Modification and Inhibition of Vancomycin Group Antibiotics by Formaldehyde and Acetaldehyde

Albert J. R. Heck,*[a] Pauline J. Bonnici,[a] Eefjan Breukink,[b] David Morris,[c] and Martin Wills[c]

Abstract: It is shown that several vancomycin group antibiotics (vancomycin, eremomycin, and avoparcin) undergo spontaneous chemical modifications when kept at room temperature at neutral pH in aqueous solutions containing traces of formaldehyde or acetaldehyde. This chemical modification predominantly results in a mass increase of 12 Da in the reaction with formaldehyde and 26 Da in the case of acetaldehyde. By using tandem mass spectrometry the modification can unambiguously be identified as originating from the formation of a ring-closed 4-imidazolidinone moiety at the N-terminus of the glycopeptide antibiotics, that is, near the receptor binding pocket of the glycopeptide antibiotics. Bioaffinity mass spectrometry shows that this ring-closure results in a dramatically decreased affinity for the peptidoglycan-mimicking D-alanyl-D-alanine receptor. Additionally, in vitro inhibition measurements on two different strains of bacteria have revealed that the modified antibiotics display reduced antibacterial activity. The ring-closure is also shown to have a dissociative effect on the dimerization of the vancomycin-analogue eremomycin. The spontaneous reaction of vancomycin with formaldehyde or acetaldehyde may have implications not only for the clinical use of this class of antibiotics, but also for the effectiveness of these antibiotics when they are used in chiral separation chromatography or capillary electrophoresis.

Keywords: antibiotics · mass spectrometry · natural products · noncovalent complexes · vancomycin

Introduction

Glycopeptides of the vancomycin group are a class of naturally occurring antibiotics produced by fermentation of microorganisms.[1, 2] They inhibit cell wall biosynthesis in bacteria by forming a complex with the C-terminal D-alanyl-D-alanine of growing peptidoglycan chains. Glycopeptides are active against Gram-positive bacteria, including the major pathogens. Of all the glycopeptides that have been discovered, only a few—such as vancomycin and teicoplanin—are on the market for clinical use. Vancomycin (vancocin, vancoled) is clinically the most widely used glycopeptide antibiotic and is administered intravenously and orally, although the absorption is weak and penetration in the cerebrospinal fluid (CSF) may be poor.

Herein we report the study of the glycopeptide antibiotics vancomycin, eremomycin, and avoparcin. These three glycopeptide antibiotics, while structurally related, have distinct characteristics that make them particularly interesting. Relative to vancomycin, eremomycin has an increased ability to dimerize,[3] which is believed to have a positive effect on its antibacterial efficacy.[4–7] Avoparcin is structurally more complex than vancomycin.[8–10] Its major application has been as an antibacterial growth-promoting food-additive, primarily for pigs and poultry. The recent appearance of vancomycin-resistant enterococci (VRE)[11, 12] has been linked to the widespread use of avoparcin by farmers. Consequently, the use of avoparcin as an animal food additive has recently been banned in the European Union.

Previous reports have shown that chemically modifying natural glycopeptides makes it possible to increase their activity against methicillin-resistant Staphylococcus Aureus and coagulase-negative Staphylococci. Numerous chemical modifications have been reported,[2, 13] several of which have been made to the N-terminus of the backbone heptapeptide

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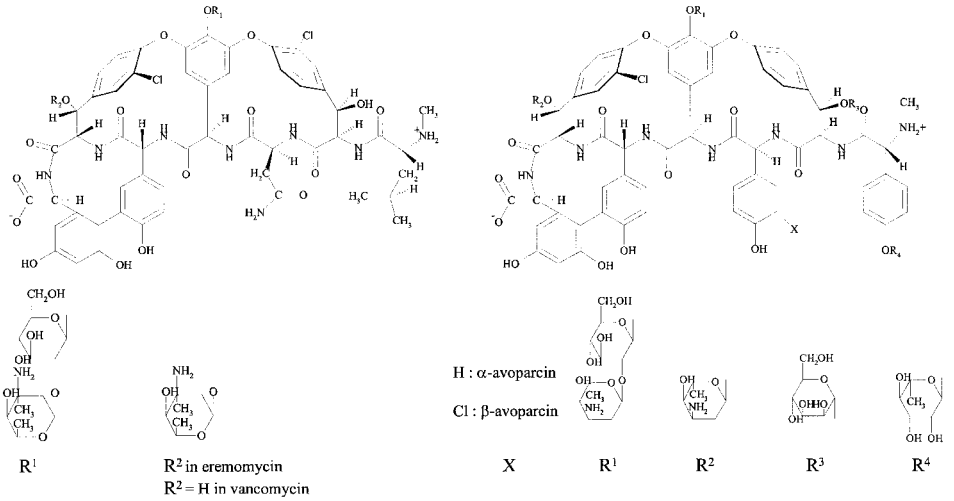
0947-6539/01/0704-0910 $ 17.50+.50/0



of the antibiotic.[14–18] In some cases, N-terminal modification of vancomycin group antibiotics led to a weak, but promising, activity against Van A Enterococci, which are highly resistant to natural glycopeptides.[15, 16] Other modifications, particularly those reducing the net charge on the N-terminus, lessened the binding to the model receptor Ac-D-Ala-D-Ala.[18] In this report, we study chemical modification of the glycopeptide antibiotics vancomycin, eremomycin, and avoparcin by formaldehyde and acetaldehyde in aqueous solutions at physiological pH. We have elucidated the chemical nature and kinetics of these modifications, primarily by mass spectrometric methods. Additionally, the effect of these chemical modifications on the antibiotics' ability to recognize model receptors, their in vitro efficacy, and their capability to dimerize have been evaluated.

Results and Discussion

Observation of spontaneous modification of avoparcin in water/methanol: In the course of ongoing studies in our laboratory on the thermal degradation of glycopeptide antibiotics[19] in aqueous/methanol solution, we observed that after storage of the solution for prolonged periods, $[M+12]$ Da adducts had been formed as products (M being the molecular weight of the antibiotic). Figure 1 shows two ESI-MS spectra of 20 μM avoparcin in water/methanol, the first taken directly after the solution was made (top spectrum) and the second after storage at room temperature for seven days (bottom spectrum). The top mass spectrum shows ions originating from doubly protonated α- and β-avoparcin (at approximately m/z 955 and 972, respectively). In the bottom spectrum, in addition to these, the $[M+12]$ Da adducts of α- and β-avoparcin (at m/z 961 and 978, respectively) were observed. These spectra revealed that, within a week, approximately half of the fresh avoparcin had been converted into the puzzling $[M+12]$ Da products. A chemical modification leading to a mass increase of 12 Da is rather rare, although not unprecedented.[20] For example, modifications leading to $[M+12]$ Da products have been reported to occur through reaction between formaldehyde and N-termini of peptides, leading to ring-closure and formation of a 4-imidazolidinone,[21–25] as depicted in Scheme 1 (in this case for an N-methylated peptide N-terminus, as found in vancomycin). Repetition of the degradation experiments in ultrapure water (using degassed water without organic modifier) did not result in the formation of the $[M+12]$ Da adducts, which led us to believe that a possible formaldehyde impurity had caused the formation of the degradation products. To establish whether this hypothesis was correct, aqueous buffered solutions of avoparcin and vancomycin were incubated at room temperature and pH = 6.8 with known concentrations of formaldehyde or acetaldehyde. Nanoflow electrospray ionization mass spectra of these

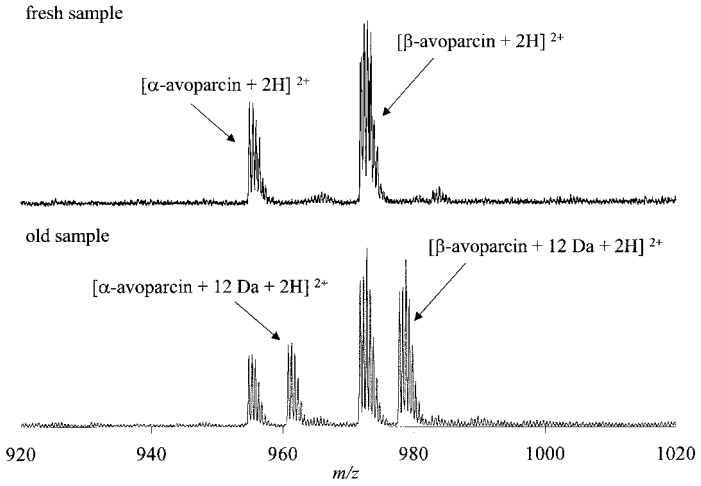
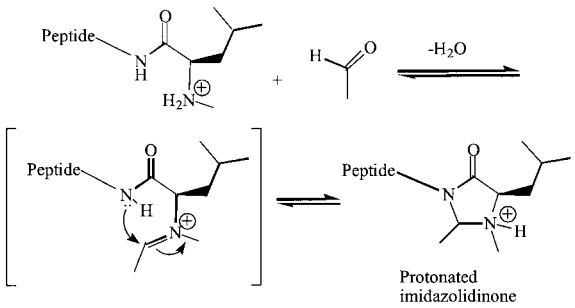


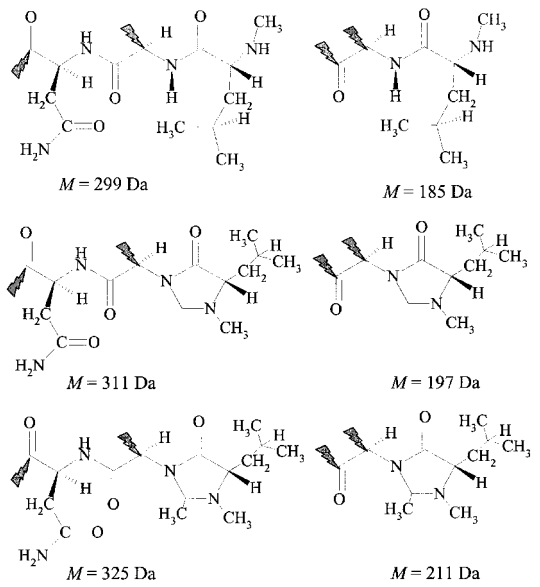
Figure 1. ESI-MS spectra of 20 μM avoparcin in water/methanol taken directly after the solution was made (fresh sample) and after storage at room temperature for seven days (old sample). The top mass spectrum shows ions originating from doubly protonated α- and β-avoparcin (at approximately m/z 955 and 972, respectively). In addition, in the bottom spectrum the $[M+12]$ Da adducts of α- and β-avoparcin (at m/z 961 and 978, respectively) were detected.



Scheme 1. Proposed scheme for the reaction between an N-methylated peptide N-terminus and acetaldehyde, proceeding through a ring closure to form a 4-imidazolidinone moiety.

solutions were taken at various time intervals. Indeed, [M+12] Da products were observed in all solutions containing formaldehyde, while [M+26] Da products were observed in solutions containing acetaldehyde.

Determination of the site of modification: To establish the site in the molecule at which these modifications had taken place, extensive comparative collision-induced dissociation MS/MS experiments were performed on the doubly protonated ions of α-avoparcin, β-avoparcin, vancomycin, and their corresponding [M+12] Da or [M+26] Da reaction products. The MS/MS spectra of these glycopeptide antibiotics were dominated by the facile loss of the carbohydrate moieties.[26] All the carbohydrate loss masses were identical in the cases both of the unmodified and the modified antibiotics, showing that the modification had not taken place in one of the carbohydrate moieties. The MS/MS spectra showed only a few marker ions, of low abundance; these could be used to identify unambiguously the site at which the modification had taken place. In the MS/MS spectra of doubly protonated vancomycin, singly charged fragment ions with m/z values of 185 Da and 299 Da were formed. In the MS/MS spectra of the corresponding [M+12] Da and [M+26] Da adducts, these fragment ions were observed at 197 and 211 Da, and 311 and 325 Da, respectively, representing mass shifts of 12 and 26 Da. The possible structures of these fragment ions are given in Scheme 2. The



Scheme 2. Mass (M) and proposed chemical structures of the marker ions observed in the collision-induced dissociation MS/MS spectra of the doubly protonated ions of vancomycin (top row) and the corresponding modified [M+12] Da (middle row) and [M+26] Da (bottom row) products. The arrows indicate the cleaved bonds.

exact mechanism of formation is probably quite complex and not completely understood, as at least two bonds would have to be broken to form these fragment ions. However, these tandem mass spectrometry results do confirm that the [M+12] Da and [M+26] Da modifications had indeed taken place around the N-terminus of the glycopeptides. We can therefore confirm that reaction with the formaldehyde/aldehyde indeed results in the formation of a ring-closed imidazolidinone moiety at the N-methylated N-terminus, as illustrated in Scheme 1. Similarly, MS/MS experiments on avoparcin and eremomycin also found fragment ions which indicated that chemical modification had taken place exclusively at the N-terminus. The 4-imidazolidinone ring formation is thought to be reversible,[22, 23] with hydrolysis proceeding by the same mechanism, but in the opposite direction. The position of this equilibrium depends, of course, on the level of aldehyde present and the pH and temperature of the solution.

Capillary electrophoresis: To establish that the ion intensities measured by ESI-MS were valid indicators of the relative quantities of vancomycin and modified vancomycin present in solution, capillary electrophoresis (CE) was used as an orthogonal method to determine the relative abundance of the two components. CE has been used extensively for the analysis of vancomycin.[18, 27] Figure 2 shows the electropherograms of vancomycin and modified vancomycin. Vancomycin (500 μM) was incubated with formaldehyde (500 μM) for a few hours in a ammonium acetate solution at pH = 7. The electropherogram of vancomycin shows one major signal, whereas the modified vancomycin solution shows a new signal at a longer migration time. CE-MS analysis verified that this new signal indeed originates from the modified [M+12] Da vancomycin. In the modified vancomycin solution, the relative abundance of the modified vancomycin is about 33%. In the ESI-MS spectrum of that particular modified vancomycin solution (not shown) the modified vancomycin ion signal contributed approximately 30% to the total ion signal, indicating that ESI-MS is in this case a good indicator for abundance of the species in solution. This assumption was confirmed by studying different mixtures of modified and unmodified vancomycin.

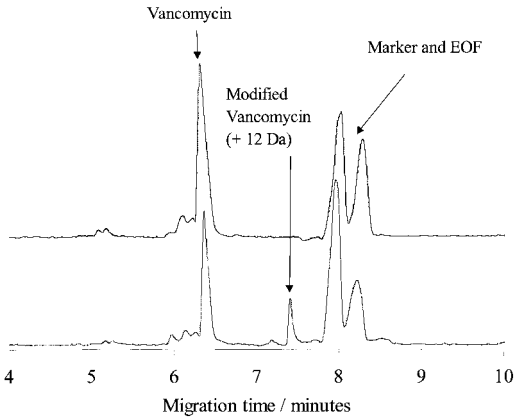


Figure 2. Top: Capillary electrophoresis electropherogram of vancomycin, revealing one major signal. Bottom : Capillary electrophoresis electropherogram of vancomycin that had been incubated for a few hours with a formaldehyde solution (approximately 500 μM). The bottom electropherogram shows a new signal at a longer migration time, originating from an [M+12] Da adduct of vancomycin.

Impact of chemical modification on antibiotic binding to receptor: In recent studies it has been shown that ESI-MS

0947-6539/01/0704-0912 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 4

may be used to evaluate quantitatively the association constants of vancomycin group antibiotics with model receptor peptides.[3, 10, 26, 28, 29] These model receptor peptides, such as *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala or *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser, have been shown to be reliable indicators of the in vivo activity of the antibiotics.[30, 31] To evaluate whether the antibiotics' efficacy could have been affected by the chemical modification, the association constants were determined by ESI-MS, using aqueous ammonium acetate (20 mM) buffer solutions. Figure 3 shows the ESI-MS spectrum of a 20 μM solution of vancomycin, partially modified by treatment with acetaldehyde for 10 h at room temperature, to which 25 μM of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala had been added immediately prior to mass analysis. The mass spectrum shows ions originating from vancomycin (approximately at *m/z* 724), the [*M*+26] Da adduct of vancomycin (approximately at *m/z* 737), and from the noncovalent complex of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (at approximately *m/z* 910). Notably, no ions could be detected for the noncovalent complex of the modified [*M*+26] Da adduct of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala, for which the doubly protonated ions should have appeared at approximately *m/z* 923 (indicated by the dashed arrow in Figure 3). Using procedures described previously,[10, 28] we derived an association constant K_a of 825 000 M^{-1} (in close agreement with literature data)[28, 29, 31] from the data in Figure 3 for the binding of vancomycin to *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala. From these data it is clear that the association constant of the modified [*M*+26] Da adduct of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala must be weak ($K_a < 500\ M^{-1}$). Similar results were obtained for avoparcin that had been treated with formaldehyde. Figure 4 shows the ESI-MS spectrum of a 10 μM solution of avoparcin, partially modified by treatment with formaldehyde for 2 hours at room temperature in an aqueous ammonium acetate (20 mM) buffer solution, to which 25 μM of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser had been added just prior to mass analysis. The mass spectrum shows ions originating from α- and β-avoparcin (at *m/z* = 955 and 972, respectively), the [*M*+12] Da adducts of α- and β-avoparcin (at *m/z* 961 and 978, respectively), and of the noncovalent complexes of α- and β-avoparcin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser (at approximately *m/z* 1150 and 1167). *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser is known generally to be a more weakly binding peptide,[10, 32] mimicking the C-terminus of the peptidoglycan present in some vancomycin-resistant enterococci (VRE) strains.[33] Again, no ions could be detected for the noncovalent complexes of the modified [*M*+12] Da adducts (their expected *m/z* values are indicated by the dashed arrows in Figure 4). ESI-MS bioaffinity results on other [*M*+12] Da and [*M*+26] Da adducts of vancomycin, eremomycin, and α- and β-avoparcin all revealed that these modifications resulted in a

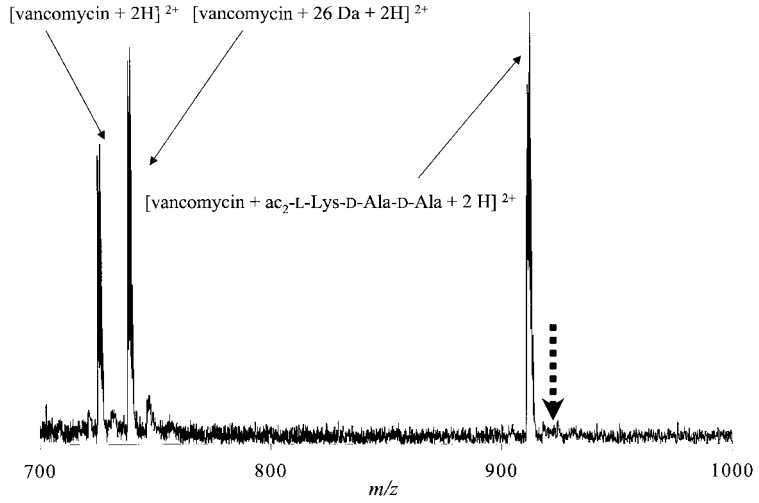


Figure 3. ESI-MS spectrum of a 20 μM solution of vancomycin, partially modified by treatment with acetaldehyde for 10 h at room temperature, to which 25 μM *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala had been added immediately prior to mass analysis. The mass spectrum shows ions originating from vancomycin (at approximately *m/z* 724), the [*M*+26] Da adduct of vancomycin (at approximately *m/z* 737), and of the noncovalent complex of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (at approximately *m/z* 910). The dashed arrow indicates where a signal would be observed for the noncovalent complex of the [*M*+26] Da adduct of vancomycin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala.

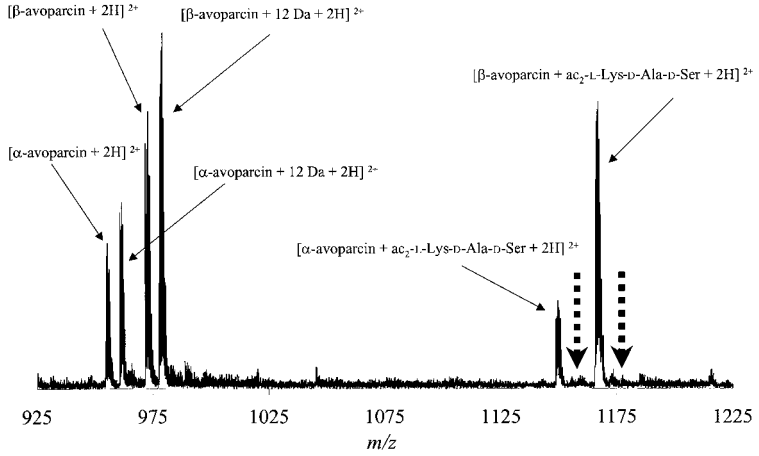


Figure 4. ESI-MS spectrum of a 10 μM solution of avoparcin, partially modified by treatment with formaldehyde for 2 h at room temperature, to which 25 μM of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser had been added just prior to mass analysis. The mass spectrum shows ions originating from α- and β-avoparcin (at *m/z* 955 and 972, respectively), the [*M*+12] Da adducts of α- and β-avoparcin (at *m/z* 967 and 984, respectively), and of the noncovalent complexes of α- and β-avoparcin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser (at approximately *m/z* 1150 and 1167). The dashed arrows indicate where signals would be observed for the noncovalent complexes of the [*M*+12] Da adducts of avoparcin with *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ser.

complete deactivation of the antibiotic towards binding to their natural peptide-mimicking receptors. A range of receptor-mimicking peptides was used, including *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Lac, which is known to be present in the peptidoglycan of the most resistant VRE strains.[11] As expected, neither the unmodified nor the modified antibiotics showed any significant binding towards the latter peptide. The deactivation of the glycopeptide antibiotics by the ring-closure is not totally unexpected in view of the dramatic structural changes in the immediate vicinity of the vancomycin binding pocket. Evidently, the ring closure results in the loss of important hydrogen bonds. In addition, as revealed by the structure of the related antibiotic A82846B bound to the pentapeptide cell wall fragment,[34] the quite flexible leucine moiety at the N-terminus of vancomycin folds around the back of the D-Ala-D-Ala receptor, probably producing stabilizing hydrophobic interactions with the methyl groups in the receptor. In the ring-closed, 4-imidazolidinone-modified vancomycin there should be more stringent geometric constraints around the leucine moiety, weakening the hydrophobic interactions.

Minimum inhibitory values of (un)modified antibiotics: The observed loss of ability to bind to the *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala receptor-mimicking peptide suggests that the modified antibiotics may have lost their antibiotic activity. In order to test this hypothesis, minimum inhibitory concentrations (MICs) were determined for the modified and unmodified antibiotics. Table 1 shows that, for both vancomycin and avoparcin, modification of the antibiotics indeed resulted in a decrease by a factor of two or three in their antibiotic activity against two Gram-positive indicator strains: *Micrococcus flavus* and *Bacillus cereus*. In these experiments, 100% of the vancomycin and at least 75% of the avoparcin had been converted (as determined by ESI mass spectrometry) to the 4-imidazolidinone ring-closed species prior to introduction into the broths. The MIC values of the modified antibiotics suggest that they still exhibit significant antibacterial activity, although they do not exhibit a high affinity for the -D-Ala-D-Ala receptor binding site. Although not studied here, we hypothesize that this may be due to reversible 4-imidazolidinone ring-opening upon addition to the broth or, alternatively, that the modified antibiotics still exhibit antibacterial efficacy.

Table 1. Minimum inhibitory concentrations (MICs) of modified and unmodified vancomycin and avoparcin, determined for two different strains of Gram-positive bacteria.

MICs [μg mL^{-1}]	*M. flavus*	*B. cereus*
vancomycin	0.25	0.75
modified vancomycin	0.75[a]	2.0[a]
avoparcin	0.08	0.08
modified avoparcin	0.16[b]	0.16[b]

[a] When introduced into the broth the vancomycin was 100% converted. [b] When introduced into the broth the avoparcin was 75% converted.

Kinetic studies: To investigate the kinetics of the conversion of vancomycin into the 4-imidazolidinone ring-closed modified species, vancomycin (20 μM) and acetaldehyde (500 μM) were dissolved in a buffered aqueous ammonium acetate (20 mM) solution (pH = 6.7) and kept at room temperature. At selected time intervals, 1 μL of this solution was analyzed by ESI-MS. From these mass spectra, the percentage conversion was calculated, using the integrals of the ion signals of the unmodified and modified [*M*+26] Da vancomycin. In Figure 5, the solid line with circles indicates the percentage conversion of vancomycin into the 4-imidazolidinone ring-closed product as a function of the reaction time. Under these conditions, the determined half-life $t_{1/2}$ of vancomycin was 26 h. This half-life is in qualitative agreement with reaction rates reported for the formation of 4-imidazolidinone adducts of several peptides in similar reactions with acetaldehyde.[22, 23] These reported studies revealed that reaction rates for 4-imidazolidinone adduct formation are highly dependent on temperature and pH, with maximum rates occurring at $6 < pH < 8$ and higher temperatures. Although no exact reaction rate constants were determined, it was found that conversion is significantly faster with formaldehyde than with acetaldehyde. In separate experiments, no significant reactivity was observed when vancomycin was allowed to react under similar conditions with other aldehydes, such as methylglyoxal, benzaldehyde, and hexylaldehyde.

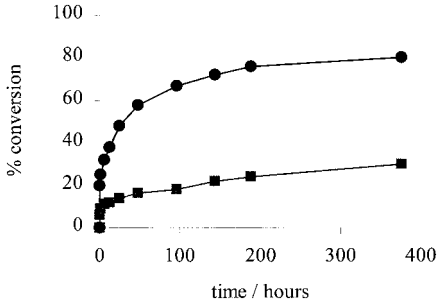


Figure 5. Kinetics of the conversion of vancomycin into imidazolidinone ring-closed vancomycin by reaction with acetaldehyde. The line with circles shows the conversion (as monitored by ESI-MS) in a buffered aqueous ammonium acetate (20 mM) solution (pH = 6.7) of vancomycin (20 μM) and acetaldehyde (500 μM). The line with squares describes the kinetics in a similar solution to which *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (15 μM) had been added at the start.

Inhibition of 4-imidazolidinone formation by binding to receptor: A kinetic study was performed similarly, albeit in the presence of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala. The conversion of vancomycin was studied in an aqueous ammonium acetate (20 mM) solution containing vancomycin (20 μM), *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (15 μM), and acetaldehyde (500 μM). The solid line with squares in Figure 5 shows the percentage conversion as a function of incubation time. It is evident from the data shown that the reaction between acetaldehyde and vancomycin is inhibited by *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala. We hypothesize that the decreased conversion rate stems from the fact that the vancomycin N-terminus is less accessible to attack by acetaldehyde when bound to the *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala ligand. A similar stabilization of vancomycin, by binding to peptidoglycan analogues, with respect to its

0947-6539/01/0704-0914 $ 17.50+.50/0 *Chem. Eur. J.* **2001**, *7*, No. 4

spontaneous isomerization into the degradation product CDP-1 has been reported.[35]

Influence of reaction with acetaldehyde on dimerization of eremomycin: Eremomycin is related to vancomycin, but has a much greater ability to dimerize,[3] which is thought to have a positive effect on its antibacterial efficacy.[4, 36] In contrast to the data for vancomycin, the ESI-MS spectra of eremomycin, when measured from an aqueous buffer at pH = 7.5, are dominated by signals originating from the triply protonated dimer.[3] Previously, we showed that such ESI-MS data can also be used to determine dimerization constants. A dimerization constant of approximately 2×10^6 M^{-1}[3] was found for eremomycin, in agreement with the literature. To investigate whether reaction with acetaldehyde would also occur in the case of eremomycin, we monitored its reaction (40 μM) with acetaldehyde (2 mM) in an aqueous ammonium acetate (20 mM) buffer at pH = 7.5 by ESI mass spectrometry. Figure 6

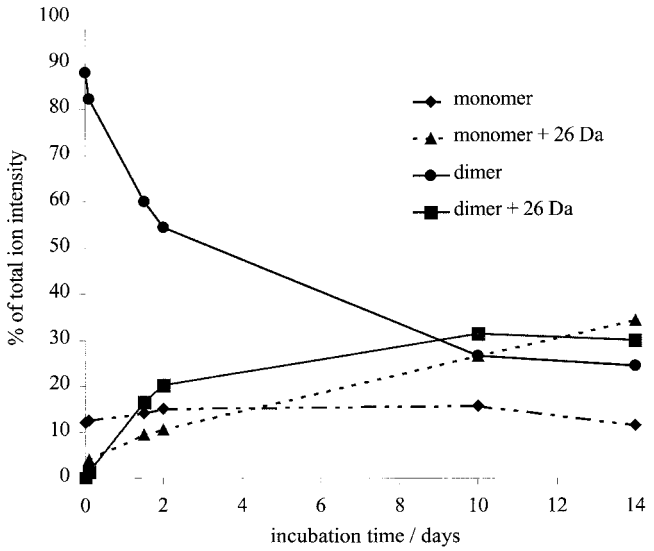


Figure 6. Relative abundance of the ions detected in the ESI-MS spectra of a solution of eremomycin (40 μM) and acetaldehyde (2 mM) in an aqueous buffer at pH = 7.5 as a function of incubation time prior to mass analysis.

shows the relative abundance of the ions observed in the ESI-MS spectra of the eremomycin and acetaldehyde solution as a function of the incubation time. At $t = 0$, only ions originating from the eremomycin monomer and eremomycin dimer were detected. At extended reaction times, new ion signals originating from the eremomycin monomer +26 Da and the eremomycin dimer + 26 Da appeared. No ion signals originating from the dimer with double addition of 26 Da could be detected. Therefore, it is evident that with eremomycin a similar reaction to that with vancomycin occurs. The reaction proceeded rather slowly as compared to vancomycin, albeit that the dimeric species were gradually converted into monomer moieties (with or without the 26 Da addition). By summing all ion signals originating from the monomer species, and all ion signals originating from the dimer species, we may determine pseudo-dimerization constants for eremomycin under these conditions. The determined dimerization constants are shown in graph form in Figure 7. It is clear that the

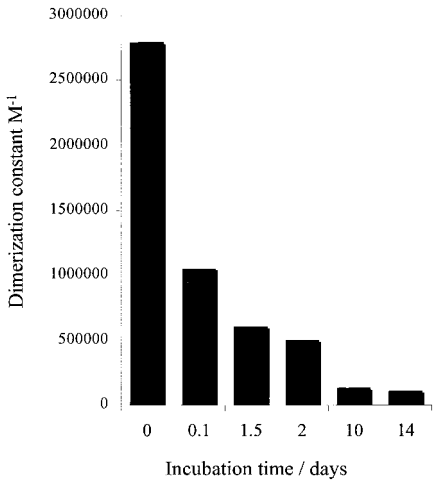


Figure 7. Eremomycin dimerization constants as determined by ESI-MS mass spectrometry (see text for details) in a solution containing eremomycin (40 μM) and acetaldehyde (2 mM) in an aqueous buffer at pH = 7.5 as a function of incubation time prior to analysis.

dimerization constant of eremomycin drops dramatically under these conditions, from a value of approximately 2 700 000 M^{-1} to 100 000 M^{-1} over a time interval of 14 days.

Potential implications: The pharmacological effects of ethanol and its metabolite acetaldehyde have been well described for numerous different drugs.[34] For instance, it is well known that acetaldehyde forms adducts with proteins, thereby altering the functions of mitochondria and of repair enzymes.[37] Additionally, the formation of imidazolidinone rings upon treatment of peptides with aldehydes has been reported for a number of specific, biologically significant, peptides including enkephalins.[22, 25] Indeed, the methodology has been employed for the preparation of aminopeptidase-resistant prodrugs.[22, 23, 25] Since acetaldehyde is a major metabolite of ethanol, and therefore likely to be present in significant levels in the liver and blood following alcohol consumption, a link has been suggested between alcohol intake and the biological activity of certain natural and non-natural peptides.[24] In addition to this, there is speculation that antibodies to imidazolidinone structures could be used to indicate levels of alcohol intake.[24] In view of this discussion, and the findings reported herein, it is possible that a high alcohol intake may reduce the efficacy of vancomycin and related antibiotics. Additionally, the spontaneous modification of vancomycin by traces of formaldehyde may have an effect on the efficiency of vancomycin, and related antibiotics, when they are used as chiral selectors in HPLC or CE, an area in which there is growing interest. These results indicate that the use of organic solvent containing traces of formaldehyde or acetaldehyde as eluent or running buffer may have an effect on chiral separation.

Conclusion

In conclusion, we have demonstrated that antibiotics of the vancomycin group may be reversibly modified at the N-terminus upon treatment with an aldehyde, through the formation of an imidazolidinone. These modifications result in the

formation of a ring-closed 4-imidazolidinone moiety at the N-terminus of the glycopeptide antibiotic. Bioaffinity mass spectrometry has indicated that this ring-closure results in a dramatically decreased affinity for the D-alanyl-D-alanine receptor. These modified glycopeptide antibiotics also exhibited a reduced efficacy against two tested strains of Gram-positive bacteria. The spontaneous reaction of vancomycin with formaldehyde or acetaldehyde at neutral pH and room temperature may have implications for the clinical use of this class of antibiotics. Additionally, the spontaneous modification of vancomycin by traces of formaldehyde may have an effect on the chiral selectivity of vancomycin, and related antibiotics, when used as chiral selectors in HPLC or CE.

Experimental Section

Chemicals: The commercially available antibiotics vancomcyin and avoparcin were a generous gift from Dr. M. Siegel (Wyeth – Ayerst, NY (USA)). The avoparcin used in this study consisted of a 1:2 mixture of two products—α- and β-avoparcin – -that differed by the substitution of a Cl atom for an H atom on one of the aromatic ring side chains. The 1:2 ratio was confirmed by capillary electrophoresis analysis using UV detection. Eremomycin was supplied by SmithKline Beecham (Harlow (UK)). Formaldehyde used was of reagent ACS grade (37% solution stabilized with 15% methanol, Acros, Geel (Belgium)). Acetaldehyde and *N,N'*-Ac_2-L-Lys-D-Ala-D-Ala were obtained from Sigma (St. Louis, MO (USA)).

Mass spectrometry: Measurements were performed on a tandem quadrupole time-of-flight (Q-Tof) instrument (Micromass Ltd., Manchester (UK)) operating in positive ion mode, equipped with a Z-spray nanoelectrospray source. Nanoelectrospray needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments Inc., Sarasota, Florida (USA)) on a P-97 puller (Sutter Instrument Co., Novato, CA (USA)). These needles were coated with a thin gold layer (approx. 500 Å), using an Edwards Scancoat six Pirani 501 sputter coater. The nano-ES needles were positioned approximately 5 mm in front of the inlet of the mass spectrometer. The potential between the needle and the inlet of the mass spectrometer was typically set to 1100 V, while a voltage of 20 V was set to the cone. Typically, 1 μL of the solutions of the glycopeptide antibiotics (at approximately 15 μM concentration) were introduced into the nanoflow needles for mass analysis. For bioaffinity measurements, samples were prepared in buffered aqueous ammonium acetate (20 mM) solutions.

An electrospray quadrupole mass spectrometer (Platform, Micromass, Manchester (UK)), equipped with a triaxial probe, was used for CE-MS analysis. The sheath flow was of 20 mM ammonium acetate in water/methanol (1:1) with 0.1% acetic acid at a flow rate of 4 $\mu L\,min^{-1}$, using dry nitrogen as nebulizing gas. It was necessary to use a higher pressure for the injection (40 kPa, 0.4 min.) when the CE was coupled to the mass spectrometer. A potential of +30 kV was applied across the capillary. The spray voltage at the mass spectrometer was approximately 4.00 kV.

Determination of antibiotic minimum inhibitory concentrations: Minimum inhibitory concentrations (MICs) of *Micrococcus flavus* DSM 1790 and *Bacillus cereus* P7 were determined by serial dilution in Mueller – Hinton (Oxoid) broth for *M. flavus* or DN broth for *B. cereus*. In Microtiter plates (U-form uncoated, Greiner) 1:1 dilution series of the appropriate antibiotic were made, and each was well inoculated with a fresh culture of bacteria (final $1-2 \times 10^5$ CFU mL^{-1}). The total volume per well was 200 μL. The plates were incubated overnight at 30 °C prior to recording MICs.

Acknowledgement

We would like to acknowledge M. Siegel (Wyett-Aherst, Pearl River (USA)) for avoparcin and vancomycin, and T. Staroske and D. H. Williams (Cambridge University (UK)) for Ac_2-L-Lys-D-Ala-D-Ser. P.J.B. and A.J.R.H. acknowledge the NWO (Dutch Science Foundation) for continuous support. A.J.R.H. and M.W. thank the British Council in the Netherlands for support through the UK – Dutch joint scientific research program (Grant JRP555).

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Received: July 17, 2000 [F2604]