin (Ψ), diacetyl-L-Lys-D-Ala-D-Ala (ac_2KAA(LDD)) and the deuterium labelled stereoisomer d_6-diacetyl-L-Lys-L-Ala-L-Ala (d_6ac_2KAA(LLL)).

external to the binding pocket of antibiotic and the preferential binding to the –D-Ala stereoisomer suggests that this ligand is tightly retained within the binding pocket. For all 14 examined pseudoaglycoristocetin anionic cluster ions, a pronounced stereoselective fragmentation was observed that closely resembled the known solution binding selectivity [52]. Even the aforementioned unexpected finding that in solution pseudoaglycoristocetin has higher affinity for binding to Ac-Gly-D-Ala than to Ac-D-Ala-D-Ala was reflected in the gas-phase by a preferential loss of the latter ligand upon CID of the cluster ion containing these two ligands. A necessary requirement for the existence of such an excellent correlation is that all ligands externally bound to the binding pocket of pseudoaglycoristocetin must have fewer and/or weaker intermolecular hydrogen bonds than the ligand located within the binding pocket. Hydrogen bonds are highly distance-dependent and directional [56] and the rigid tetracyclic structure of pseudoaglycoristocetin restricts the possibility for adapting to a favorable geometry for the hydrogen bonds formed with the externally bound ligand. For larger and/or more flexible receptors, however, the likelihood increases for generating an optimal hydrogen bonding network to an externally bound ligand. This phenomenon was indeed observed when we analysed anionic cluster ions containing ristocetin, which has extra saccharide substituents (a tetra- and a monosaccharide). These cluster ions exhibit a stereoselective fragmentation that do not parallel the known solution binding selectivity thereby indicating that the flexible saccharide moieties provide the energetically preferred binding site. Thus, in these complexes the bioactive binding pocket has a less favorable mode of binding. A similar observation was made in a recent blackbody infrared radiative dissociation (BIRD) investigation of protein-carbohydrate complexes [58], where it was demonstrated that the bioactive recognition site was not energetically preferred. This and other observations [57] highlight the problems associated with obtaining information about biological relevant intrinsic binding affinities in desolvated biomolecular complexes. The first requirement for such investigations is that the specific interactions in solution must be retained in the gas-phase. The second requirement is that polar regions of the complex, which are hydrogen bonded to water molecules in solution should not form strong intermolecular hydrogen bonds upon desolvation. Such non-specific bonds should be weak and not contribute significantly to the intermolecular electrostatic interaction energy for the gaseous complex. The cationic antibiotic–peptide complexes did not fulfill the first requirement, whereas the anionic ristocetin-peptide ions did not fulfill the second requirement. However, there is strong experimental evidence supporting that both requirements are satisfied in the anionic complexes formed between –D-Ala peptides and vancomycin, aglycovancomycin or pseudoaglycoristocetin [52].

3.3. Gas-phase H/D exchange of glycopeptide antibiotic—peptide complexes

In contrast to CID, gas-phase hydrogen/deuterium exchange is a non-destructive method, which can be used to probe gas-phase structures of non-covalent complexes. H/D exchange can potentially probe several sites in a complex if the multiple exchanges observed can be correlated with the different available reactive sites. We investigated the gas-phase structures of protonated vancomycin antibiotics as well as their complexes with –D-Ala peptides using ND_3 as deuterating reagent. In particular, we addressed whether and how complexation affects the reactivity of the antibiotics.

Gas-phase H/D exchange of various peptides and amino acids with ND_3 has shown that amino, backbone amide, hydroxylic and carboxylic hydrogens are exchanged rapidly [59–61]. Doubly-protonated vancomycin contains 20 of such hydrogens known to exchange readily, but we only observed exchange of seven hydrogens [62]. In peptides, amino and carboxylic hydrogens are known to be the most reactive hydrogens towards exchange with ND_3 [59,63]. Six of these hydrogens are present in doubly-protonated vancomycin (assuming a non-zwitterionic gas-phase structure with protonated amino groups). Thus, most of the amide and hydroxylic hydrogens in vancomycin are unexpectedly not available for exchange. Doubly-protonated pseudoaglycoristocetin and ristocetin were found to exchange 10 and 12 hydrogens, respectively [62]. This indicates that the two additional sugar groups (containing 15 hydroxylic hydrogens) on ristocetin only play a very limited role in the H/D exchange reactions for the free antibiotic. The mechanisms involved in H/D exchange reactions of small peptides have been the subject of several studies [59–61,64,65]. The mechanism proposed by Campbell et al. [59] for exchanging amide hydrogen involves proton transfer from the N-terminal am-

monium ion to the amide carbonyl oxygen in concert with transfer of the amide proton to ammonia (ND_3) to form an ammonium ion (ND_3H^+) solvated by the tautomerized peptide. This mechanism, supported by semiempirical calculations, was proposed to explain the H/D exchange reactions of a number of glycine oligomers. The mechanism requires close proximity between the N-terminus (or another protonated amino group in the peptide) and the amide carbonyl oxygen. Another requirement is the formation of several intermolecular hydrogen bonds between ND_3H^+ and the tautomerized peptide. These requirement are easily met by glycine oligomers since they are very flexible peptides, and they can adopt the conformations which are needed for the tautomer mechanism to be operative. However, the vancomycin antibiotics are very rigid molecules and therefore not likely to be able to adopt such conformations. Thus, the conformational rigidity of the vancomycin antibiotics may 'deactivate' some of the amide hydrogens for exchange.

The vancomycin (V) complexes $[V+Ac\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$ and $[V+Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$ are distinguishable by different levels of hydrogen exchange. Surprisingly, the former complex containing fewer exchangeable hydrogens exchanges more hydrogens (13) than the latter complex (12) [62]. In addition, the hydrogens of $[V+Ac\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$ exchange faster than the those of $[V+Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$. In contrast, the corresponding eremomycin-peptide ligand complexes are not distinguishable by different levels of hydrogen exchange (both 15) and there is no difference in their kinetic behavior. Complexation of ristocetin (R) and pseudoaglycoristocetin (Ψ) with peptide ligands affects their H/D exchange reactions very differently, e.g., $[R+Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$ exchanges 23 hydrogens, whereas $[\Psi+Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala+2H]^{2+}$ only exchanges 9 hydrogens. In case of ristocetin, the ligand makes more sites on the antibiotic capable of taking part in H/D exchange relative to the uncomplexed antibiotic. In contrast, the complexation of pseudoaglycoristocetin makes fewer sites on the antibiotic capable of taking part in H/D exchange relative to the free antibiotic. This indicates that the saccharide substituents on ristocetin are involved in intermolecular hydrogen bonding with the ligand.

It is generally assumed that intermolecular hydrogen bonding may induce H/D exchange, e.g., in the so-called onium mechanism intermolecular hydrogen bonding makes endothermic proton transfer energetically feasible thereby facilitating H/D exchange [59]. However, intermolecular hydrogen bonding may also inhibit H/D exchange as observed in the pseudoaglycoristocetin complex. An analogous case of inhibition of H/D exchange because of inter-and intramolecular hydrogen bonding was reported by Green et al. [66] and Campbell et al. [59], respectively. Campbell et al. [59] observed a dramatic lack of reactivity of glycine oligomers larger than Gly_3 with the exchange reagents D_2O and CD_3OD. Gly_3 readily exchanged its labile hydrogens whereas Gly_4 and Gly_5 were virtually non-reactive. For H/D exchange to occur, the energy recovered by forming the exchange intermediate must compensate for the loss in intramolecular solvation energy. Gly_4 and Gly_5 have a more extensive solvation of the charge site (N-terminus) than Gly_3, which makes proton transfer more endothermic, thereby reducing the extent of H/D exchange. A similar situation may exist in the pseudoaglycoristocetin complex.

3.4. High energy CID and electron capture dissociation of antibiotic–peptide complexes

Electron capture dissociation (ECD) can be used to probe the extent of intramolecular non-covalent bonding in polycationic proteins [67–69]. Capture of an electron at a protonated site leads to fast clevage of the polypeptide main-chain. If, however, the cleaved main-chain is joined by non-covalent bonds, the two fragments will appear as a reduced molecular ion, $[M+nH]^{\bullet(n-1)+}$. This phenomenon may be responsible for the absence of fragment ions when large (>17 kDa) multiply protonated proteins capture electrons. Conversely, electron capture for an unfolded protein conformation will result in two separate product ions. Thus, the occurrence or absence of fragment ions may provide information about the non-covalent bonding within gaseous protein ions. We employed a similar strategy to probe the gas-phase structure of a diprotonated vancomycin–tripeptide complex.

ECD of the doubly-protonated complex formed between vancomycin and $Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala$ caused covalent bond cleavage with retainment of the non-covalent complex [70]. Breakage of covalent bonds without dissociation of the intermolecular bonds have not been observed at low-energy CID of cationic and anionic vancomycin+$Ac_2\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala$ complexes [52]. Two fragmentations of vancomycin occurred without dissociation of the complex upon ECD: (1) loss of methyl amine from the N-terminal *N*-methyl-D-leucin residue; (2) a concomitant loss of the disaccharide moiety and a chlorine substituent. Interestingly, already nearly two decades ago, Williams et al. [71] also reported loss of chlorine from vancomycin as a result of one electron reduction occuring in the FAB ionization process. ECD also caused fragmentation of the tripeptide ligand (loss of one acetyl group) without complex dissociation. The abundant loss of the dissaccharide moiety and the acetyl group of the ligand indicate that they are not involved in strong hydrogen bonds with the remainder of the complex. Furthermore, these results demonstrate that ECD can cleave covalent bonds without dissociation of weak non-covalent bonds nearby. Similar observations have been made upon ECD of protein ions [68]. Also high-energy CID (E_{LAB} = 100 keV) of the corresponding dianionic complex caused covalent fragmentation reactions without dissociating the complex [54]. For avoparcin–tripeptide complexes, however, competition between non-covalent and covalent dissociation was observed at low collision-energy

[53]. Breakage of covalent bonds with retainment of the non-covalent complex upon low-energy CID has been observed in a number of cases [72–77]. From such studies, it has been demonstrated that slow heating favors covalent breakage [74,75]. However, with ECD and high energy CID of the vancomycin–tripeptide complex, a different situation prevails, since for this particular complex covalent bond cleavage occurs exclusively in ECD and high-energy CID where the activation occurs on a very short time-scale (i.e., fast heating). In high-energy CID, the collisional electron detachment reaction, that precedes covalent bond cleavage, is most likely a vertical process [54]. Likewise, in ECD, the fragmentation reaction is believed to occur before the internal energy (acquired by the ion upon electron capture) is redistributed among all internal degrees of freedom (i.e., a non-ergodic process) [78].

It is noteworthy that ECD and CID of vancomycin antibiotics at high and low collision energy yields only few types of fragment ions, most of which are formed by cleavage in the saccharide moieties [52,54,79]. The resistance of the peptide back-bone of the antibiotics towards fragmentation is due to the extensive cross-linking of the amino acid side-chains. Within the cross-linked structure at least two covalent bond cleavages are required to create two fragments.

4. Future perspectives

In this manuscript, we have highlighted, by using the glycopeptide antibiotic vancomycin–cell-wall mimicking peptide system, some of the potentials of bio-affinity mass spectrometry. The technique is powerful as it requires very little sample, but mostly as it is able to observe and detect different molecular species in solution in a multiplexed manner. Although, care must always be taken to validate mass spectrometry (gas-phase)-based data when monitoring solution-phase properties, it has been by now well established that mass spectrometry may be a valuable complementary tool in structural biology of protein–protein and protein–ligand interactions as further evidenced and highlighted by recent reviews [80,81].

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