The Formation of Heterodimers by Vancomycin Group Antibiotics

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Abstract: The formation of heterodimers in mixtures of glycopeptide antibiotics has been detected by electrospray ionization mass spectrometry (ESI-MS), and dimerization constants have been determined. By using NMR spectroscopy, it has been shown that these heterodimers indeed exist in aqueous solution. The dimerization constants obtained by NMR spectroscopy are in good agreement with those determined by ESI-MS. Structural information on the heterodimer interface of some of the heterodimers is obtained by using two-dimensional NMR techniques and reveals that these heterodimers are similar in structure to the homodimers.

Keywords: antibiotics · heterodimers · mass spectrometry · NMR spectroscopy · vancomycin

Introduction

In previous work, we have shown that antibiotics of the vancomycin group, with the exception of teicoplanin, form homodimers.[1] Several studies have indicated that these homodimers may be important in promoting antibiotic activity.[2] Over a range of antibiotics (Figure 1), a large variation in the dimerization constants is observed (Table 1). Specifically, a homodimer can bind on a bacterial cell surface by initial attachment (of one half of the dimer) to a mucopeptide precursor terminating in –D-Ala-D-Ala. The binding of a second mucopeptide precursor into the other half of the dimer is then effectively an intramolecular event, and this is enhanced because of the chelate effect. The surface-enhanced binding which results from this effect has been experimentally demonstrated.[2] Although the possibility that a mixture of two glycopeptide antibiotics might contain heterodimers is evident, the analysis of proton NMR spectra to investigate this phenomenon is a complex task, due to the very large numbers of proton resonances involved. Other convenient techniques such as UV difference spectrophotometry and microcalorimetry lack the specificity to enable the unambiguous detection of heterodimers in antibiotic mixtures. However, since mass spectrometry gives a direct measure of the masses of the species involved (which differ for the various possible dimers), this technique has a clear potential in an examination of the problem. Using electrospray ionization mass spectrometry (ESI-MS), we now show that when mixtures of two glycopeptide antibiotics are introduced into the mass spectrometer source, peaks arise because of the formation of heterodimers (as well as the anticipated peaks due to the homodimers). By using methods previously described,[3] it is possible to determine dimerization constants for these dimers and to compare them with values previously published for the formation of homodimers in aqueous solution (Table 1). We also use ^{1}H NMR spectroscopy to show that heterodimers exist in solution, and the corresponding dimerization constants are determined by using this method.

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Results and Discussion

Determination of the association constants with electrospray ionization mass spectrometry: The quantitative use of ESI-MS for the determination of the association constants

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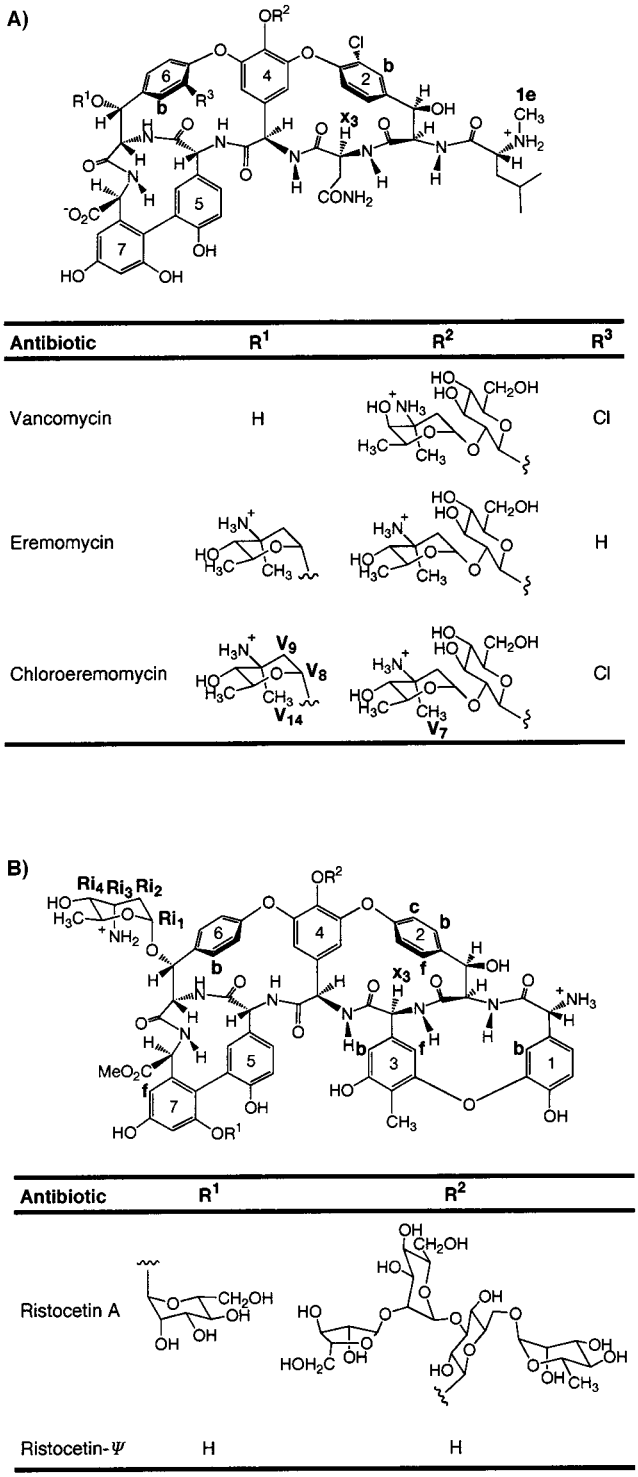

Figure 1. Structures of some antibiotics of the vancomycin group.

depends on a few instrumental parameters such as interface voltages (cone voltage, tube lens voltage, and desolvation voltage), which were all optimized according to procedures described in detail previously.[3] The desolvation voltage is one of the most critical parameters and was set to a previously optimized value of 55 V, which was just sufficient to desolvate all the solvent molecules of the ions generated by ESI-MS. When vancomycin (25 μM in 5 mM ammonium acetate buffer) was introduced by ESI into the mass spectrometer, the mass

Table 1. Dimerization constants of some glycopeptide antibiotics and their mixtures.

Antibiotic 1	Antibiotic 2	K_{dim} [M^{-1}]		
		ESI-MS	NMR	Literature
Vancomycin	Vancomycin	< 1000		49,[7] 700,[8] 4.0×10^3 [9]
Vancomycin	Eremomycin	7.2×10^4	3.7×10^4[a]	
Vancomycin	Ristocetin-A	2.0×10^4	8.0×10^3	
Vancomycin	Ristocetin-Ψ	3.1×10^4	2.5×10^4	
Eremomycin	Eremomycin	7.5×10^5		3.9×10^5,[10] 3.0×10^6[1]
Eremomycin	Ristocetin-A	2.1×10^5	8.7×10^4	
Eremomycin	Ristocetin-Ψ	4.9×10^4	2.2×10^4	
Ristocetin-A	Ristocetin-A	< 1000		150,[11] 500,[12] 4000[9]
Ristocetin-Ψ	Ristocetin-Ψ	< 1000		50[8]
CE[b]	CE	7.0×10^4		1.5×10^4 [10]
CE	Eremomycin	1.7×10^6		
CE	Vancomycin	1.5×10^4	5.4×10^3[a]	
CE	Ristocetin-A	3.5×10^4	1.1×10^4	
CE	Ristocetin-Ψ	9.0×10^4	1.8×10^4	

[a] *N*-Demethylvancomycin was used instead of vancomycin (see text). [b] CE = Chloroeremomycin.

spectrum was dominated by ion signals that correspond to the doubly protonated monomer of vancomycin (data not shown).[3] The ESI mass spectrum of eremomycin taken under identical conditions is shown in the bottom section of Figure 2. Ion signals are observed that correspond to the doubly and triply protonated monomer of eremomycin. In addition, ions corresponding to the triply and possibly quadruply protonated dimer of eremomycin (which has the same m/z value as the doubly protonated monomer) are observed. The mass resolution of the instrument used is sufficient to allow relative quantification of the amount of doubly protonated monomers and quadruply protonated dimers (through the resolution of isotopic peaks). In previous studies on binding of bacterial cell-wall precursor peptides to vancomycin antibiotics, we have shown that the relative intensities of the ion signals in ESI mass spectra may be used to calculate the relative concentrations of the species present in solution and, thus, the affinity constants.[3] This method relies on the assumption that there should be a one-to-one correlation between the ion abundances and the solution-phase concentrations. For this assumption to be true, the ionization probability of the monomer species should be similar to the ionization probability of the dimer species. In the ESI spectrum of eremomycin shown in the lowest panel of Figure 2, the ratio of abundances of ions (measured by peak areas) of the monomer and dimer species is 30:100. The equilibrium equation is given in Equation (1). The above-mentioned assumption implies that for each 30 molecules of

$$A + A \rightleftharpoons AA \quad (1)$$

free eremomycin there are $2 \times 100 = 200$ molecules of eremomycin involved in forming 100 dimer molecules. With a total initial concentration of 25 μM of eremomycin monomer it can be calculated that there is at equilibrium 3.4 μM of free eremomycin and 10.8 μM of eremomycin dimers. Thus, from Equation (2) for the equilibrium constant for the formation of the homodimer (K_{AA}; [AA] is the concentration of the

$$K_{AA} = \frac{[AA]}{[A]^2} \quad (2)$$

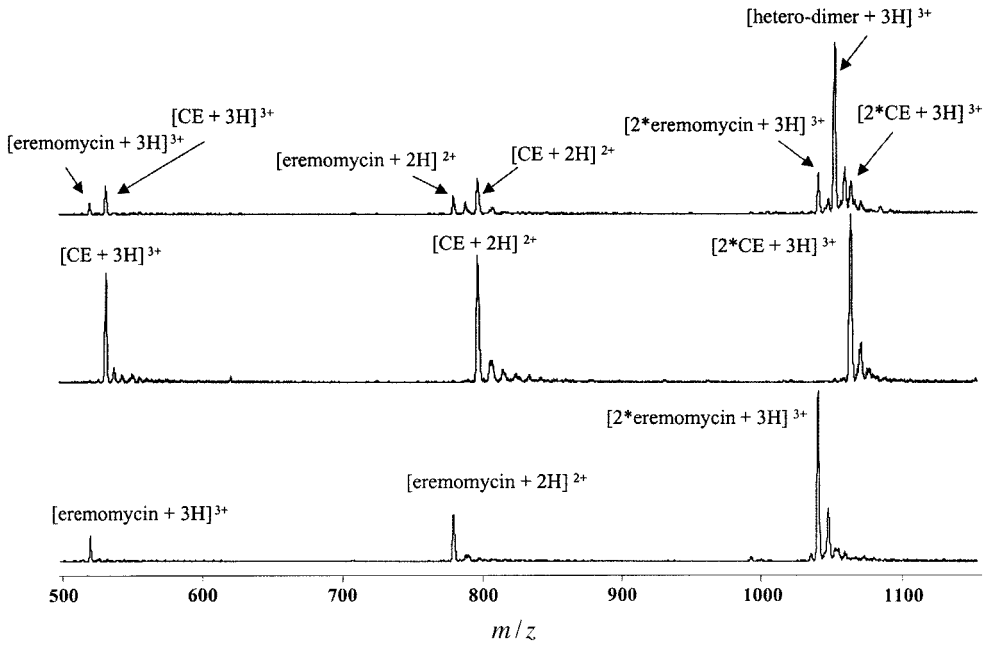

Figure 2. Top: ESI-MS spectrum of an equimolar mixture (25 μM) of eremomycin and chloroeremomycin (CE). Middle: ESI-MS spectrum of chloroeremomycin (25 μM). Bottom: ESI-MS spectrum of eremomycin (25 μM).

homodimer and [A] is the concentration of the antibiotic monomer), the binding constant from the spectrum shown at the bottom of Figure 2 is obtained as $9.3 \times 10^5\,\text{M}^{-1}$.

The ESI mass spectrum of chloroeremomycin (CE) is shown in the middle panel of Figure 2. It is immediately evident that relatively more monomeric ions are observed in this spectrum when compared with the spectrum of eremomycin (bottom panel). Using the same approach, a dimerization constant for chloroeremomycin of $8.0 \times 10^4\,\text{M}^{-1}$ was calculated. The top panel of Figure 2 displays the spectrum observed when an equimolar (25 μM) mixture of eremomycin and chloroeremomycin is introduced into the mass spectrometer. Besides ion signals that can be attributed to ions also observed in the spectra of each individual antibiotic (the lower two panels), additional, abundant ion signals are observed for heterodimer species. Strikingly, the signal due to the heterodimer of eremomycin and chloroeremomycin is the most intense signal. From a single measurement, the top ESI mass spectrum allows direct determination of the three different association constants, that is, the dimerization constant of eremomycin, the dimerization constant of chloroeremomycin, and the heterodimerization constant of the eremomycin/chloroeremomycin complex. The values obtained from the top spectrum of Figure 2 are 7.4×10^5, 9.3×10^4, and $1.67 \times 10^6\,\text{M}^{-1}$, respectively. The two values for the homodimers of the two individual antibiotics are in reasonable agreement with the data obtained from the individual measurements (see above). In a similar manner association constants of homo- and heterodimers were obtained for a whole range of antibiotics. Table 1 summarizes the association constants, averaged over several different experiments on pure, as well as on mixtures of antibiotics.

Determination of the association constants by ^{1}H NMR spectroscopy: In the light of the above results, mixtures of glycopeptide antibiotics were investigated by ^{1}H NMR spectroscopy. ^{1}H NMR titrations were carried out by the addition of aliquots of a solution of one glycopeptide antibiotic to a solution of a second glycopeptide antibiotic until one-to-one mixtures were obtained. The result of one such titration is shown in Figure 3. On addition of eremomycin to a solution of

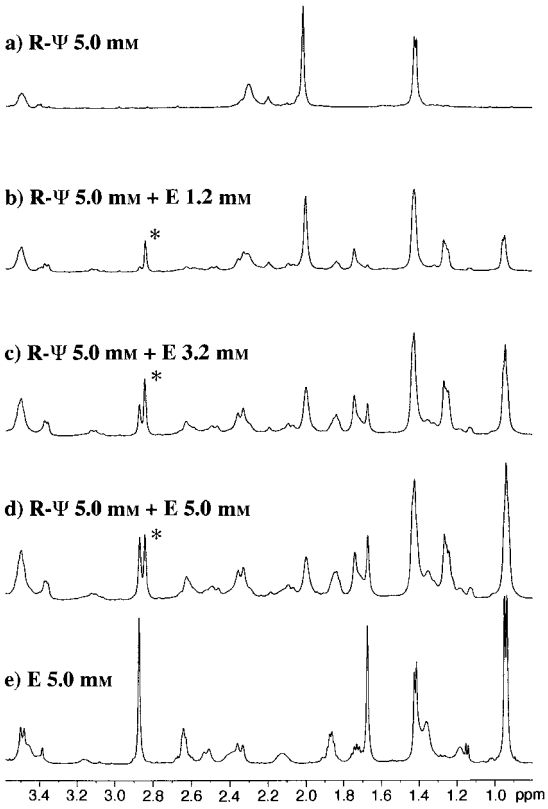

Figure 3. Portion of the ^{1}H NMR spectra of ristocetin-Ψ (R-Ψ), eremomycin (E), and mixtures of these antibiotics (D_2O, pH 4.5, 300 K). The peak due to the *N*-methyl group of the heterodimer is labeled with an asterisk.

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ristocetin pseudoaglycone (ristocetin-Ψ, 5.0 mM in D_2O), a singlet appears at $\delta \approx 2.8$, which corresponds to the *N*-methyl group of residue 1 of eremomycin (Figure 3b, signal marked with an asterisk). With increasing concentration of eremomycin, a second singlet can be seen at $\delta \approx 2.9$ (Figure 3c). At equal concentrations of the two antibiotics, the ratio of the two singlets is approximately 1:1 (Figure 3d). Comparison with the spectrum of eremomycin (5.0 mM, Figure 3e) shows that the peak at $\delta \approx 2.9$ is due to the *N*-methyl group of eremomycin homodimer. Therefore, the other singlet most likely corresponds to the *N*-methyl group of eremomycin in the ristocetin-Ψ/eremomycin heterodimer. As one would expect, in the course of the titration the heterodimer peak appears first, and, with increasing concentration of eremomycin, the peak due to the homodimer of this antibiotic increases in intensity.

In order to gain more evidence for the formation of heterodimers and to determine their geometry, NOESY spectra were obtained from the one-to-one mixtures of antibiotics in aqueous solution. The heterodimers were expected to be of the same geometry as homodimers, that is, antiparallel back-to-back aggregates (Figure 4). Table 2 lists some of the protons of chloroeremomycin and ristocetin-Ψ that showed significant changes in chemical shift upon heterodimer formation. Most of these protons are situated at the heterodimer interface (e.g., x_3, 2b, 3b, V_9, V_{14}, Ri_2, Ri_3), but also protons which do not point towards the interface are affected (e.g., 6b and 1e). On formation of the heterodimer, protons Ri_{2e} and Ri_3 of ristocetin-Ψ are probably situated near the carbonyl group of residue 2 of chloroeremomycin leading to the relatively large downfield shift. NOESY crosspeaks were observed between protons of the sugar attached to residue 6 of chloroeremomycin and residues 2 and 3 of ristocetin-Ψ. Furthermore, crosspeaks between the ristosamine moiety (Ri_1, Ri_2) of ristocetin-Ψ and proton 2b of chloroeremomycin were detected (Table 2), indicating that the geometry is indeed as shown in Figure 4.

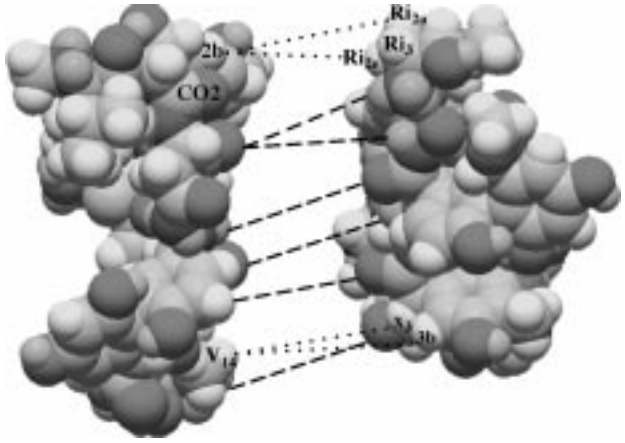

Figure 4. Exploded view of a chloroeremomycin (left)/ristocetin-Ψ (right) heterodimer. Dashed lines represent hydrogen bonds and dotted lines represent observed NOEs between the two halves of the dimer (see text, Figure 1B and Table 2). Protons 2b, 2c, and Ri_1 of ristocetin-Ψ and V_8 and V_9 of chloroeremomycin are covered by other atoms. Therefore, NOEs between these protons are not shown in the figure. The amide group involved in the hydrogen bond second from the bottom is also covered by other atoms.

Table 2. Chemical shifts of some protons of chloroeremomycin and ristocetin-Ψ (R-Ψ; see Figures 1 and 4) in antibiotic homodimers/monomers and heterodimer and NOEs between the two halves of the heterodimer (D_2O, pH 4.5, 300 K).

	Chloroeremomycin				Ristocetin-Ψ	
	homodimer	heterodimer	NOEs to R-Ψ		mono[a]	heterodimer[b]
1e	2.86	2.85		**1b**	6.92	7.05
2b	7.34	7.40	Ri_1, Ri_2	**2b**	7.30	7.20
$\mathbf{x_3}$	4.98	4.79		**2c**	7.08	7.05
6b	7.81	7.76		**3b**	6.50	6.46
$\mathbf{V_7}$	1.44	1.30		$\mathbf{x_3}$	5.41	5.61
$\mathbf{V_8}$	5.07	5.16	2b, 2c	**3f**	6.13	6.34
$\mathbf{V_{9a}}$	2.39	2.36	2b, 2c	**6b**	7.47	7.50
$\mathbf{V_{9e}}$	2.55	2.47	2b, x_3	$\mathbf{Ri_1}$	5.16	5.12
$\mathbf{V_{14}}$	1.68	1.74	3b, x_3	$\mathbf{Ri_{2a,e}}$	2.33	2.34, 2.54
				$\mathbf{Ri_3}$	3.88	4.24
				$\mathbf{Ri_4}$	3.73	3.79

[a] Since the dimerization constant of ristocetin-Ψ is very small ($50\ M^{-1}$) and monomer and homodimer are in fast exchange, these shifts correspond to the average of monomer and homodimer (at a ristocetin-Ψ concentration of 5.0 M ca. 25% of the antibiotic exists as dimer). [b] These chemical shifts are averages as ristocetin-Ψ in the heterodimer is in fast exchange with monomer/homodimer.

In most mixtures of glycopeptide antibiotics, the heterodimers were found to be in slow exchange (on the NMR timescale) with the respective monomers or homodimers. Therefore, the populations of the heterodimers could be determined from integration of suitable peaks resulting from heterodimers in ^1H NMR spectra. Thus, in Equation (3) for

$$K_{AB} = \frac{[AB]}{[A][B]} \tag{3}$$

the equilibrium constant for the formation of the heterodimer (K_{AB}), the concentration of the heterodimer ([AB]) was obtained from integration of the peak corresponding to the *N*-methyl group (1e, see Figure 1) in antibiotics which have such a group (vancomycin, eremomycin, chloroeremomycin). The concentrations of the monomeric antibiotics ([A] and [B], respectively) can be calculated as follows. For the known equilibrium constant of the formation of the homodimer (K_{AA}), we have Equation (2), in which [AA] is the concentration of the homodimer. For the total concentration of the antibiotic ($[A]_t$) we can use Equation (4), rearrangement of which and substitution of the value for [AA] from Equation (2) gives us Equation (5). The solution of this quadratic

$$[A]_t = [A] + [AB] + 2[AA] \tag{4}$$

$$K_{AA} = \frac{0.5([A]_t - [A] - [AB])}{[A]^2} \tag{5}$$

equation affords the concentration of the monomeric antibiotic as shown in Equation (6). In a similar manner, the concentration of the other monomeric antibiotic (B) can be derived from the equation for the known equilibrium constant of the other homodimer [K_{BB}, Eq. (7)].

$$[A] = \frac{-0.5 + [0.25 - 2K_{AA}([AB] - [A]_t)]^{1/2}}{2K_{AA}} \tag{6}$$

$$K_{BB} = \frac{[BB]}{[B]^2} \tag{7}$$

Thus, having determined [AB], [A], and [B], K_{AB} can be calculated from Equation (3). It must be noted that in deriving K_{AB} for the eremomycin/vancomycin and chloroeremomycin/vancomycin heterodimers (where both halves of the heterodimer possess a *N*-methyl group), *N*-demethylvancomycin was used instead of vancomycin. This was to ensure that only one half of the heterodimers contains a *N*-methyl group in order to circumvent the problem of overlapping signals. The equilibrium constants K_{AB} for some glycopeptide antibiotic mixtures are listed in Table 1.

In the NMR spectra of a ristocetin-Ψ/vancomycin mixture, the heterodimer was found to be in fast exchange (on the NMR timescale) with the monomer and homodimer of ristocetin-Ψ and vancomycin. In this case the concentration of the heterodimer could not be derived from integration of ^{1}H NMR signals (as above), because we found no signals corresponding to only hetero- and homodimer, but only averaged signals. Therefore, the concentration of heterodimer ([AB]) was calculated by using the Equation (8), in which δ is

$$[AB] = |[(\delta - \delta_{mono})/(\delta_{hetero} - \delta_{mono})]| [R\text{-}\Psi]_t \quad (8)$$

the chemical shift of a suitable ristocetin-Ψ signal (2f, 7f, Ri_1, see Figure 1) derived from a solution containing ristocetin-Ψ (5.0 mM) and vancomycin (5.0 mM). $[R\text{-}\Psi]_t$ is the total concentration of ristocetin-Ψ (5.0 mM). The chemical shift for the ristocetin-Ψ monomer (δ_{mono}) was derived from a diluted ristocetin-Ψ solution (0.5 mM), in which the amount of homodimer is less than 10 %. The chemical shift for ristocetin-Ψ in the heterodimer (δ_{hetero}) was taken from a solution (0.6 mM ristocetin-Ψ, 5.0 mM vancomycin) in which ristocetin-Ψ was assumed to exist only as heterodimer with vancomycin. Thus, having determined [AB], K_{AB} could be determined from Equations (6) and (3), as described for the slow-exchange case.

An approximate thermodynamic basis for the formation of heterodimers is presented in Figure 5. From this diagram, it may be seen that although a price in free energy has to be paid to split a strong homodimer, it is possible, in principle, for the heterodimer to be populated to a significant extent if the destruction of a weak homodimer occurs concurrently and a heterodimer is formed whose equilibrium constant is somewhere near the geometric mean of the equilibrium constants for the homodimers. Although the approximation gives a clear understanding of the physical basis of the observations, it is not a quantitative approach, and indeed several measured heterodimerization constants are significantly higher than the geometric mean value of the corresponding homodimers.

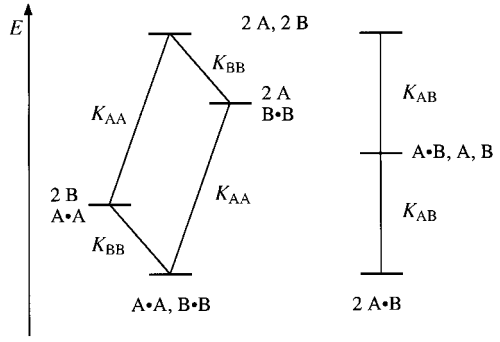

Figure 5. Energy diagram showing how two monomers of an antibiotic A (2 A) and two monomers of an antibiotic B (2 B) can form two homodimers (A · A and B · B) or two heterodimers (2 A · B). K_{AA}, K_{BB} and K_{AB} are equilibrium constants of the formation of A · A, B · B and A · B, respectively. The figure makes the assumption that the standard free energy of formation of two heterodimers is the same as that of formation of two homodimers. The experimental data suggest that this assumption is a useful starting approximation, but there are deviations (see text).

Minimum inhibitory concentrations (MICs): Glycopeptide antibiotics bind to cell-wall precursors at the surface of Gram-positive bacteria; this leads to cell death.[4, 5] Dimerization plays an important role in achieving stronger binding to these cell-wall precursors.[2] In order to find out whether the formation of heterodimers leads to an improvement in the biological activity of glycopeptide antibiotics, one-to-one mixtures of antibiotics were tested against a vancomycin-susceptible and a vancomycin-resistant strain of *Enterococcus faecium*. As can be seen from Table 3, when mixtures of antibiotics are employed, there is no significant increase in activity of the mixture relative to the more active antibiotic (of the mixed pair) alone.

Table 3. MICs of glycopeptide antibiotics and their 1:1 mixtures against vancomycin-susceptible and -resistant *Enterococcus faecium*.

Antibiotic 1	Antibiotic 2	MIC [μM]	
		susceptible	resistant
Vancomycin		2	512
Vancomycin	Eremomycin	0.5 – 1	1024
Vancomycin	Ristocetin-A	2	> 512
Vancomycin	Ristocetin-Ψ	0.5	> 64
Eremomycin		1	> 1024
Eremomycin	Ristocetin-A	1 – 2	> 512
Eremomycin	Ristocetin-Ψ	0.5	> 128
Ristocetin-A		2	> 512
Ristocetin-Ψ		0.25 – 0.5	> 64
CE[a]		0.5	128
CE	Ristocetin-A	1	512
CE	Ristocetin-Ψ	nd[b]	nd
CE	Eremomycin	nd	nd
Teicoplanin		4 – 8	256
Teicoplanin	Vancomycin	0.5	512
Teicoplanin	Eremomycin	0.5	256
Teicoplanin	CE	0.5	256
Teicoplanin	Ristocetin-Ψ	0.5	> 64

[a] CE = chloroeremomycin. [b] nd = not determined.

Conclusion

We have used ESI-MS to detect the formation of heterodimers in mixtures of glycopeptide antibiotics. Further investigations using NMR spectroscopy showed that these heterodimers indeed exist in aqueous solution, and NOESY spectra indicate the geometry of the heterodimers is similar to that found in homodimers, that is, they exist as antiparallel, back-to-back aggregates. Further, both techniques have been used to estimate dimerization constants for the formation of these heterodimers. The dimerization constants calculated by ^{1}H NMR spectroscopy are in good agreement with those

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determined by ESI-MS, although the latter are greater by a factor of 2–3 than those determined by NMR spectroscopy. This agreement between the two methods is remarkably close, bearing in mind that similar variations are not uncommon where binding constants are repetitively measured by a given technique, or by various conventional methods (see Table 1).

The vancomycin/eremomycin and vancomycin/chloroeremomycin heterodimers have dimerization constants somewhat near the geometric means of the equilibrium constants for the homodimers. A price in free energy has to be paid to split the strong homodimer of eremomycin or chloroeremomycin, but the destruction of the relatively weak vancomycin homodimer to form a heterodimer of moderate stability significantly compensates this price in free energy. Interestingly, both techniques give dimerization constants for vancomycin/ristocetin-A and vancomycin/ristocetin-Ψ heterodimers that are more than one order of magnitude higher than the dimerization constants for the vancomycin and ristocetin homodimers. The dimerization constant for the eremomycin/chloroeremomycin dimer measured by ESI-MS is also significantly higher than the geometric mean of the equilibrium constants of the homodimers. It is also noteworthy that, as determined both by ESI-MS and ^{1}H NMR spectroscopy, eremomycin recognizes ristocetin-A favorably over ristocetin-Ψ by a factor of 4–5, whereas this behavior is reversed in the case of chloroeremomycin, which recognizes ristocetin-Ψ favorably over ristocetin-A by a factor of 2–3 (see Table 1).

Determination of MICs of one-to-one mixtures of antibiotics has shown that heterodimer formation does not lead to a significant gain in biological activity relative to pure antibiotics.

Experimental Section

Chemicals: The antibiotics were kindly supplied by several people/institutions (see the Acknowledgment). Ristocetin-Ψ was obtained by acid methanolysis from ristocetin-A, as described previously.[6]

Mass spectrometry: Electrospray ionization mass spectra were recorded on a Finnigan TSQ700 triple quadrupole mass spectrometer. All samples were introduced by using a nanoflow electrospray source (Protana, Odense, DK). Solutions of antibiotics at concentrations of 25 μM (for each component) were made up in aqueous 5 mM ammonium acetate, pH = 5.1 (acidified with acetic acid), 298 K. Approximately 2 μL of these solutions were used and introduced into a nanoflow electrospray needle. Mass spectra were averaged over typically 100 scans. The determined dimerization constants were averaged over several experiments, also recorded at different concentrations of the antibiotics. The errors were estimated to be approximately 20%.

NMR spectroscopy: Prior to NMR analysis, the antibiotics were dissolved in D_2O, lyophilized, and redissolved in D_2O. The pD was adjusted to 4.5 with NaOD or DCl. All pD sample readings were measured with a Corning pH meter equipped with a combination glass electrode, and no corrections were made for an isotope effect. In titration experiments, the stock solution containing the second antibiotic was prepared from a solution identical to that of the first antibiotic in the NMR tube, so as not to change the pD or concentration of the first antibiotic.

NMR spectra were recorded on a Bruker DRX500 spectrometer at 288 K or 300 K. Chemical shifts were measured with respect to an external sodium 3-trimethylsilyl-2,2,3,3-[D_4]propionate solution (TSP, 6 mM in D_2O, $\delta = 0.00$ ppm). Suppression of solvent was achieved by presaturation. One-dimensional spectra were recorded with 32k complex data points. Two-dimensional NOESY and TOCSY spectra were recorded in phase-sensitive mode by using Time Proportional Phase Increment (TPPI) to give quadrature detection in f_1. 2048 complex data points were recorded in f_2 and 450–512 real points in f_1 with mixing times of 60–300 ms. Zero filling was used to give a final transformed matrix of 2048×2048 real points.

The errors in the dimerization constants were estimated to be approximately 10% where the heterodimers were in slow exchange, and approximately 20% where the heterodimers were in fast exchange. The following dimerization constants for the homodimers were used to calculate the dimerization constants for the heterodimers: vancomycin ($700\,M^{-1}$), eremomycin ($3.9 \times 10^5\,M^{-1}$), chloroeremomycin ($1.5 \times 10^4\,M^{-1}$), ristocetin-A ($500\,M^{-1}$), ristocetin-Ψ ($50\,M^{-1}$).

Determination of antibiotic minimum inhibitory concentrations: Minimum inhibitory concentrations (MICs, in μM) of *Enterococcus faecium* 207 (vancomycin resistant) and *Enterococcus faecium* 211 (vancomycim susceptible) were determined by serial dilution in BHY broth at 37 °C. Tubes containing broth (4 mL) and antibiotic were inoculated with a 3–4 h culture of *E. faecium* 207 or *E. faecium* 211 (250 μL, ca. 10^8 cfu) and incubated with gentle shaking for 18–20 h prior to recording MICs.

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