transition-metal systems for their reactivity toward CO_2 through the use of CO_2(l).

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Registry No. $IrCl(N_2)(PPh_3)_2$, 15695-36-0; $IrMe(CO)(PPh_3)_2$, 53248-08-1; $IrPh(CO)(PPh_3)_2$, 25037-18-7; $IrMo(O_2)(CO)(PPh_3)_2$, 82469-62-3; $IrPh(O_2)(CO)(PPh_3)_2$, 53401-83-5; $[RhH(PCy_3)_2]_2(\mu\text{-}N_2)$, 69302-51-8; $[Ni(PCy_3)_2]_2(\mu\text{-}N_2)$, 21729-50-0; $Pd(PCy_3)_2$, 33309-88-5; $Pt(PCy_3)_2$, 55664-33-0; $Mo(CO)_3(PCy_3)_2$, 73690-53-6; $W(CO)_3(PCy_3)_2$, 73690-56-9; $[Co(PPh_3)_3]_2(\mu\text{-}N_2)$, 33541-16-1; $[Co(PEt_2Ph)_3]_2(\mu\text{-}N_2)$, 33540-72-6; $Na[Co(N_2)(PEt_2Ph)_3]$, 33540-73-7; CO_2, 124-38-9; $IrMe(OCO_3)(CO)(PPh_3)_2$, 82469-63-4; $IrPh(OCO_3)(CO)(PPh_3)_2$, 82469-64-5; $Ni(CO_2)(PCy_3)_2$, 57307-01-4; *trans*-$PtH(O_2COH)(PCy_3)_2$, 82469-65-6; $WH(O_2COH)(CO)_3(PCy_3)_2$, 82482-84-6.

Fission Fragment Ionization Mass Spectrometry of Alamethicin I

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Abstract: Positive and negative Cf-252 fission fragment ionization spectra have been obtained for a natural and a synthetic sample of alamethicin I. The positive spectra of the two samples are identical to within an experimental error, and the negative spectra are identical except for the presence of a trace amount of impurity in the natural sample. The spectra provide strong evidence that the natural and synthetic samples are identical and thus that natural alamethicin I has the same structure as the known structure of the synthetic sample. The fragmentation occurring in the positive ion spectra is analyzed, and several series of sequence ions are identified. Reaction mechanisms producing the several series of sequence ions are suggested. Two of the sequence ions series involve addition of sodium ion to the alamethicin I molecule followed by fragmentation with retention of the sodium in the charged fragment.

Alamethicin (ALA)[2] is a peptide antibiotic produced by the fungus *Trichoderma viride*.[3] The primary interest in alamethicin stems from the fact that it affects the electrical properties of artificial bilayer membranes.[4,5] Some controversy has surrounded the elucidation of the structure of natural alamethicin,[6] which is a mixture of closely related compounds. Using the solid-phase technique,[7] one of us has recently concluded the chemical synthesis of the major component of alamethicin (ALA I) and has demonstrated identity of the synthetic with the natural product.[8]

Rinehart and co-workers[9] have made an extensive investigation of alamethicin using various mass spectrometric procedures, and their work provides strong evidence concerning the amino acid sequences in alamethicin I and II. Our main analytical goal in the work reported here is to demonstrate by fission fragment ionization mass spectrometry that separated and purified alamethicin I from natural sources is identical with synthesized alamethicin I. Such a comparison provides further evidence about the structure of natural alamethicin I. Fission fragment ionization mass spectrometry[10,11] has been little used for the determination of the identity and structure of peptides, and consequently we give the results of our findings. In addition, we present details of the novel and interesting ionic chemistry giving rise to the observed spectra.

At present there exists only a very limited set of published data on polypeptides using fission fragment ionization mass spectrometry[10-12] or the related ionization techniques involving bombardment by keV ions[13] and intense laser pulses.[14] Macfarlane and co-workers have given in the open literature the spectra of two peptides, β-endorphin[12] and gramicidin A.[10] The spectrum of the 31-residue peptide β-endorphin covers the mass range from 900 to the quasi-molecular ion region at m/z 3487 ($(M + Na)^+$). This spectrum is remarkable in that a distinct $(M + Na)^+$ ion is observed (signal to noise fluctuation ratio of approximately 20:1), while virtually no fragmentation is seen above m/z 900. The hints of fragment ion peaks that are to be seen have very low signal to noise fluctuation ratios (~1:1). The spectrum given for gramicidin A (15 residues) is restricted to the molecular ion region where $(M + Na)^+$ ions at m/z 1904 and 1918, respectively, are observed for the two variants studied. Macfarlane and co-workers also describe[10] certain aspects of the spectra of γ-glutathione (six residues) and a series of di- and tripeptides.

Positive and negative mass spectra for the tripeptide Ala-Ala-Ala have been obtained in this laboratory.[11] Quasi-molecular ions are observed in the positive ion spectrum, and the fragmentation is representative of the peptide structure. By contrast,

(1) (a) The Rockefeller University. (b) University of Maryland. Deceased January 3, 1982.

(2) Abbreviations: ALA, alamethicin; Aib, α-aminoisobutyric acid; Phol, phenylalaninol.

(3) Meyer, C. E.; Reusser, F. *Experientia* **1967**, *23*, 85–86.

(4) Mueller, P.; Rudin, D. O. *Nature (London)* **1968**, *217*, 713–719.

(5) (a) Eisenberg, M.; Hall, J. E.; Mead, C. A. *J. Membr. Biol.* **1973**, *14*, 143–146. (b) Boheim, G. *Ibid.* **1974**, *19*, 277–303. (c) Boheim, G.; Hall, J. E. *Biochim. Biophys. Acta* **1975**, *389*, 436–443.

(6) (a) Payne, R.; Jakes, R.; Hartley, B. S. *Biochem. J.* **1970**, *117*, 757–766. (b) Melling, J.; McMullen, A. E. ISC-IAMS Proceedings *Sci. Council Jpn.* **1975**, *5*, 446–452. (c) Jung, G.; Dubischar, N.; Leibfritz, D. *Eur. J. Biochem.* **1975**, *54*, 395–409. (d) Gisin, B. F.; Kobayashi, S.; Hall, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 115–119. (e) Marshall, G. R.; Balasubramanian. In "Peptides, Structure and Biological Function"; Gross, E., Meienhofer, J., Eds.; Pierce Chemical Co.: Rockford, IL, **1979**, pp 639–646.

(7) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.

(8) Gisin, B. F.; Davis, D. G.; Borowska, Z. K.; Hall, J. E. Kobayashi, S. *J. Am. Chem. Soc.* **1981**, *103*, 6373.

(9) Pandey, R. C.; Cook, J. C., Jr.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1977**, *99*, 8469–8483.

(10) Macfarlane, R. D.; Torgerson, D. F. *Science (Washington, D.C.)* **1976**, *191*, 920–925.

(11) Chait, B. T.; Agosta, W. C.; Field, F. H. *Int. J. Mass Spectrom. Ion Phys.* **1981**, *39*, 339–366.

(12) Macfarlane, R. D. "Biochemical Applications of Mass Spectrometry, First Supplementary Volume"; Waller, G. R.; Dermer, O. C., Eds.; Wiley: New York, 1980; p 1209.

(13) Benninghoven, A.; Sichterman, W. K. *Anal. Chem.* **1978**, *50*, 1180–1184.

(14) Posthumus, M. A.; Kistemaker, P. G.; Meuzelaar, H. L. C.; Ten Noever de Brauw, M. C. *Anal. Chem.* **1978**, *50*, 985–991.

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the negative ion spectrum yields little structural information, although a moderately intense $(M - H)^-$ ion is observed.

Recently a number of papers have described the use of fast atom bombardment (usually 2–8-keV argon atoms) to obtain the spectra of peptides[15–19] (selected list of leading references).

Experimental Section

The spectra were obtained with the Rockefeller University ^{252}Cf fission fragment ionization time-of-flight mass spectrometer.[11] The ion accelerating voltage was ±10 kV, and the electrostatic particle guide was maintained at ∓12 V with respect to the grounded 3-m flight tube. The Cf-252 source with a strength of ~7 μCi yielded a flux of approximately 1000 fission fragments per second through the sample foil. The ion collection rates ranged between 400 and 650 ions/s, and the acquisition time per spectrum ranged from 3 to 6 h. The ion flight times were accumulated in 18 000 time bins with a width of 6.25 ns. At the conclusion of the data acquisition, the computer examined the data to identify peaks, and the centroids of the peaks other than the quasi-molecular ion peaks were calculated with a three-point algorithm. The centroids of the quasi-molecular ion peaks were calculated with a five-point algorithm. These peak centroid times were converted to ion masses by using the two-constant equation and the procedure described in ref 11.

The samples of alamethicin I were electrosprayed[20] onto thin (6.3 μm) aluminized polyester film (Alexander Vacuum Research, Inc., Greenfield, MA) to a thickness of 10 μg/cm², which corresponds to 5 nmol/cm² of alamethicin I.

In this work two separate samples of alamethicin I were measured. One sample was obtained from a commercial preparation of alamethicin produced by biological processes by the Upjohn Co., Kalamazoo, MI (Upjohn Code U-22324 8831-CEM-93.3). This preparation contains alamethicin I and other compounds, and the alamethicin I was isolated by preparative silica gel chromatography followed by Sephadex G-10 chromatography in ethanol–water.[8] The alamethicin I isolated in this way will be referred to here as natural alamethicin I. The other alamethicin I sample was prepared ab initio by synthetic methods using the solid support peptide synthesis technique. The alamethicin I sample prepared in this way will be referred to as synthetic alamethicin I. The synthesis of this compound, its purification, and its characterization by techniques other than mass spectrometry are given in ref 8.

Results and Discussion

Comparison of the Spectra of Natural and Synthetic Alamethicin I. Because the appearance of fission fragment ionization time-of-flight mass spectra differ in some important ways from spectra obtained with the more familiar magnetic deflection and quadrupole instruments, we shall first discuss briefly some general features to be found in the spectra we shall present. Perhaps the feature that most causes the appearance of the spectra to differ from those of conventional spectra is the fact that isolated peaks often have a shape consisting of a sharp central portion and a winglike or taillike lower portion. Such peaks have quite a distorted shape by magnetic deflection mass spectrometric standards, but these distortions are reproducible in our mass spectrometer; they are characteristic of the material being studied; and they contain potentially useful information. We have shown[21] that the sharp component arises from ions that retain their identity during flight, while the wings, tails, and distortions arise from ions that undergo metastable decompositions in flight.

When many peaks are present in a spectrum, as is usually the case, the broad lower portions overlap and produce a continuous background, which rises to high intensities in the low mass regions.

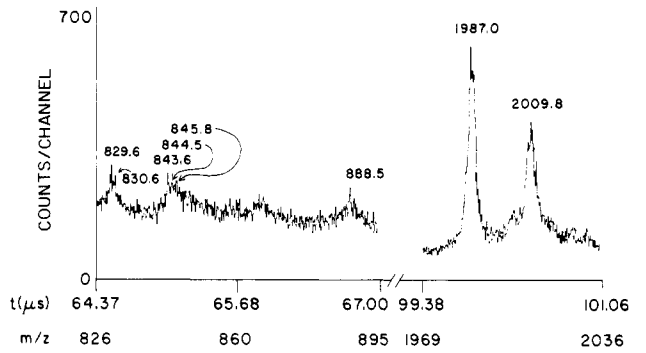


Figure 1. Portions of positive ion time-of-flight spectrum of synthetic alamethicin I. Reproduction of computer graphic output. Left display: portion of fragmentation region; right display: quasi-molecular ion region.

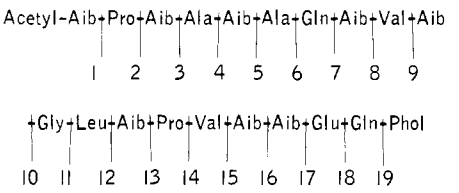


Figure 2. Amino acid sequence of synthetic alamethicin I. Monoisotopic M_r = 1963.10. Aib, aminoisobutyric acid; Phol, phenylalaninol.

The sharp components of the peaks are usually well enough defined so that the flight times corresponding to the peak centroids can be accurately determined in spite of the wings or tails. This is true even if the intensity of the sharp peaks relative to the background is small. We show on the left side of Figure 1 the portion of the synthetic alamethicin I spectrum between flight times of 64.37 and 67.00 μs. The mass spectrometer computer was instructed to recognize as mass peaks channels that have a net intensity greater than 4 times the statistical fluctuation in the background. The statistical fluctuation was calculated as follows. The 18 000 channels in which the mass spectral data are stored are divided into subgroups containing equal numbers of channels, usually 1000. The average number of ion counts per channel is calculated by using the expression $\sum_{i=n}^{i=n+1000} C_i/1000$ (assuming a subgroup of 1000 channels). The distribution of the number of ion counts over the channels may be taken as Poisson, and if for such a distribution the average number of occurrences is μ, the standard deviation of the number of occurrences is $\mu^{1/2}$.[22] Then in our experiments the threshold for accepting ion counts in a channel as significant is $4(\sum_{i=n}^{i=n+1000} C_i/1000)^{1/2} + \sum_{i=n}^{i=n+1000} C_i/1000$.

In the spectrum on the left side of Figure 1, the peaks annotated by m/z values were identified by using this procedure. One observes that for adjacent peaks the masses assigned by the computer differ by values close to unity. Indeed, the average value of the mass excess for the six annotated peaks is 0.5_9 with a standard deviation of 0.1_1. This mass excess is appropriate for ions with the hydrogen atom content that these have. These results are typical, and they lead us to believe that the apparatus consistently identifies real mass peaks even when the signal to noise ratio is as low as that shown in Figure 1. This method of peak identification was used for all of the results given in this paper.

When the mass of the peak being studied is above the inherent resolution of the apparatus, even the sharp components of adjacent peaks overlap, and a mass value that is intermediate between the masses of the component ions is obtained for the composite peak. The synthetic alamethicin I $(M + Na)^+$ QM ion peak shown on the right side of Figure 1 is an example of such an unresolved cluster of peaks. Here the major components are the ^{12}C and the ^{13}C isotopic peaks. The experimental m/z value of this $(M + Na)^+$ peak is 1987.0_3 daltons, which corresponds closely to the mean

(15) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc., Chem. Commun.* **1981**, *7*, 325–327.
(16) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tetler, L. W. *Org. Mass Spectrom.* **1981**, *16* 256–260.
(17) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N.; Whalley, E. T. *Biomed. Mass Spectrom.* **1981**, *8*, 337–342.
(18) Williams, D. H.; Bradley, C.; Bojesen, G.; Santikarn, S.; Taylor, L. C. E. *J. Am. Chem. Soc.* **1981**, *103*, 5700–5704.
(19) Rinehart, K. L.; Gaudioso, L. A.; Moore, M. L.; Pandey, R. C.; Cook, J. C.; Barber, M.; Sedgwick, R. D.; Bordoli, R. S.; Tyler, A. N.; Green, B. N. *J. Am. Chem. Soc.* **1981**, *103*, 6517–6520.
(20) McNeal, C. J.; Macfarlane, R. D.; Thurston, E. L. *Anal. Chem.* **1979**, *51*, 2036.
(21) Chait, B. T.; Field, F. H. *Int. J. Mass Spectrom. Ion Phys.* **1981**, *41*, 17–29.

(22) Parzen, E. "Modern Probability Theory and Its Applications"; Wiley: New York, 1960; Chapter 5.

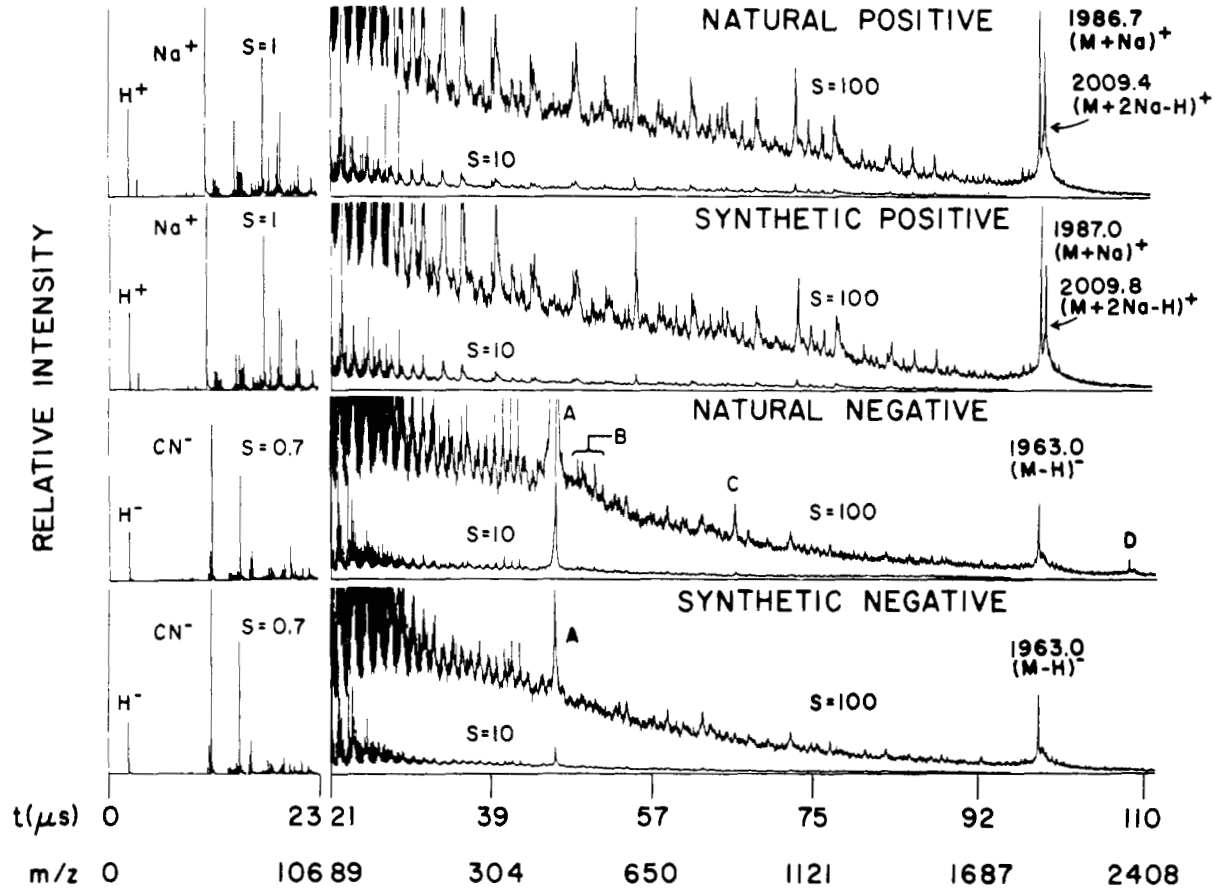


Figure 3. Spectra of alamethicin I. Fourfold compression of mass scale. S = sensitivity of intensity display.

value m/z 1986.9_6 daltons calculated with the three most abundant isotopic components and an appropriate assumed structure for alamethicin I (see below).[23]

To attack the problem of the amino acid sequence of alamethicin I, an amino acid sequence was hypothesized[8] and a compound with this sequence was synthesized as described in the Experimental Section. This sequence is shown in Figure 2, where the peptide bonds are numbers from the N terminal end of the molecule. Positive and negative fission fragment ionization mass spectra were obtained for the synthetic and the natural compounds under the same experimental conditions.

Representations of the four spectra are given in Figure 3. These representations involve 4-fold compressions of the original data in order to depict on a single page spectra that are approximately 2500 daltons wide. In so doing, some detail is lost, but a significant pattern remains.

It is immediately evident from Figure 3 that there is a very high degree of correspondence between the positive ion spectra of the natural and synthetic compounds, and indeed, above m/z 200 the spectra are identical with respect to the peak identities, their relative intensities, and their shapes. Below m/z 200 the peak identities are similarly correspondent; however, a few variations are evident in relative intensities. Most of the peaks for which these variations are observed are produced by inorganic substances. The mass measurement accuracy of the fission fragment ionization method enables one to obtain information on the atomic composition of ions comprising the spectra,[10,11] and we commonly observe the presence of salts as contaminants in organic samples, especially those that have been subjected to certain purification techniques. Thus the few variations that are observed do not refer to alamethicin I itself. The excellent correspondence of the patterns of the positive ion spectra provides evidence that the natural and synthetic alamethicin I samples are identical.

Detailed considerations of the spectra confirm that the samples are identical. A strong, high mass ion is observed in the synthetic alamethicin I spectrum at m/z 1987.0_3 (isotopically averaged mass), which corresponds to a monoisotopic m/z of 1986.1_7. This m/z value is 23.1 daltons higher than the value of the molecular weight of the structure shown in Figure 1 (1963.1_0), and thus the ion is the $(M + Na)^+$ ion. The experimental m/z value for the corresponding ion in the natural alamethicin I is 1986.7_4, which is identical with the value for the synthetic compound to within experimental error. Thus the monoisotopic molecular weight of the natural compound is also 1963.1_0, which constitutes evidence that the amino acid composition of the natural compound is identical with that of the synthetic compound.

Figure 3 (see also Figure 1) shows that the spectra of both samples contain an ion with experimental m/z values of 2009.7_5 and 2009.4_4, respectively, for the synthetic and natural compounds. These are 22.8 and 22.7 daltons, respectively, higher than the m/z values for the $(M + Na)^+$ ions. $(M + 2Na - H)^+$ ions are sometimes observed in fission fragment ionization mass spectrometry,[10,11] and our m/z 2009–2010 ions most likely have this identity. However, such ions should be 22 daltons higher than the $(M + Na)^+$ ions, and our higher experimental mass differences indicate either the occurrence of unexpected experimental errors or perhaps the formation of some $(M + 2Na)^+$ ions, a previously unreported species. However, the ions are without doubt a kind of quasi-molecular ion, and their existence and m/z values provide further evidence that the molecular weight of the natural alamethicin I is 1963.

The fission fragment instrument yields a wealth of detailed spectral information comprising intensities and m/z values with 0.02% accuracy and separation of peaks with unit mass difference

(23) The calculated average m/z value is obtained by using the isotopes ^{12}C, ^{13}C, ^{14}N, ^{15}N, ^{16}O, and ^{1}H. The isotopic ions included are M, M + 1, and M + 2. Let n = number of $^{12}C + {}^{13}C$ atoms, x = number of ^{13}C atoms, P = probability of ^{13}C, Q = probability of ^{12}C, and $P(x)$ = probability of having x ^{13}C atoms in the ion. Then $P(x) = [n!/(x!(n - x)!)]P^xQ^{n-x}$. Analogously for ^{15}N, with $P(y)$ representing the probability of having y ^{15}N atoms in the molecule. For M + 1, for example, the total probability is $P(M + 1) = P(x = 1)P(y = 0) + P(x = 0)P(y = 1)$; analogously for M and M + 2. Then average MW = $[MW_0P(M) + MW_1P(M + 1) + MW_2P(M + 2)]/[P(M) + P(M + 1) + P(M + 2)]$.

up to about m/z 1000. This information is very voluminous since it covers the whole spectral range from 1 to 2500 daltons. It is not feasible because of space limitations to present tables of these data here. We have compared the detailed positive spectra of natural and synthetic alamethicin I, and the detailed correspondence of the two spectra is of a very high order. The identity of the total spectra provides evidence that the amino acid sequences in the two samples are the same. Thus our positive ion spectra provides strong evidence that natural alamethicin I has the amino acid sequence shown in Figure 1.

We now consider the negative mass spectra of alamethicin I depicted in the lower half of Figure 3. The spectra of the natural and synthetic compounds exhibit a high degree of similarity but with some differences. Thus a very strong peak labeled A is present in the natural alamethicin I spectrum with 10 times greater intensity than that of the corresponding peak (also labeled A) in the synthetic alamethicin I spectrum, and peaks labeled B, C, and D are present in the former spectrum that are not observed in the latter spectrum. We have been able to establish that the peaks labeled A, B, C, and D result from the presence in the peptide sample of a trace amount of anionic detergent, a type of compound for which our method is known from past experience to be highly sensitive. The major peaks in the spectrum of an authentic sample of detergent (Linbro) were identical with the impurity peaks in Figure 3. Peak A is the detergent anion, and we shall represent it by the symbol A^-. Peaks B result from the association of A^- with various inorganic impurities, C corresponds to $(2A + Na)^-$, and D corresponds to $(M + A)^-$, where M represents alamethicin I. The formation of these association ions is not unreasonable in the light of prior experience with fission fragment ionization mass spectrometry in this laboratory.[11]

Omitting ions A–D from the considerations, the agreement of the spectra of the two samples above the m/z of ion A is excellent. Below the m/z of ion A, differences of intensities (but not m/z values) occur for four peaks with flight times around 39 μs. The differences are small; we have no explanation for them; and they warrant mentioning only because of the high order of agreement otherwise observed with our spectra.

As with the positive ion spectra, comparison of the detailed tabulated negative ion spectra for the natural and synthetic samples shows excellent agreement with the exception of the ions resulting from the detergent impurity. The experimental m/z values for the quasi-molecular ions at $t \simeq 100$ μs are 1963.0_0 and 1962.9_5 for the synthetic and natural samples, respectively. These are, of course, isotopically averaged values. These ions are without question the $(M - H)^-$ ion, and the results lead to a molecular weight of the natural alamethicin I of 1963.1_0. This indicates that the amino acid compositions of the two samples are identical; the excellent agreement of the fragmentation pattern indicates that the amino acid sequences are the same. The negative ion spectra and the positive ion spectra are completely concordant, and the total mass spectrometric support for the alamethicin I structure given in Figure 1 is very strong.

We are not aware of any previous use of this natural–synthetic comparison method of determining peptide identity, especially for a peptide of this size. It should be one of considerable generality and power.

Analysis of Fragmentation Reactions and Implications for Sequencing. We first consider the positive ion spectra. An inspection of the spectra of alamethicin I reveals the presence of a number of ion series, and the chemical identities of the ions comprising the series are the same as or analogous to those that have been observed in the past in the isobutane chemical ionization mass spectra of peptides.[24] Nominal structures that have been suggested for the ion types that we have observed are seen in Chart I. A fourth type of ion, *C*-ammonium ion, found in chemical ionization mass spectra, was not observed in these spectra. All four types of ions appear in the spectrum of Ala-Ala-Ala obtained previously in this laboratory.[11]

(24) Mudgett, M.; Bowen, D. V.; Field, F. H.; Kindt, T. J. *Biomed. Mass Spectrom.* **1977**, *4*, 159.

Chart I

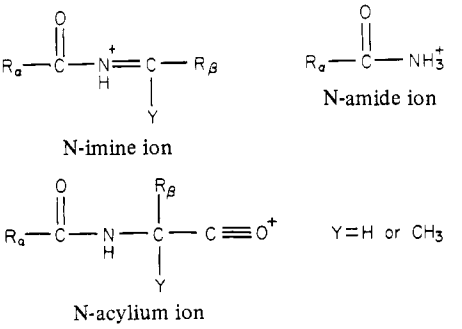


Table I. Imine Fragment Ion Series

bond broken[a]	ion modification	m/z measd	m/z calcd[b]	intensity[c] (net counts)
1	none	100.10	100.08	20700
2	none	197.10	197.13	2604
3	+Na − H	304.16	304.16	526
4	+Na − H	375.22	375.20	458
5	+Na − H	460.30	460.25	337
6	+Na − H	531.20	531.29	329
7	+Na − H	659.38	659.35	274
8	+Na − H	774.34	744.40	443
9	+Na − H	843.6_4	843.47	241
10	+Na − H	928.6_7	928.52	385
11				
12	+Na − H	1098.9_0	1098.63	245
13	+Na − H	1183.8_9	1183.68	317
14	+Na − H	1281.3_8	1280.74	178
15	+Na − H	1380.1_3	1379.80	175
16	+Na − H	1465.4_5	1464.86	240
17	+Na − H	1550.4_8	1549.91	172

[a] Bond numbering given in Figure 2. Actual bond broken is C_α–CO. [b] Monoisotopic value. [c] Intensity in excess of background.

We give in Table I the imine ions observed in the positive spectrum of synthetic alamethicin I. The bonds broken to form the observed ions are listed in column 1, where the bond numbering is that given in Figure 2. The second column reflects the fact that in one way the imine ions formed in fission fragment ionization mass spectrometry are markedly different from those observed in isobutane chemical ionization mass spectrometry. For the ions formed by breaking bonds 1 and 2, the m/z values observed could result from the *N*-imine ion structure given in Scheme I. However, for the ions produced by breaking bonds higher than the second, the observed m/z values jump by 22 daltons, and we postulate that the ions having these masses are imine ions wherein one of the hydrogens has been replaced by a sodium. The occurrence or nonoccurrence of this replacement is represented by the entry in column 2 of Table I.

The occurrence of this H–Na replacement reaction has some precedence in fission fragment ionization mass spectrometry. First, the formation of $(M + 2Na - H)^+$ ion was reported by Macfarlane and co-workers early in the history of the technique,[10] and we have also observed the phenomenon in this laboratory.[11] Indeed, we pointed out above that this type of ion appears in the alamethicin I spectrum. Hydrogen–sodium exchange is observed in fragment ions in the $(B + H + Na)^+$ and $(B + 2Na)^+$ ions found in the fission fragment ionization spectrum of guanosine.[11] These ions are the H–Na exchange analogues of the well-known $(B + 2H)^+$ ions produced from nucleosides by a variety of ionization techniques. We have observed four different types of fragment ions containing either Na or Li in the fission fragment ionization spectra of polyethers [poly(ethylene glycols)].[25] We point out that the quasi-molecular ion observed in our positive ion spectra of alamethicin I is always the $(M + Na)^+$ ion, and for practical

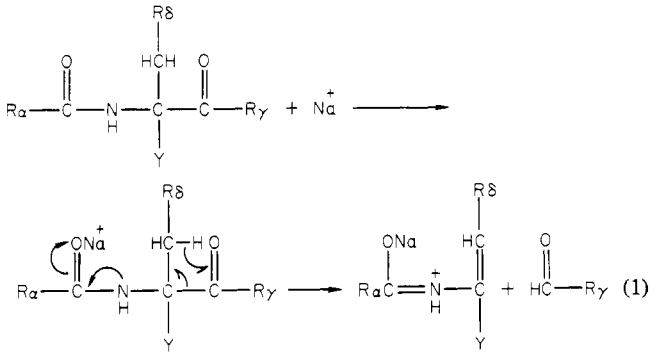
(25) Unpublished finding in this laboratory.

Table II. Variant Imine Fragment Ion Series

bond broken[a]	ion modification	m/z measd	m/z calcd	intensity[c] (net counts)
7	$+Na - CH_2CONH_2$	602.36	602.33	1068
9	$+Na - CH_3$	829.6_2	829.46	290
12	$+Na - CH(CH_3)_2$	1056.9_3	1056.58	625
15	$+Na - CH_3$	1366.0_4	1365.79	91
18	$+Na - CH_2COOH$	1622	1620.95	104

[a] Bond numbering given in Figure 2. Actual bond broken is C_α-CO. [b] Monoisotopic value. [c] Intensity in excess of background.

purposes no $(M + H)^+$ quasi-molecular ion is produced. We suggest that some of this cationization brings about fragmentation of the resulting quasi-molecular ion, and we further suggest as a possible reaction mechanism that given in eq 1.



(1)

Y = H or CH_3

All of the amino acid residues in alamethicin I save one have side-chain hydrogen atoms available for the four-center reaction depicted in eq 1. Glycine is the residue that does not have such a hydrogen atom, and we shall see that this affects the results. The imine ion given in eq 1 has a different structure (aside from the replacement of H by Na) than that originally postulated to occur in isobutane chemical ionization (Chart I), and the new structure is used because it is difficult to rationalize H–Na exchange by using the structure of Chart I.

One can write a reaction analogous to eq 1 involving protonation of the molecule and subsequent fragmentation to produce an imine ion without H–Na exchange. Two such ions are included in Table I, and we do not understand this division between protonated and Na-exchanged forms. As was pointed out above, no significant amount of protonated quasi-molecular ion is formed, and this raises a question about the origin of the protonated imine ions.

The third and fourth columns of Table I give the measured and calculated m/z values. The latter are monoisotopic values, and at the lower m/z values the measured m/z values given correspond to those of the ^{12}C monoisotopic peaks. At the higher m/z values the resolution is insufficient to separate the ^{12}C peaks and the first ^{13}C peaks, as was described above. One observes that up to about m/z 800, the agreement between the measured and the calculated m/z values is generally closer than 0.1 dalton, but above m/z 800, the measured value begins to exceed the calculated values because of peak overlap. However, the overall agreement between the measured and calculated m/z values is excellent and provides convincing evidence that all of the peaks tabulated refer to the same type of ion. The intensities are tabulated in the fifth column, and one sees that the intensities of the first two peaks (the protonated imine ions) are significantly greater than those of the H–Na exchanged imine peaks. However, it is noteworthy that with one exception the intensities of the sodium-exchanged peaks decrease very gradually with increasing mass. The series extends through the ion formed by breaking bond 17, and the intensities are large enough that ab initio sequencing would be possible if this were necessary. The ion formed by breaking bond 11 is absent from the sequence. The residue to the left of this bond is glycine, and we ascribe the absence of the ion to the fact that glycine has

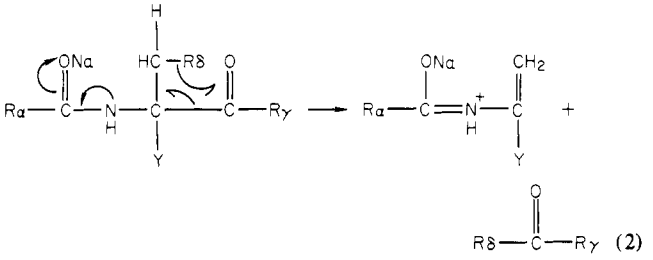
Table III. *N*-Acylium Fragment Ion Series

bond broken[a]	m/z measd	m/z calcd[b]	intensity[c] (net counts)
1	128.12	128.07	5750
2	225.15	225.12	1427
3	310.52	310.18	749
4	381.29	381.21	328
5	466.43	466.27	282
6	538.16	537.30	220
7			
8	750.27	750.42	309

[a] Bond numbering given in Figure 2. Actual bond broken is CO-N. [b] Monoisotopic value. [c] Intensity in excess of background.

no hydrogen that can take part in the four-center decomposition depicted in eq 1.

We also observe five ions that are imine ions but which do not fall into the series given in Table I. They are produced by a variant of reaction 1, namely,

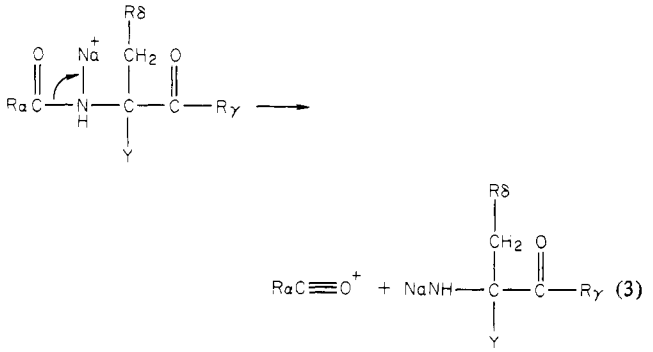


(2)

Y = H or CH_3

The observed ions of this type are given in Table II. The symbols R_δ used in eq 1 and 2 can stand for an H atom or a more extensive group. Of the residues appearing to the left of bond 18 in the alamethicin molecule (Figure 2), R_δ is H for the aminoisobutyric acid and alanine residues. The variant imine ions found in the spectrum and given in Table II correspond to fragmentations at each residue where R_δ is an entity larger than H. The upper limit with this fragmentation process is at bond 18. This type of fragmentation is obviously of much potential use for identifying the amino acids present in a peptide.

The third series of ions found in the alamethicin I spectrum is the *N*-acylium series, and the ions observed have the same structure as the *N*-acylium ion depicted in Scheme I; that is, no H–Na exchange occurs. By analogy with the reaction mechanisms postulated for the formation of the imine and amide series, we suggest as a possible reaction for the formation of the *N*-acylium ions the mechanism shown in eq 3. Obviously the formula we give for the neutral fragment in eq 3 is a guess.



(3)

Y = H or CH_3

The *N*-acylium ions are given in Table III, from which it may be seen that this is an incomplete series consisting of only seven members produced by breaking bonds toward the amine end of the molecule. High-time tails were especially prominent on these peaks, and at least partly as a consequence of peak distortions, the agreement between the measured and calculated m/z values

Table IV. Amide Fragment Ion Series

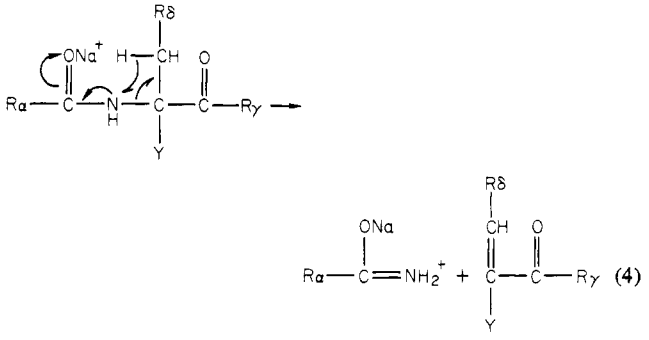
bond broken[a]	m/z measd	m/z calcd[b]	intensity[c] (net counts)
1	167.09	167.08	1109
4	420.19	420.22	267
5	505.28	505.28	132
7	704.38	704.37	299
9	888.49	888.49	232
12	1143.9_1	1143.65	278
14	1325.9_3	1325.76	74
17	1595.6_3	1594.93	86

[a] Bond numbering given in Figure 1. Actual bond broken is N–C_α. [b] Monoisotopic value. [c] Intensity in excess of background.

is somewhat poorer for the *N*-acylium ions than for the imine ions. We have shown[21] that such high-time tailing results from fragmentation of parent ions during acceleration, and we have found that if we reduce the voltage between the sample foil and the first grid of the apparatus (increase the time in the acceleration region) the peaks of the acylium ions are sharpened and their intensities are increased.

The last ion series that can be identified in the alamethicin I spectrum involves an analogue of the amide ion shown in Scheme I. However, all of the amide ions produced by fission fragment ionization have experienced H–Na exchange. The series is quite incomplete.

We suggest as a reaction for the formation of the (tautomeric) exchanged amide ion that seen in eq 4. When glycine is the



(4)

Y = H or CH_3

residue involved, this reaction encounters the same difficulties with the four-center decomposition that were pointed out in connection with eq 1. In addition, proline as a residue constitutes a problem, for with it the formation of an amide ion like that in eq 4 requires the occurrence of another four-center decomposition reaction. The presence of glycine and proline accounts for the absence of ions produced by breaking bonds 10 and 13. The other gaps in the sequence are unexplained.

The sequence information contained in the tables of sequence ions (Tables I–IV, and particularly I and II) provides an abundant amount of ab initio information concerning the sequence of alamethicin I, and this information largely confirms the sequence given in Figure 1. Thus our mass spectrometric measurements with positive ions show that the sequences in the natural and synthetic alamethicin I samples are identical, and they may be taken independently largely to confirm the structure postulated for synthetic alamethicin I and to confirm the validity and integrity of the solid-phase synthesis of our sample of alamethicin I.

The negative ion spectrum does not provide useful information about the amino acid sequence. We have been able to identify clusters of ions that contain ions that could be considered as sequence ions, but the absolute intensities are never very high, and the putative sequence ions are seldom distinguished from their neighbors. We can offer two possible explanations that could work together to produce this result. First, one observes from the negative ion spectra given in Figure 3 that the ion in the spectrum with the greatest intensity is the CN^- ion, and another ion with higher *m/z* and an intensity about 75% that of the CN^- is also present. This ion is probably the CNO^- ion at *m/z* 42. In previous work we have found[11] that CN^- ion is a very intense ion in fission fragment ionization negative ion mass spectra of nitrogen containing organic molecules. The CNO^- ion is sometimes also quite intense. For example, in the negative ion spectra of Ala-Ala-Ala the CN^- ion comprises 31% of the total ionization and the CNO ion comprises 12%. An inspection of Figure 3 leads one to the conclusion that the sum of the CN^- and CNO^- intensities in alamethicin I comprises more than half the total ionization. Thus the fragmentation tends to be very extensive in the sense that it proceeds to produce large intensities of two ions with very small *m/z* values. This must serve to deplete the intensities of the more informative fragment ions with large *m/z* values. Second, we have shown that negative ions produced by chemical ionization tend to undergo facile four-center reactions, which split out H_2, H_2O, and other small molecules.[26] Multiple loss of hydrogen molecules can give rise to clusters of fragment ions, which could be the explanation for the clusters we observe in the alamethicin I spectrum. Thus we are suggesting that the intensities of fragment ions with relatively large *m/z* values are intrinsically small because of the extensive amount of fragmentation to form CN^- and CNO^-, and in addition the intensities are distributed over a number of *m/z* values because of multiple hydrogen loss. Our prior experience has been that the negative ion spectra of peptides are uninformative (except that the $(M - 1)^-$ ions provides molecular weight information), and our present experience with alamethicin I is in keeping with this experience.

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(26) Smit, A. L. C.; Field, F. H. *J. Am. Chem. Soc.* **1977**, *99*, 6471–6483.