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Nuclear Magnetic Resonance Studies on the Antibiotic Avoparcin

Conformational Properties in Relation to Antibacterial Activity

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SUMMARY

Avoparcin is a commercially important glycopeptide antibiotic which is active against Gram-positive bacteria. Recently, β-avoparcin, the major component of the avoparcin mixture, and several other analogues have been structurally characterized and tested for their antibacterial activity. Varying degrees of activity were observed for only minor structural differences. For example, β-avoparcin and epi-β-avoparcin, which differ only in the stereochemistry at position 1′ of the NH_2-terminal phenylsarcosine subunit, exhibit a 10- to 100-fold difference in antibacterial activity. Following the supposition that the conformational properties of these molecules may explain the differences in their antibacterial activity, we have analyzed the conformations of β-avoparcin, epi-β-avoparcin, and other avoparcin analogues in aqueous solutions using NMR methods. On the basis of an analysis of the ^1H chemical shifts, ^1H-^1H nuclear Overhauser enhancement data, and pH titration experiments, it was concluded that the conformations of all of the analogues are similar at the COOH terminus. However, for β-avoparcin and epi-β-avoparcin, conformational differences were observed in the NH_2-terminal region of the molecules. In this paper, we present a detailed description of the over-all conformations of these two glycopeptides as deduced from an in-depth analysis of their corresponding ^1H NMR spectra and discuss the possible relationships this may have with their markedly different antibacterial activities.

INTRODUCTION

Avoparcin, a commercially important animal feed antibiotic, consists primarily of two structurally similar glycopeptides referred to as α and β. On the basis of chemical and spectroscopic evidence, their chemical structures were elucidated as well as the structures of other, more minor, components of the avoparcin mixture (Fig. 1) (1–3). The isolated compounds were found to be structurally similar to other members of this class of antibiotics (vancomycin, ristocetin, and actinoidin) (4) and to have varying degrees of antibacterial activity. Mild degradation products of avoparcin have also been isolated, identified, and tested for antibacterial activity. For example, epi-β-avoparcin, which was formed upon heating an aqueous solution of avoparcin (pH 5–8) at 80° for 16 hr, was found to be a 10- to 100-fold less active than β-avoparcin against Gram-positive bacteria. The only difference in structure between these two molecules is in the stereochemistry of the α-methine (1′) position of the NH_2-terminal phenylsarcosine subunit. Using the method of Lomakina as described by Harris and Harris (5), β-avoparcin was found to have the R configuration and epi-β-avoparcin the S configuration at position 1′ (6).

Although the structures of β-avoparcin and its analogues have been well characterized, little is known about the conformational properties of these molecules. The conformational features of these glycopeptides may be important in determining their antibacterial activities by affecting the manner in which these molecules interact with mucopeptide precursors of bacterial cell walls, their proposed site of action (4). In previous studies using NMR methods, the conformations of related glycopeptide antibiotics (vancomycin and ristocetin) have been elucidated in dimethyl sulfoxide (7, 8). The studies have shown that these two structurally related antibiotics have similar conformations at the COOH-terminal end of the molecules. At the NH_2 terminus, however, the amino group of ristocetin A is in a relatively fixed position owing to the structural constraints imposed by the phenyl ether linkage. In contrast, the NH_2-terminal end of vancomycin is not covalently linked in the same manner and is free to adopt several different conformations.

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In the present study, we have undertaken a detailed conformational analysis of β-avoparcin and its analogues in aqueous solution using 1- and 2-dimensional ^{1}H NMR methods at 500 MHz. Our investigations have focused on two of the avoparcin antibiotics, β-avoparcin and epi-β-avoparcin. These two molecules exhibit significant differences in antibacterial activity for only minor differences in structure and thereby provide an excellent probe for investigating the structure-activity relationships of the glycopeptide antibiotics.

EXPERIMENTAL PROCEDURES

NMR samples were prepared in ^2H_2O at concentrations of 1–4 mM. The pH of the samples was adjusted with NaOD and DCL and is reported as the uncorrected meter reading. All ^{1}H NMR spectra were recorded on a Bruker WM-500. Typical acquisition parameters for 1-dimensional spectra were as follows: 4500 Hz sweep width, 16K data points, 90° pulse angle, and a 2.0-sec repetition rate. Chemical shifts are reported relative to 2,2-dimethyl-2-sila-5-pentane sulfonate, which was used as an external reference.

For the ^{1}H-^{1}H NOE[3] experiments (9), a presaturation pulse of low power (20×10^{-5} W) was applied to a particular resonance prior to accumulation of the FID. After collecting and storing eight transients, a different resonance was irradiated and the data were stored in a separate location of memory. Similarly, after several of the resonances were irradiated, a control spectrum was recorded after presaturating at a frequency where no resonance appeared in the spectrum. This procedure was recycled several times (typically 128, therefore 1024 scans for each irradiation frequency). To process the data, the control FID was subtracted from the other FIDs that were collected, and the difference FID was exponentially multiplied (line broadening = 2 Hz), Fourier-transformed, and phase-corrected. Using this method, only the changes in intensity were observed, and the effects of long-term magnet instabilities were minimized. A presaturation time for obtaining good signal-to-noise without significant contributions from spin diffusion effects was found to be 0.8–1.0 sec. No effort has been made to extract accurate internuclear distances from the observed NOEs. Rather, the observation of an NOE was used to identify those protons in close proximity (<2.7 Å).

Two-dimensional SECSY (10) was performed using a (90°-½t_1-90°-½t_1-acquire)n pulse sequence as previously described (10). In a typical experiment, a total of 128 FIDs containing 4K data points were collected for each increment of t_1. After zero-filling, the free induction decays were multiplied by a shifted sine-bell window function in both dimensions before being Fourier-transformed. Using the above parameters, a digital resolution of 1.22 Hz/pt in the f_1 dimension and 1.00 Hz/pt in the f_2 dimension was obtained.

RESULTS

Figure 1 depicts the structures of the avoparcin analogues and their corresponding antibacterial activities. As a first step in the elucidation of their solution conformations, the ^{1}H resonances of the compounds shown in Fig. 1 must be assigned. These assignments were made on the basis of an analysis of the scalar coupling, ^{1}H homonuclear decoupling, NOE difference, 2-dimensional SECSY, and chemical shift comparisons between the compounds. An exhaustive treatment of these experimental results is not presented herein. Instead, in this paper, we have chosen to highlight the NMR methods employed and present some of the ^{1}H data that illustrate the important conformational features of the compounds studied.

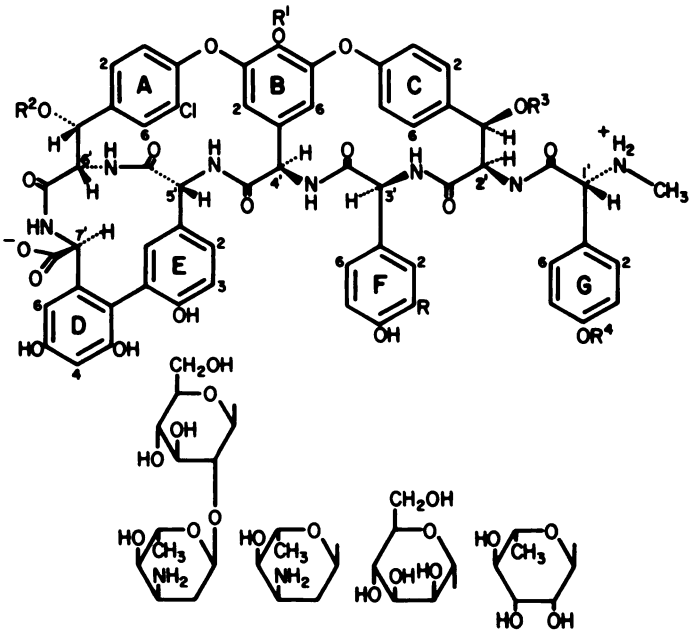
Figure 2 illustrates the use of the 2-dimensional SECSY experiment which was used to establish the scalar coupling (J) connectivities between the resonances. A response in this data set is represented by cross-peaks in the contour map corresponding to resonances on the central horizontal line representing the normal 1-dimensional spectrum. The *dotted lines* connecting the cross-peaks identify those protons that are scalar-coupled. For comparison, a 1-dimensional spectrum of β-avoparcin has been plotted on the same scale below the contour map. In the 2-dimensional data set, a small f_1 window was chosen to optimize resolution and data acquisition time. As a consequence, some of the peaks appear as foldovers but they can still be analyzed. For example, the peak corresponding to the anomeric proton of ristosamine (R^{1b}) should be located at the top of the contour plot and connected to the peak corresponding to the CH_2 protons (R^{1b}-2) of ristosamine (also folded over), which should be at the bottom of the plot. Other foldover peaks include R^1-5, R^1-6, R^4-5, and R^4-6. A particular advantage of this method, compared with the conventional techniques for determining the scalar connectivities, is that the SECSY experiment does not require the application of a selective homonuclear decoupling pulse, which is often technically difficult in complicated regions of the spectra. Furthermore, the 2-dimensional technique identifies all scalar connectivities within a single experiment.

To assign the protons that are in close proximity but do not experience a through-bond scalar interaction or which exhibit only small scalar couplings, ^{1}H-^{1}H NOE methods were employed. An example of two such data sets is shown in the difference spectra of Fig. 3A and B. The *arrows* indicate the sites of preirradiation and the negative peaks correspond to the ^{1}H resonances which experience an NOE effect. For comparison, the normal 1-dimensional spectrum is shown in Fig. 3C. From these data the C_{BZ}, C_2, 2′, and R^3-1 protons could be assigned.

Procedures such as those described above were used to assign the ^{1}H chemical shifts for the avoparcin analogues shown in Fig. 1. All of the compounds (α-avoparcin, β-avoparcin, ε-avoparcin, β-avoparcin-CDP-I, and epi-β-avoparcin) were found to have similar ^{1}H chemical shifts for the protons located at the COOH terminus, suggesting that the structural differences among these compounds do not have a significant effect on the conformational features at the COOH-terminal end of these molecules. In contrast, the ^{1}H chemical shifts of the protons at the NH_2-terminus (C, F, and G rings, C_{BZ}, NCH_3) of β-avoparcin and epi-β-avoparcin were very different (Table 1), reflecting different conformations at the NH_2-terminal end of these molecules.

In addition to aiding in the ^{1}H resonance assignments, ^{1}H-^{1}H NOE experiments provided detailed conformational information. Figure 4A depicts an NOE difference spectrum in which the 6′ proton of β-avoparcin-CDP-I was irradiated. Based on the negative NOEs observed, the conformation at the COOH-terminal end of the molecule must satisfy the condition that 6′ is near A_6, E_6, A_{BZ}, and 5′ as shown in the structure *inset* to the

[3] The abbreviations used are: NOE, nuclear Overhauser enhancement; FID, free induction decay; SECSY, spin-echo correlated spectroscopy.



COMPOUND	R^1	R^2	R^3	R^4	R	MIC(μG/ML)
β-AVOPARCIN	"	"	"	"	Cl	2
EPI-β-AVOPARCIN*	"	"	"	"	Cl	128
α-AVOPARCIN	"	"	"	"	H	4
ε-AVOPARCIN	"	"	H	"	Cl	2
β-AVOPARCIN-CDP-I	H	"	"	H	Cl	0.5

*EPIMERIZED AT THE 1' POSITION

FIG. 1. *Chemical structures and antibacterial activities (minimum inhibitory concentration in micrograms per milliliter for Staphylococcus Smith) of the glycopeptide antibiotics used*

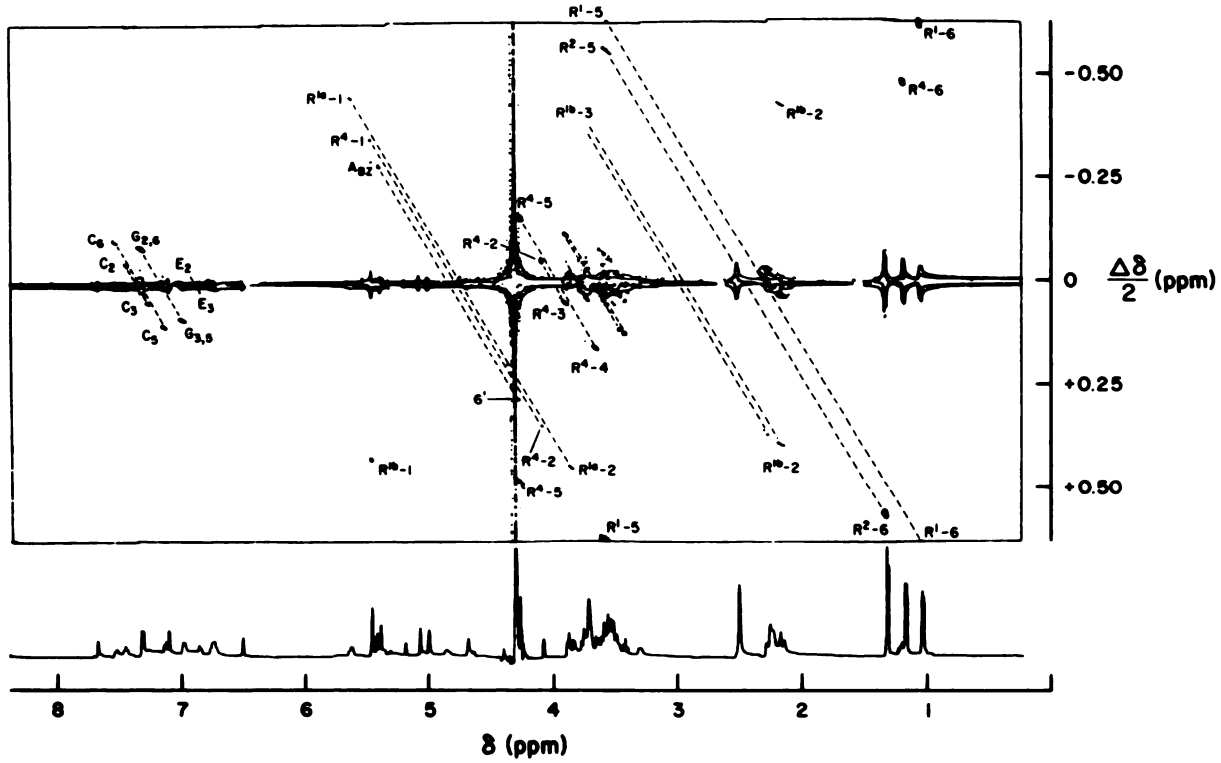


FIG. 2. *Two-dimensional contour plot of β-avoparcin (4 mM) acquired by SECSY*

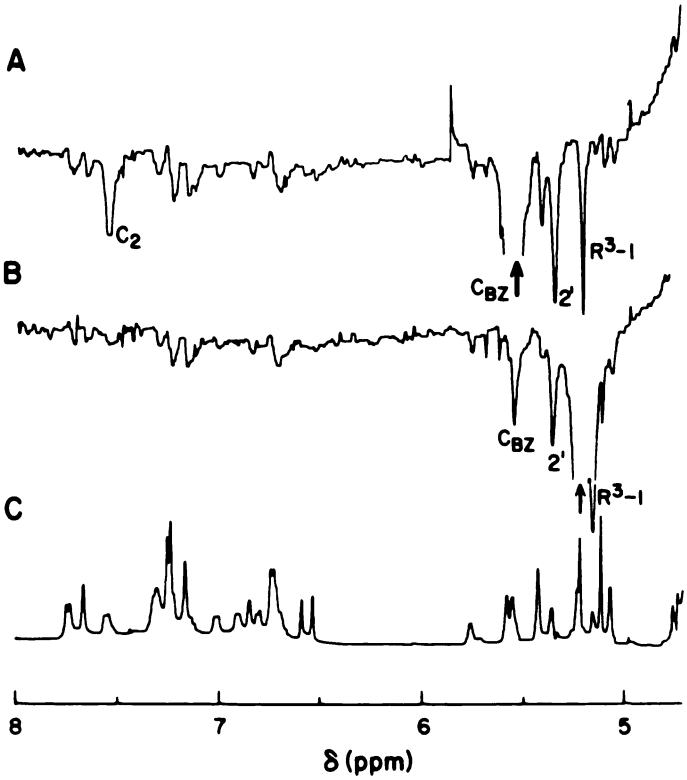


FIG. 3. *NOE and NMR spectra of β-avoparcin-CDP-I*

A and B. ^1H-^1H NOE difference spectra (500 MHz) of β-avoparcin-CDP-I (4 mM) in ^2H_2O at 40°. The *arrows* indicate the position of the irradiation frequency. The control irradiation frequency was to the far left of the spectrum (not shown). C. ^1H NMR spectrum of β-avoparcin-CDP-I acquired under the same conditions.

right of the spectra. Although similar NOEs were observed for all of the molecules at the COOH terminus, differences were observed for the NOEs displayed by β-avoparcin and epi-β-avoparcin for the protons near the NH_2 terminus. For example, when the $G_{2,6}$ protons of epi-β-avoparcin were irradiated, an NOE was observed at $G_{3,5}$, NCH_3, and the mannose protons, R^3-4 and R^3-6, suggesting a close proximity of the mannose to the G-ring. In contrast, for β-avoparcin no NOE was observed for the mannose protons when $G_{2,6}$ was irradiated. Furthermore, the NOE data for β-avoparcin suggests that the F_2 protons are in close proximity to C_6. An NOE between F_2 and C_6 was not observed, however, with epi-β-avoparcin. These results clearly reflect differences in the conformations at the NH_2-terminal end of the molecules. In particular, the orientation of the mannose sugar with respect to the NH_2-terminal side chain (NCH_3 and G-ring) is different in β-avoparcin and epi-β-avoparcin, and the F-ring exhibits a preference for the pseudoaxial conformation in the 16-membered ring containing the amino acids B, C, and F in β-avoparcin and for the pseudoequatorial conformation in epi-β-avoparcin.

Figure 5 depicts the pH dependence of the chemical shifts of the NCH_3 resonance for β-avoparcin and epi-β-avoparcin. A pK_a of ~6.5 was calculated for the NCH_3 group of β-avoparcin, whereas the pK_a for the same group in epi-β-avoparcin was found to be shifted ~1 pH unit to the alkaline. These results indicate that the NCH_3 group experiences a different chemical environment in β-avoparcin as compared with epi-β-avoparcin and provide further evidence in support of a difference in their conformations at the NH_2 terminus.

DISCUSSION

The conformational properties of β-avoparcin and all of its analogues were found to be similar at the COOH-terminal end of the molecules. Based on the observed NOEs (Fig. 4), the 6′, 5′, E_6, A_{BZ}, and A_6 protons must be on the same face of the molecules in an orientation pictured in the *inset* to the right of Fig. 4 with a *cis*-amide linkage between amino acid residues 5 and 6. It is perhaps noteworthy that the same COOH-terminal conformation was found for vancomycin (7), ristocetin (8), and β-avoparcin (3) in dimethyl sulfoxide, a factor which may be correlated with their related biological activity.

Although the geometries of the molecules were found to be similar at the COOH terminus, the conformational properties of β-avoparcin and epi-β-avoparcin are very different at the NH_2-terminal end. The NOE data suggest that the G-ring of the NH_2-terminal side chain of epi-β-avoparcin is in close proximity to the mannose, which is not the case in β-avoparcin. In addition, the higher pK_a of the NCH_3 group observed with epi-β-avoparcin indicates that it is more difficult to pull off these protons, as would be the case if the protonated NCH_3 group of epi-β-avoparcin were involved in additional interactions, possibly with the mannose. This hypothesis is supported by our NOE data which indicate the close proximity of the mannose to the NH_2-terminal

TABLE 1

Chemical shifts (δ) of the ^1H resonances for β-avoparcin and epi-β-avoparcin (2 mM) in ^2H_2O (pH ~3) at 50°

Peak	β-Avoparcin	Epi-β-avoparcin
	ppm	*ppm*
NCH_3	2.71	2.64
6′	4.37	4.40
5′	4.76	4.73
R^2-1	5.09	5.10
R^3-1	5.21	5.13
2′	5.33	5.48
A_{BZ}	5.47	5.49
R^4-1	5.54	5.77
C_{BZ}	5.54	5.41
R^{1b}-1	5.54	5.58
R^{1a}-1	5.68	5.71
E_3	6.96	6.96
E_2	7.05	7.05
$G_{3,5}$	7.08	7.38
$G_{2,6}$	7.44	7.56
C_6	7.78	7.57
A_6	7.76	7.82
C_2	7.62	7.41
E_6	7.19	7.22
C_3	7.42	7.27
C_5	7.26	7.41
R^2-6	1.41	1.42
R^4-6	1.27	1.30
R^{1b}-6	1.12	1.13

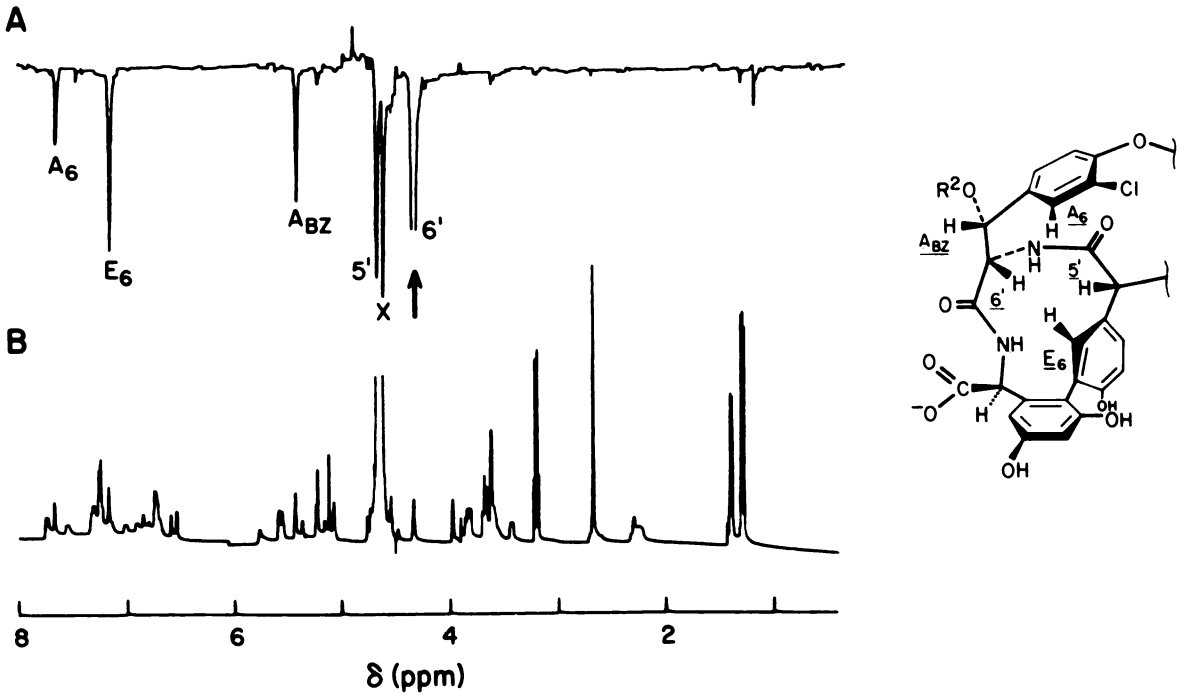


FIG. 4. *NOE and NMR spectra of β-avoparcin-CDP-I*

A. ^1H-^1H NOE difference spectrum (500 MHz) of β-avoparcin-CDP-I (4 mM) in ^2H_2O at 40°. The *arrow* indicates the position of the irradiation frequency. The assignment of the resonances is below the difference spectrum and corresponds to the protons shown to the right of the spectra. B. ^1H NMR spectrum of β-avoparcin-CDP-I acquired under the same conditions.

side chain of epi-β-avoparcin. A more detailed analysis of the structure is complicated, however, because of the flexibility of the NCH_3 side chain, which may allow for a family of conformational states. Nevertheless, at least two of the possible conformations support our data and place the mannose in close proximity to the G-ring and amino moiety of epi-β-avoparcin: one in which the amino group is facing away from the network of aromatic rings and another in which the amino moiety is facing toward the rings.

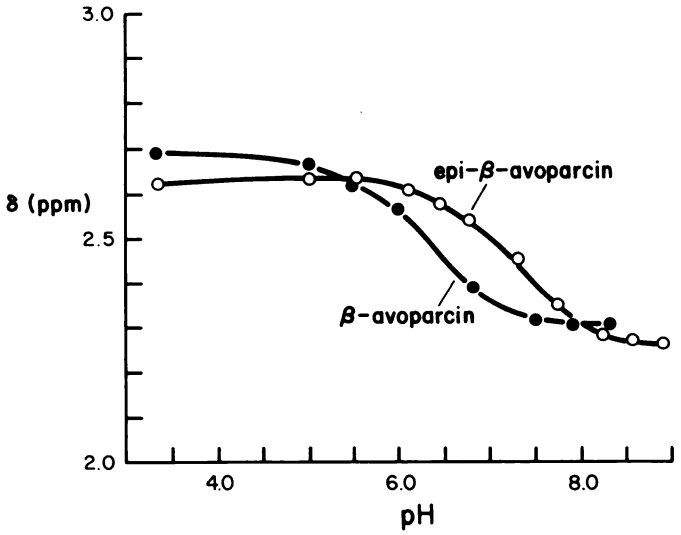


FIG. 5. *^1H chemical shift versus pH for the NCH_3 protons of the NH_2-terminal end of β-avoparcin (●) and epi-β-avoparcin (○)*

In an effort to determine whether the mannose is responsible for the large difference in pK_a observed between β-avoparcin and epi-β-avoparcin, a pH titration experiment was performed with a mixture of ε-avoparcin and epi-ε-avoparcin. The compounds of this mixture, which were formed upon heating a solution of ε-avoparcin (pH 6.5) at 75° for 18 hr, have the same stereochemical differences as β-avoparcin and epi-β-avoparcin at position 1′, but lack the mannose residue. At a pH of 2.70, the chemical shifts for the NCH_3 protons of ε-avoparcin (2.70 ppm) and epi-ε-avoparcin (2.65 ppm) are only slightly different. As the pH of the mixture is increased to 5.6, an upfield chemical shift is observed for the NCH_3 resonances from both compounds. Both begin to titrate at the same pH, with ε-avoparcin exhibiting a slightly greater chemical shift sensitivity to pH. Although the exact pK_a values could not be determined because of precipitation of the compounds (pH ~6.6), the pH titration curves, as far as they could be measured, appeared very similar. These data support the equivalence, or near equivalence, of their pK_a values and substantiate our earlier hypothesis that the large differences in pK_a observed for the NCH_3 groups of β-avoparcin and epi-β-avoparcin are due to the presence of the mannose.

It is also interesting to note that, for all of the avoparcin analogues that contain a mannose, the epi-diastereomer is the preferred product (70/30) under conditions in which epimerization occurs (80°, pH 5–8). However, for ε-avoparcin, which lacks mannose, a 50/50 mixture

of diastereomers is formed under similar conditions (6). The displacement from the equilibrium mixture observed for the mannose-containing avoparcin analogues directly implicates the mannose in altering the NH_2-terminal conformations of β-avoparcin and epi-β-avoparcin.

CONCLUSIONS

From extensive NMR experiments with β-avoparcin and its analogues, the conformations for all of the molecules at the COOH terminus were found to be similar and resembled that reported for vancomycin and ristocetin, suggesting a common structural feature required by all of the glycopeptide antibiotics. In contrast, the NH_2-terminal conformations of β-avoparcin and epi-β-avoparcin were found to be very different and appear to be related to the differences in the orientation of the NH_2-terminal side chain with respect to the mannose. The differences in the NH_2-terminal conformations observed between epi-β-avoparcin and β-avoparcin could alter their ability to interact with bacterial mucopeptides and thus explain their differences in antibacterial activity. This possibility is investigated further in the following paper (11), where we have studied the interactions of β-avoparcin and epi-β-avoparcin with acetyl-D-alanyl-D-alanine and diacetyl-L-lysyl-D-alanyl-D-alanine, which serve as models for mucopeptide precursors.

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