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Structure of Alamethicin in Solution: Nuclear Magnetic Resonance Relaxation Studies†

Utpal Banerjee and Sunney I. Chan*

ABSTRACT: An NMR relaxation study at 500 MHz of the icosapeptide antibiotic alamethicin is reported. This study lends further support to the partly helical, partly extended, amphiphilic, and dimeric structure recently proposed for this peptide in methanolic solutions [Banerjee, U., Tsui, F. P., Balasubramanian, T. N., Marshall, G. R., & Chan, S. I. (1983) *J. Mol. Biol. 165*, 757]. The *N*-acetyl methyl groups toward the N terminus of alamethicin in this solvent system were found to exhibit unusual NMR relaxation behavior. The decay of the transverse magnetization due to these protons was nonexponential, but the spin–lattice relaxation recovery of the longitudinal magnetization was exponential. In a solution saturated with urea, however, both decays were exponential. These observations are shown to be consistent with the proposed structure. Studies in water yielded qualitatively similar but more complex results. The transverse relaxation times suggest further aggregation in water and indicate that the larger aggregates in water may be made up of the smaller units observed in methanol.

In our previous paper of this series (Banerjee et al., 1983), we reported the 500-MHz NMR spectra of the icosapeptide alamethicin (Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol) in two solvent systems, viz., methanol and water. Two-dimensional NMR was used in combination with double-resonance experiments to obtain a complete assignment of the resonances to the protons in the molecule. Our work confirmed and extended the earlier NMR results of Martin & Williams (1976) and Davis & Gisin (1981). From the peptide amide coupling constants and two-dimensional nuclear Overhauser effect (NOE) results, we deduced the conformation of the molecule in methanol that was consistent with these data as well as a line of other experimental observations.

The proposed dimeric structure in methanol is partly extended and partly helical. In accordance with the values of the NMR coupling constants and two-dimensional NOE results, in our model (a) the amide protons of residues 15–20 are intermolecularly hydrogen bonded with the corresponding residues of the opposing molecule to create a rigid, extended parallel β-pleated structure for the C-terminal end of the molecule, (b) the proline at position 14 breaks the continuity of this structure, and amino acids 10–14 are forced into an open, non-hydrogen-bonded conformation, and (c) amino acids 3–9 are folded into an α helix, with the Gln-7 side chains from the two strands in the right juxtaposition to facilitate a hydrogen bond between them. The resultant structure is highly amphipathic: one face is completely hydrophobic with the aliphatic side chains exposed, whereas the other face is primarily hydrophilic with polar side chains and peptide groups lining the extended β-sheet region.

In the above structure, the two helices at the N-terminal end of the proposed dimer are held rigidly together and are energetically stabilized by a side-chain amide hydrogen bond between the two Gln-7's. Extensive hydrophobic interaction between the side groups in the helical region no doubt contributes to the stability of the structure as well. Since one helical strand must be necessarily twisted slightly with respect to the other in this structure, an interesting consequence is that the two acetyl methyl groups at the N termini of the dimer are inequivalent in terms of the angle subtended by them with respect to the long axis of the dimer. Since this is the principal rotor axis, it follows on the basis of the Woessner et al. (1969) treatment of the relaxation of symmetric top methyl groups attached to the side chain of an anisotropically tumbling ellipsoid that the two sets of *N*-acetyl methyl protons should exhibit different spin–spin relaxation times (T_2's). The C termini of the dimer are also inequivalent, but since the individual units are related to one another by a 2-fold screw axis, the side groups in this part of the molecule are symmetrically displaced with respect to the principal rotor axis of the aggregate. Protons from the two aromatic rings on the individual strands, for example, subtend the same angle to the rotor axis and should therefore exhibit equal spin–spin relaxation times.

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In this paper, we report NMR relaxation measurements that are consistent with the above expectations. We have undertaken T_1 and T_2 experiments for selected protons of alamethicin in methanol, in water, and in the presence of urea under conditions that would break up intermolecularly hydrogen-bonded oligomers. On the basis of these relaxation measurements, estimates of the correlation times and orientations of the acetyl groups in the molecule have been obtained.

Materials and Methods

Alamethicin used in this study is a generous gift of Dr. G. B. Whitfield, Jr., of the Upjohn Co. The experiments were repeated and confirmed with the highly purified "fraction 4" kindly supplied by Professors G. R. Marshall and T. M. Balasubramanian and with alamethicin purchased from PHLS England. D_2O was purchased from Aldrich Chemical Co. Inc., and CD_3OD was from Merck, Inc. Perdeuterated urea was prepared by repeated evaporation of a solution in D_2O.

All solutions of alamethicin were made in NMR tubes to prevent loss in handling. Typically, the concentration of the peptide used was in the millimolar range. The measured pD value of an alamethicin solution was typically 6.84 in CD_3OD and 6.52 in D_2O. In view of the closeness of these values to neutrality, no pD adjustments were made. Prior to T_2 measurements, oxygen was eliminated from the solution by bubbling dry gaseous nitrogen into the sample. Samples for T_2 measurements in the presence of urea were made by dissolving alamethicin in saturated solutions of perdeuterated urea in CD_3OD or D_2O.

The 500-MHz NMR spectra were recorded on a Bruker WM 500 spectrometer operating at a field strength of 11.74 T, and 200-MHz NMR spectra were acquired on a Varian XL-200 spectrometer operating at a field strength of 4.69 T. All spectra were recorded at 298 K. NMR spectra were acquired in the Fourier-transform (FT) mode by using quadrature-phase detection and a cycling time between pulses of 5 s. 32K and 8K data points and spectral widths of 6000 and 2500 Hz were used to acquire the 500- and 200-MHz spectra, respectively.

The Carr–Purcell–Meiboom–Gill (CPMG) repetitive two-pulse sequence (Farrar & Becker, 1971) $90°_x[-\tau-180°_y-\tau]_n$ was used for the measurement of spin–spin relaxation time (T_2). The values of τ used were 0.5 and 2 ms for the experiments at 500 and 200 MHz, respectively. The advantages of the CPMG method over the simple Hahn echo technique are the following: (a) the short value of τ minimizes the effects of molecular diffusion on the measurement of T_2; (b) the phase shift in the 180° pulse with respect to the 90° pulse eliminates errors introduced by inaccuracies in the measurement of the length of the 180° pulse. Using this technique, echo signals were generated at increasing time intervals. When a NMR peak is describable by a single spin–spin relaxation time (T_2), the intensity derived from Fourier transformation of the echo signal is expected to decay exponentially with a time constant T_2 (Farrar & Becker, 1971), i.e.

$$I(t) = A \exp(-t/T_2)$$

where $I(t)$ is the intensity of the resonance derived from Fourier transformation of the nth echo signal and $t = 2n\tau$ for the nth echo. For a composite peak described by two component T_2's, the time decay of total intensity is nonexponential and may be described by

$$I(t) = C_A \exp(-t/T_{2A}) + C_B \exp(-t/T_{2B})$$

The Fourier-transformed spectra were scaled in the "absolute intensity mode". No spectral resolution enhancements or line broadenings were used. Sample tubes were not spun during spectral acquisition to prevent artifactual modulation of T_2's introduced by sample spinning. Accurate measurement of intensity was accomplished by cutting out peaks and weighing them on a Mettler balance.

Spin–lattice relaxation times (T_1) were determined by the inversion recovery method by using the standard $180°-\tau-90°$ pulse sequence. Data manipulation was accomplished by the automatic T_1 determination routine available in the Bruker software.

Results

The proton NMR spectra of alamethicin in methanol and in water have been assigned (Banerjee et al., 1983). In this paper, we emphasize the *N*-acetyl methyl protons at 2.05 ppm and the aromatic ring protons of phenylalaninol centered around 7.2 ppm. As expected, the measured ratio of the intensity of these two peaks is 5:3.

Since the *N*-acetyl methyl protons on the two strands of the proposed dimer are not symmetrically positioned with respect to the principal tumbling axis, they were expected to show differences in spin–spin relaxation times. On the other hand, the more symmetrically disposed aromatic groups at the extended C-terminal end of the molecules should relax with essentially the same time constant. In Figure 1a, we plot the time dependence of the logarithm of the intensity of the acetyl and aromatic protons of alamethicin in methanol as derived from the Fourier transforms of the various echo signals in the CPMG experiment. These measurements were made at 500 MHz. It is clear from the plot that while the contribution to the echo signal from the aromatic protons followed an exponential time course, that from the acetyl resonance was nonexponential, describable only by a minimum of two component T_2's. The number of protons contributing to the slower decay may be ascertained by linear extrapolation of this component to zero time. If the intensity of the signal from the aromatic residues is normalized to five protons, we find that the long time constant component of the acetyl group resonance corresponds to 1.5 protons only. Thus, it seems that half of the *N*-acetyl protons have a significantly longer spin–spin relaxation time than the other half. The acetyl resonance relaxation data were readily fitted to a sum of the two exponentials. A three-parameter constrained fit and a four-parameter unconstrained fit yielded similar results. The relative weights of the two exponentials needed to fit the *N*-acetyl relaxation data are equal, and the average values (from several measurements) of the time constants are 457 and 168 ms. In contrast, the aromatic proton relaxation data were readily fitted to a single exponential with a time constant of 570 ms.

Similar measurements of the spin–spin relaxation times were also made at 200 MHz with methanol as a solvent and at 500 and 200 MHz in water. The aromatic protons showed a normal single-exponential relaxation behavior in all cases while the acetyl resonance showed two component T_2's in both solvents and at both fields. The two T_2's in methanol at 200 MHz are 465 and 153 ms. In water, however, additional aggregation reduces the T_2's further, and the corresponding values at 500 MHz are 213 and 96 ms. Whereas in methanol the two relaxation components contributed equally to the intensity of the echo signal, in water the situation was more complicated. Within the error limits in the measurement of intensity of broad and overlapping lines, the best least-squares fit to the relaxation data was obtained when the relative contribution of the slower relaxation component (longer T_2) is 1.56 times that of the faster one.

The above results may be taken as direct evidence in support of our dimer model for alamethicin in methanolic solutions.

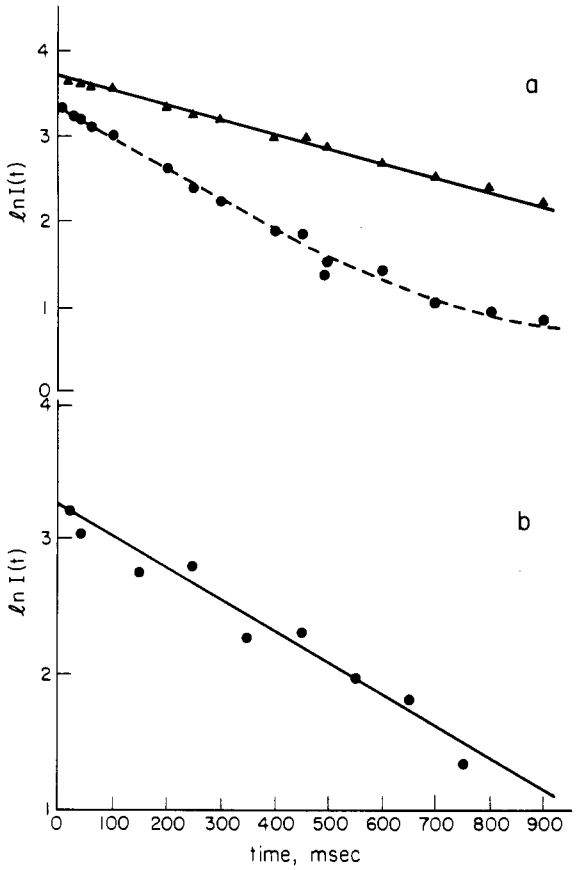

FIGURE 1: (a) Semilog plots of the intensity of the *N*-acetyl methyl protons (●) and the phenylalaninol aromatic protons (▲) of alamethicin as measured from Fourier-transformed spectra of the echo signals obtained for various total time intervals between pulses in a Carr–Purcell–Meiboom–Gill T_2 experiment. Solvent, methanol; temperature, 298 K. The broken curve for the *N*-acetyl methyl protons gives the results of a least-squares fit of the T_2 data to the sum of two exponentials: $I(t) = C_A \exp(-t/T_{2A}) + C_B \exp(-t/T_{2B})$. The values of the best-fit parameters are $C_A = 12.9$, $C_B = 14.2$, $T_{2A} = 457$ ms, and $T_{2B} = 168$ ms. (b) Effect of a saturated solution of urea in methanol on the decay of the transverse magnetization of the *N*-acetyl methyl protons in a Carr–Purcell–Meiboom–Gill (CPMG) experiment. The decay of the transverse magnetization was determined by measuring the intensity of the *N*-acetyl resonance in Fourier-transformed spectra of the echo signal at various total time intervals between the excitation pulses.

We verified that the component T_2's arise from the acetyl residues on the two strands of a tightly bound asymmetric dimer by undertaking similar spin–spin relaxation measurements in a saturated solution of urea at 308 K. The results of this experiment are plotted in Figure 1b. In the saturated urea solution, we found that the contribution to the echo signal from the *N*-acetyl methyl resonance followed a simple single-exponential time course. The slope of this plot indicated a T_2 of 295 ms. A similar experiment in water gave identical results although the fit to the data is not as good, probably on account of incomplete disruption of the secondary structure in this solvent system.

In contrast to the T_2's, the spin–lattice relaxation behavior for the *N*-acetyl methyl group followed a single exponential time course. At 500 MHz, the T_1 for the *N*-acetyl methyl group in methanol was determined to be 1.42 ± 0.1 s.

Discussion

In this work, we have undertaken a relaxation study of the methyl protons of the N-terminal acetyl group of alamethicin. Our NMR data revealed nonexponential decay for the transverse magnetization due to these protons. In methanol, the decay can be decomposed into two exponential components of equal weight, and from the amplitude of these components relative to that of the phenyl ring protons, we surmise that the smallest aggregate unit of the molecule that is consistent with these observations is the dimer in this solvent system. This result reinforces our structural conclusions derived from analysis of the amide coupling constants and the two-dimensional NOE data, including the invariance of the amide coupling constants with the enhanced aggregation noted in water. Another important corollary of the nonexponential T_2 decay observed for the *N*-acetyl methyls, for our picture to be totally self-consistent, is that the dimer must dissociate only slowly on the time scale of these experiments. This slow dissociation is expected for a structure like the one proposed here since there are seven hydrogen bonds, in addition to hydrophobic interactions, holding the two subunits together.

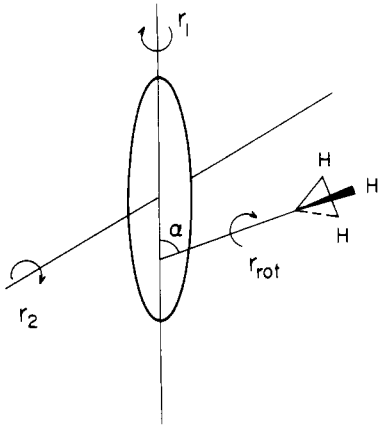

FIGURE 2: Principal axes of rotation of a methyl group rigidly attached to an ellipsoid. r_1, r_2, and $r_{rot.}$ represent the rates of rotation about the axes indicated.

As it was clear from the inspection of a Corey–Pauling–Koltun (CPK) model of the dimer structure proposed, the methyl groups that are attached to the side chains on the N termini of the two molecules are distinct from one another in terms of the angle subtended with respect to the long axis of the aggregate. The two *N*-acetyl groups may be visualized as a pair of rapidly rotating symmetric tops attached by short and rigid stems to a bulky and slowly tumbling prolate ellipsoid. The relaxation behavior of such symmetric top methyl groups attached to an asymmetrically tumbling ellipsoid has been well studied (Woessner et al., 1969). Here, the primary mechanism for relaxation is the magnetic dipole–dipole interaction among the proton spins of the rotor, and the spin–lattice and spin–spin relaxation times are given by (Woessner, 1962)

$$\frac{1}{T_1} = \frac{9}{8}\gamma^4\hbar^2 R_0^{-6}[J_1(\omega_0) + J_2(2\omega_0)]$$

$$\frac{1}{T_2} = \frac{9}{32}\gamma^4\hbar^2 R_0^{-6}[J_0(0) + 10J_1(\omega_0) + J_2(2\omega_0)]$$

where γ is the gyromagnetic ratio of the proton spins, R_0 is the interproton distance, and $J_n(n\omega_0)$ denotes the spectral densities at frequencies $n\omega_0$ ($n = 0, 1, 2$).

In Figure 2, we represent the alamethicin molecule as a prolate ellipsoid and indicate the principal motions that may contribute to the relaxation of the methyl protons. We denote by r_i the rates of reorientations about the specified axes. α is the angle subtended by the axis of a methyl rotor relative to the long axis of the molecule. Following Woessner et al.

(1969), the relevant correlation times are

$$\frac{1}{\tau_A} = 6r_2$$

$$\frac{1}{\tau_B} = r_1 + 5r_2$$

$$\frac{1}{\tau_C} = 4r_1 + 2r_2$$

$$\frac{1}{\tau_{i2}} = \frac{1}{\tau_i} + r_{rot.}$$

where i = A, B, C. A considerable simplification of the Woessner results obtains for the present problem since (i) the relaxation vectors connecting the protons of the methyl group are perpendicular to the rotor axis and (ii) for a macromolecule the size of alamethicin the reorientation rates r_1 and r_2 for the whole molecule are much slower than the rate of fast reorientation of the methyl group, $r_{rot.}$. Under these conditions, the spectral densities are given by

$$J_0(0) = \frac{1}{10}\left[\frac{(3\cos^2\alpha - 1)^2}{6r_2} + \frac{3\sin^2 2\alpha}{r_1 + 5r_2} + \frac{3\sin^4\alpha}{2(2r_1 + r_2)}\right]$$

$$J_n(n\omega_0) = \frac{1}{60}\left\{\frac{(3\cos^2\alpha - 1)^2}{6}\frac{1/r_2}{1 + [n\omega_0/(6r_2)]^2} + 3\sin^2 2\alpha\frac{1/(r_1 + 5r_2)}{1 + n^2\omega_0^2/[(r_1 + 5r_2)^2]} + 3\sin^4\alpha\frac{1/(4r_1 + 2r_2)}{1 + n^2\omega_0^2/[(4r_1 + 2r_2)^2]} + 12\frac{1/r_{rot.}}{1 + (n\omega_0/r_{rot.})^2}\right\}$$

where n = 1, 2.

Given the rotational diffusion times anticipated for the macromolecule, i.e., $r_{rot.} \gg r_1, r_2$, we expect $J_0(0) \gg J_1(\omega_0)$ and $J_2(2\omega_0)$. This is verified experimentally by the observations that (i) $T_1 \gg T_2$ and (ii) T_2's at 200 and 500 MHz for the acetyl resonance are identical within errors of measurement. Also for a long prolate ellipsoid, r_2 is much slower than r_1. Making this assumption and substituting values for the internuclear distance between protons and the gyromagnetic ratio, we obtain the following expressions for T_1 and T_2:

$$\frac{1}{T_1} = \frac{1.5 \times 10^{10}}{r_{rot.}}\left[\frac{1}{1 + (\omega_0/r_{rot.})^2} + \frac{1}{1 + (2\omega_0/r_{rot.})^2}\right]$$

$$\frac{1}{T_2} = (7.9 \times 10^7)\frac{(3\cos^2\alpha - 1)^2}{r_2}$$

We now consider T_1 and T_2 in turn.

T_1. The above T_1 expression in conjunction with the T_1 data for the N-acetyls yields a reorientation rate $r_{rot.}$ of 2.0×10^{10} s^{-1}. This reorientation rate places the motion in the extreme narrowing limit, and a frequency dependence of T_1 is not expected as long as $(\omega_0/r_1)^2$, $(\omega_0/r_2)^2 \gg 1$. This is in accordance with the experimental observations. Finally, since the spin–lattice relaxation rates are dominated by the rapid rotation of the methyl groups, the spectral densities $J_1(\omega_0)$ and $J_2(2\omega_0)$ are independent of α. We have observed rather similar T_1's for all the N-acetyl groups.

T_2. The above expression for $1/T_2$ predicts that T_2 would depend on α, the angle subtended by the rotor axis of the N-acetyl group vis-ã-vis the long axis of the prolate ellipsoid. This angle is not known a priori for either N-acetyl group of the alamethicin dimer. However, a relation between them may be deduced from the T_2 data since

$$\frac{T_{2A}}{T_{2B}} = \frac{(3\cos^2\alpha_A - 1)^2}{(3\cos^2\alpha_B - 1)^2} = \frac{457}{168} = 2.7$$

The angles α_A and α_B can be estimated from a CPK model of alamethicin; they are approximately 70° and 80°, respectively. This would predict a T_2 ratio of ~2, in reasonable agreement with the measured value of 2.7. Substituting these angles in the T_2 expression, we derive $r_2 \sim 1.3 \times 10^7$ s^{-1} and $\tau_A = 1/(6r_2) \sim 1.2 \times 10^{-8}$ s. This correlation time for the reorientation of the molecule about the short axis is in excellent agreement with the value of 1×10^{-8} s predicted for a 45-Å-long ellipsoid in methanol from hydrodynamic theory by using Perrin's equation for long ellipsoids to calculate the frictional coefficients (Perrin, 1934).

Independent confirmation of the above interpretation of the NMR relaxation results has come from the urea experiments. In the presence of urea, the decay of the x–y plane magnetization becomes exponential, indicating that the nonexponential decay in methanol is a consequence of the secondary structure of the molecule in this solvent system. In contrast to the urea results, the relaxation data in water indicate greater complexity. This is not surprising as we expect a further aggregation of the dimer units by the sequestering of the hydrophobic units away from water. A detailed analysis of T_2 in water in terms of motional parameters would involve more correlation times than have been considered for the case of methanol and would not be warranted by our limited data.

Finally, it should be emphasized that we have based our conclusions on relaxation measurements on samples from three different sources including the highly purified fraction 4 obtained from Marshall and Balasubramanian (Marshall & Balasubramanian, 1979; Vodyanoy et al., 1982). All of these alamethicin samples showed nonexponential T_2's in methanol for the terminal acetyl methyl group, thus excluding the possibility of contribution from an impurity.

Conclusions

The NMR relaxation measurements reported in the present study lend further support to the dimeric structure of alamethicin proposed in our recent paper (Banerjee et al., 1983). In the latter work, coupling constant measurements indicated that the N terminus of the molecule (amino acids 1–9) is α helical and that the C terminus (amino acids 15–20) is in an extended β-pleated sheet conformation. The "bend region" (amino acids 10–14) has peptide groups forced into a non-hydrogen-bonded extended structure. Our conclusions regarding the N terminus are consistent with the crystal structure of alamethicin recently reported (Fox & Richards, 1982) as well as recent ^{13}C NMR studies by Jung and co-workers (Oekonomopulos et al., 1982) on analogues of alamethicin. However, there seems to be some ambiguity about the secondary structure of the C terminus. The X-ray crystal structure indicated α helix for the C terminus as well, with the expected break of continuity at Pro-14. The NMR coupling constants measured by us for this end of the molecule (J_{NH-CH} = 7.6–9.1 Hz) are not consistent with an α helix. These data suggest instead that the C terminus exists in a more extended structure. Jung and co-workers (Oekonomopulos et al., 1982) have proposed that the C terminus in alamethicin analogues might be in a more disordered secondary structure, and our present results are largely in agreement with this suggestion. However, we have presented evidence here that alamethicin exists as a dimer in methanolic solution and that

the β-pleated sheet toward the C terminus is most probably stabilized by intermolecular hydrogen bonding between the two opposing molecules.

The disparity between the solution results and the crystal structure may not be so surprising and emphasizes the importance of solvent in determining the secondary structure of peptides. Earlier reflection spectroscopy studies (Fringeli & Fringeli, 1979) have indicated that alamethicin is all helical in the dry state, but the molecule undergoes a helix to β-sheet transition in the presence of hydrated lipids, an observation which we believe can be accounted for simply by the solvation of the peptide. Our structure is consistent with CD measurements in ethanol, which indicated that only 40% of the molecule is α helical (McMullen et al., 1971).

The structure proposed by the NMR studies is highly amphiphilic; it has one face that is completely hydrophobic, while the other is partly lined with polar groups (Banerjee et al., 1983). The additional aggregation observed in water is consistent with these features of the structure. Such an amphiphilic unit may play an important role in the pore formation and antibiotic characteristics of alamethicin.

Acknowledgments

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Registry No. Alamethicin, 59588-86-2.

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