CHEMICAL-IONIZATION MASS SPECTROMETRY OF β-LACTAM ANTIBIOTICS

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(Received for publication May 26, 1975)

Chemical-ionization (CI) mass spectra are described for methyl esters of eight clinically significant penicillins and their breakdown products. These substances give spectra with very few fragment ions and contain easily discernible protonated molecule ions. The main cleavage reaction is postulated to involve a retro 2 + 2 DIELS-ALDER-type fragmentation of the β-lactam ring liberating one fragment ($m/e=174$) that is characteristic of the penicillin nucleus and a second fragment that is molecule specific, as it contains the elements of the side chain. The other fragment ions, though interesting, are of minor intensity. The free acids, on the other hand, fragment more extensively because of their relative instability and lack of volatility. These spectra resemble electron impact spectra more closely and, though they encode more structural information, are less reproducible from run to run. The ease with which the esters can be made and the relative simplicity of their CI mass spectra make this method significant for the identification and characterization of β-lactam antibiotics.

Interest in the physical and chemical properties of the β-lactam family of antibiotics, the penicillins and cephalosporins, continues unabated because of their remarkable therapeutic utility and clinical acceptance. In addition to the many naturally occurring antibiotics, literally thousands of semisynthetic analogs have been prepared.[1] The use of physical measurements has played a significant role in facilitating this work. There have been, however, only two papers devoted primarily to mass spectrometry of bioactive penicillins, and these were concerned with electron-impact (EI) ionization.[2,3] We have recently demonstrated the utility of chemical-ionization (CI) mass spectrometry for the analysis and rapid microidentification of macrolide antibiotics and related model substances.[4,5,6,7] In this paper, we report that chemical ionization is also readily applicable to β-lactam antibiotics and their methyl esters.

Experimental

Sodium cloxacillin, sodium methicillin, and hetacillin were gifts of Bristol Laboratories (U. S.A.); ampicillin, potassium phenoxymethyl penicillin, and potassium benzyl penicillin were from Wyeth Laboratories; and 6-aminopenicillanic acid was purchased from Aldrich Chemical Company. These were converted to their protonated form by dissolving 50mg of the salt form in a biphasic system of 25 ml each of water and ethyl acetate. A Sargent glass electrode was im-

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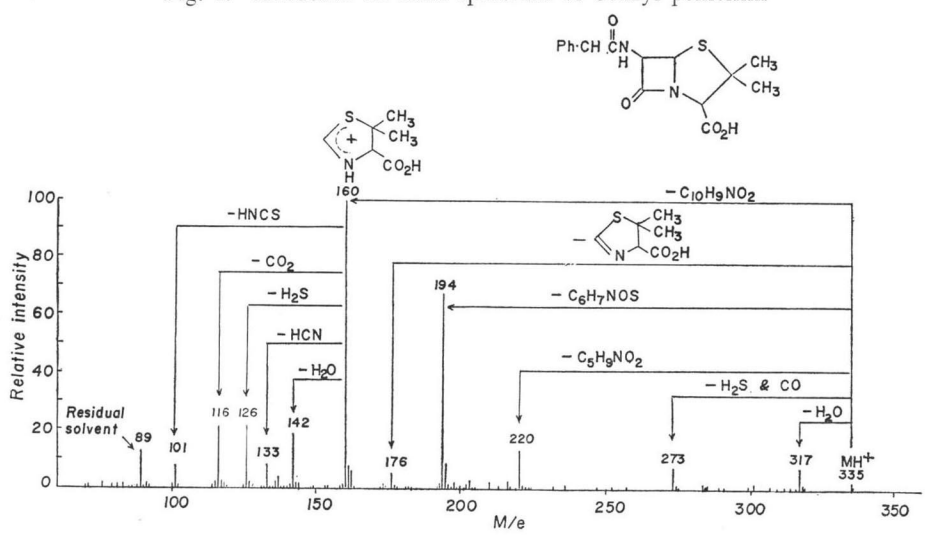
mersed into the chilled (0°C) mixture and 5% aqueous HCl solution was added to the appropriate pH (benzyl penicillin and methacillin, pH 3.5; phenoxymethyl penicillin, and cloxacillin pH 3.0; and cephalothin, pH 2.5). The separated organic layer was washed with cold H_2O, dried (anhydrous Na_2SO_4), and evaporated *in vacuo* at 25°C to an oily residue, which was dried at 1.0mm at 25°C for at least 10 minutes. The needed methyl esters were prepared by a literature procedure[8] using pyridine, N, N'-dicyclohexylcarbodimide, and absolute MeOH in cold, dry CH_2Cl_2. The products were isolated by preparative t.l.c. (silica gel G), and residual dicyclohexylurea was removed by successive crystallization from 50 percent benzene-petroleum ether. The products were characterized by i.r., p.m.r., and mass spectrometry. The penicilloic esters were prepared by reaction of methanol solutions of the free acids with ethereal diazomethane.

The low-resolution chemical-ionization mass spectra were obtained using a Finnigan Model 3200 quadrupole mass spectrometer equipped for chemical ionization. A Systems Industries 150 data system was used to control the mass analyzer voltages and to process and display the spectral data. Samples were introduced into the ion source by means of a direct insertion probe. While repetitatively scanning the mass range 50 ~ 450 amu, the samples were heated from approximately 100~300°C. The ion-source temperature was maintained at approximately 160°C. The pressure of the reagent gas in the ion source was adjusted to approximately 0.5 torr. The various ion-source voltages were adjusted for maximum sensitivity but were generally close to the following values: electron voltage, 150 v; ion energy, 10 v; ion repeller voltage, 0 v; and lens voltage, 30 v. The high-resolution chemical-ionization data were obtained with an A.E.I. MS-9 doublefocusing instrument equipped with a Scientific Research Instruments Corporation Chemspect CIS-2 chemical-ionization ion source. A technique suggested by Dr. T. L. CHANG* was used, consisting of introduction of the reagent gas (isobutane) into the ion source at a pressure considerably below the pressure normally used to obtain CI mass spectra. Under these conditions the spectrum consists of a combination of both EI- and CI-generated ions. The former are necessary to provide the reference ions from perfluorotributylamine, which the computer uses to establish the mass versus time calibration. The experimentally measured masses discussed in the text are within 10ppm of the masses calculated for the specified elemental compositions.

Results and Discussion

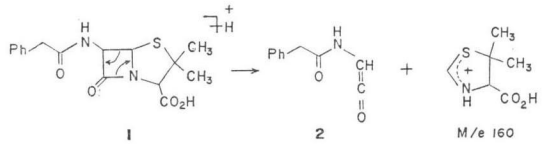
Our initial study of the chemical-ionization mass spectra of penicillins used isobutane as a

Fig. 1. Isobutane CI mass spectrum of benzyl penicillin



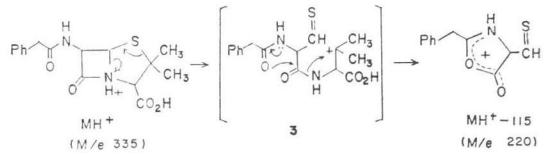
* Personal communication, Dr. T.L. CHANG, Wyeth Laboratories, Inc., Philadelphia, Pa.

relatively mild proton-transfer reagent gas. The samples were introduced into the ion source in the free-acid form by means of a heated probe. The resulting spectra were surprisingly complex and did not show the abundant protonated molecule ions typical of isobutane CI mass spectra. Although plausible structures can be proposed for most of the abundant fragment ions, their relative intensities vary considerably while heating the samples in the ion source. Fig. 1 shows the isobutane CI mass spectrum of benzyl penicillin recorded at a sample temperature of approximately 200°C. This spectrum is representative of the many we have run in this class. The protonated molecule ion (MH^+) is relatively weak and varies considerably in intensity with experimental conditions. Ion *m/e* 160 is dominant, usually the base peak, and is characteristic of the penicillin nucleus. The accurately measured mass of the *m/e* 160 ion indicated a composition of $C_8H_9NSO_2$, which is consistent with formation of the ion via a reverse 2+2 DIELS-ALDER cleavage of the protonated molecule ion:[2,3]



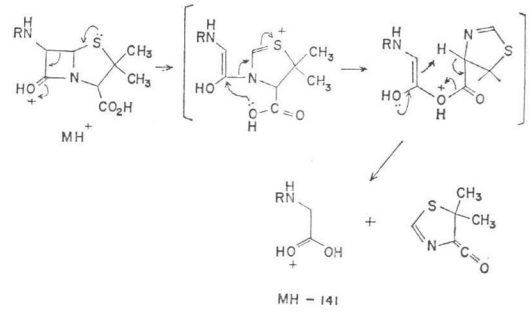
The protonated counterpart of 2 contributes a low-intensity ion at *m/e* 176. All of the lower mass ions with relative intensities of greater than a few percent can be rationalized as resulting from loss of small neutral molecules (H_2O, HCN, H_2S, CO_2, and HNCS) from the *m/e* 160 ion (Fig. 1).

Most of the penicillin free-acid CI mass spectra show prominent and structurally diagnostic peaks corresponding to MH^+-115 and MH^+-141. Loss of a 115 fragment from the protonated molecule ion can be rationalized as follows:



The substantial resonance possible in the MH^+-115 ion helps account for the intensity of this ion. The intermediate tertiary carbonium ion (**3**) expels a neutral aziridine moiety to lead to the final fragment.

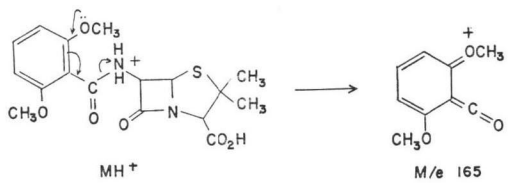
The MH^+-141 ion in the isobutane CI mass spectrum of benzyl penicillin has the elemental composition $C_{10}H_{12}NO_3$ (measured mass, 194.0828; calculated 194.0817). Formation of an ion with this composition must involve rupture of the β-lactam ring, with migration of one of the carboxyl oxygens to the side-chain fragment. A possible mechanism for the formation of the MH^+-141 ion is given aside. It is not certain to what extent thermal fragmentation contributes to the formation of this ion.



The remaining ions of significant intensity

result from loss of small neutral molecules (H_2O, CO_2, H_2S) from MH^+, giving a cluster of ions at high mass that are characteristic of a given penicillin and so provide a "fingerprint" for a given antibiotic.

The isobutane CI mass spectrum of methicillin shows an abundant ion at *m/e* 165, resulting from cleavage of the dimethoxybenzamide substituent in a manner similar to the mechanism proposed for the hydrolysis of certain hindered aromatic esters:[9]



As previously mentioned, the relative intensities of the MH^+ and fragment ions in the CI mass spectra of the penicillins vary considerably as the sample is heated in the ion source. For example, plots of the ion-current intensities at *m/e* 160 and the protonated molecule ion (*m/e* 335) for benzyl penicillin versus increasing sample temperature show two distinct maxima (Fig. 2). Surprisingly, the *m/e* 160 ion-current maximum occurs at a lower temperature than the maximum for the protonated molecule ion. The other penicillin free acids examined were found to behave in a similar manner. This suggests that generation of the *m/e* 160 ion is at least partially a

Fig. 2. Ion-current intensities at *m/e* 335 (MH^+) and *m/e* 160 versus temperature in isobutane CI analysis of benzyl penicillin

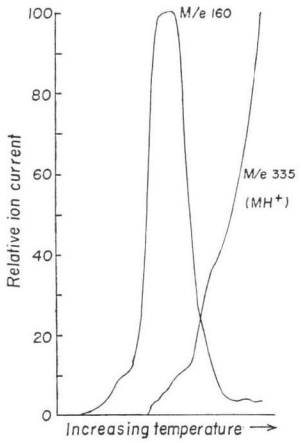
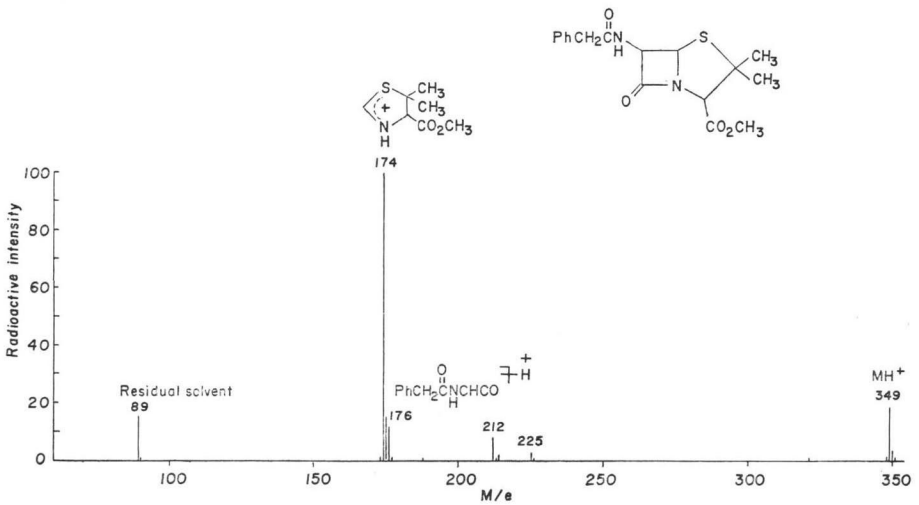


Fig. 3. Isobutane CI mass spectrum of benzyl penicillin methyl ester



result of thermal fragmentation prior to ionization. It is likely that some of the newer techniques for obtaining spectra of thermally labile compounds, such as field desorption[10] or "direct" chemical ionization,[11,12] will give simpler and more reproducible spectra of the free penicillins. However, for the time being we chose to convert the penicillins to their corresponding methyl esters. Not only do the penicillin methyl esters undergo volatilization at lower temperatures than the corresponding free acids, but their isobutane CI mass spectra are far more typical of what we have come to expect from CI mass spectra of antibiotics.

The isobutane CI mass spectrum of benzyl penicillin methyl ester (Fig. 3) is reproducible and structurally informative. Only a few ion peaks stand out, and they are typical of the fragmentations observed in the spectra of other penicillin methyl ester molecules. The protonated molecule ion is the second most abundant ion in the spectrum, constituting 10% of the total ion current. Fragmentation of the protonated molecule ion is limited to the β-lactam ring. The base peak (*m/e* 174, 55% of total ion current) corresponds to the protonated thiazolidine ion (**5a**) just as in the CI mass spectrum of the free acid and the EI mass spectrum of the methyl ester.[2,3] Direct formation of **5a** from MH^+ is indicated by a metastable ion peak at *m/e* 86.8. The protonated counterpart of the remaining portion of the molecule contributes a moderately abundant ion, **6**. In the case of benzyl penicillin methyl ester, *m/e* 176 is a doublet and high-resolution mass analysis was required to resolve **6** ($C_{10}H_{10}NO_2 = 176.071$) from the ^{34}S-isotope peak of **5** ($C_7H_{12}NO_2{}^{34}S = 176.055$). The peak at *m/e* 225 is due to the protonated molecule ion of dicyclohexyl urea, an impurity formed from dicyclohexylcarbodiimide during preparation of the methyl esters. Loss of CO from the protonated molecule ion gives rise to a peak at *m/e* 321. Although of minor importance here, for certain other penicillin derivatives, such as 6-aminopenicillanic acid, expulsion of CO from the protonated molecule ion is a major fragmentation process.

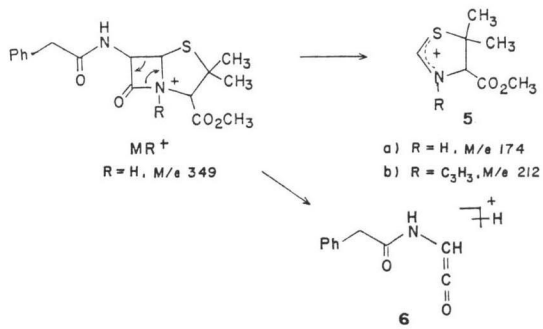
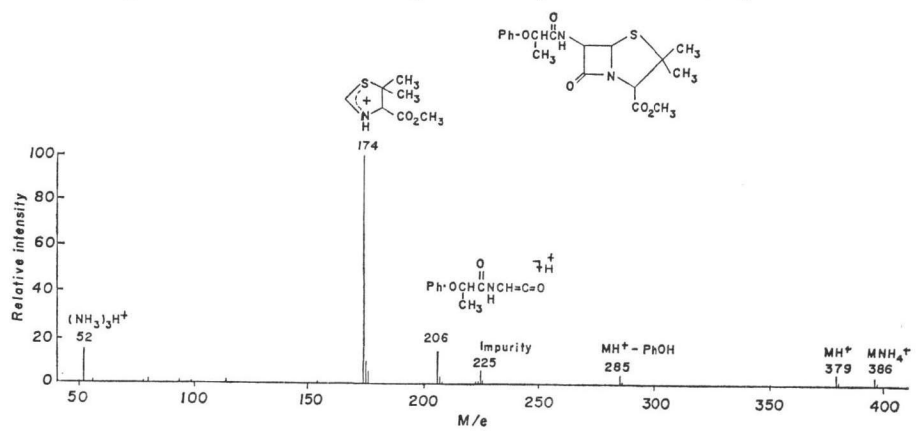


Fig. 4. Ammonia CI mass spectrum of phenethicillin methyl ester

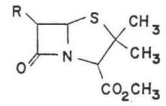


The only remaining ion in the isobutane CI mass spectrum of benzyl penicillin methyl ester with a relative intensity greater than 1 percent occurs at *m/e* 212. The occurrence of this ion remains a puzzle. High-resolution mass measurement suggests a composition of $C_{10}H_{14}NO_2S$ (calculated 212.0745; measured, 212.0771). Formation of an ion of this composition (**5b**) could occur by attachment of $C_3H_3^+$ instead of a proton to a molecule of benzyl penicillin methyl ester, with subsequent rupture of the β-lactam ring. The occurrence of weak C_3H_3-attachment ions is common in isobutane CI mass spectra. However, we would expect to see a comparable peak at *m/e* 387 corresponding to $(M+C_3H_3)^+$, and no such ion peak is observed. The ion is observed in the isobutane CI mass spectra of all of the penicillin methyl esters, and is absent in the ammonia CI mass spectra.

For some classes of compounds the use of ammonia as reagent gas results in less fragmentation than when using isobutane. This appears to be the case for the penicillin methyl esters, although differences in the spectra are not great. The ammonia CI mass spectrum of phenethicillin methyl ester (Fig. 4) is representative. The protonated molecule ion (*m/e* 379) is accompanied by an ammonium attachment ion (*m/e* 386). A metastable ion peak at *m/e* 372.1 shows that at least some of the protonated molecule ion results from loss of NH_3 from the ammonium-attachment ion. Formation of the *m/e* 285 ion can be rationalized as resulting from protonation of the ether oxygen followed by elimination of phenol. The two remaining ions (*m/e* 174 and 206) result from the previously discussed fragmentation of the β-lactam ring.

Tables 1 and 2 summarize the masses and abundances of the prominent ions in the isobutane and ammonia CI mass spectra of six penicillin methyl esters and the isobutane CI mass

Table 1. Chemical-ionization mass-spectral data for penicillin methyl esters



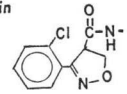
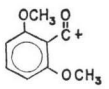
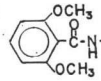
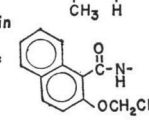
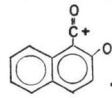
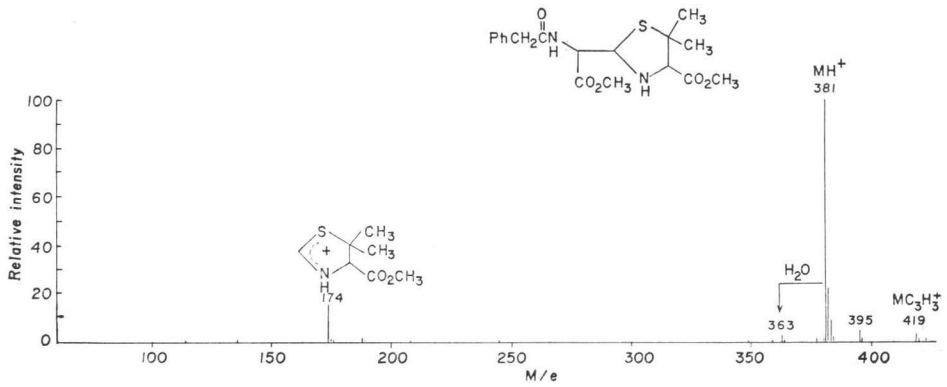
Compound	Molecular weight	Reagent gas	Intensities relative to base peak				
			MH^+	MNH_4^+	m/e 174	MH^+-173	Other prominent ions m/e
Benzylpenicillin	348	i-Butane	20%	–	100%	7%	212 (7%)
R = Ph-CH_2CO-NH-		NH_3	16	47	100	1.5	
Phenoxymethyl penicillin	364	i-Butane	7	–	100	1	212 (6%); 337 (2%) $MH^+ - CO$
R = Ph-OCH_2CO-NH-		NH_3	7	12	100	1	
Cloxacillin	449	i-Butane	24	–	100	4	212 (3%)
R = 							
Methicillin	394	i-Butane	8	–	100	38	165 (17%)
R = 		NH_3	6	<1	100	22	165 (2%)
Phenethicillin	378	i-Butane	7	–	100	13	285 (7%); MH^+-PhOH; 212 (7%)
R = Ph-O-CH(CH_3)-CO-NH-		NH_3	5	4	100	14	285 (4%)
Nafcillin	428	i-Butane	13	–	100	68	199 (22%)
R = 		NH_3	<7	1	100	14	199 (12%)
							 , 212 (4%)

Table 2. Chemical-ionization mass-spectral data for penicilloic acid dimethyl esters

Compound	Molecular weight	Reagent gas	MH^+	*m/e* 174	MH^+-173	Other prominent ions
Benzyl penicilloic acid R = Ph-CH_2C(=O)N(H)-	380	*i*-Butane	100%	15%	1%	*m/e* 363 (2%) MH^+-H_2O
Phenoxymethylpenicilloic acid R = $PhOCH_2$C(=O)N(H)-	396	*i*-Butane	100	19	2	*m/e* 152 (8%) $PhOCH_2C(=NH)OH$

Fig. 5. Isobutane CI mass spectrum of benzyl penicilloic acid dimethyl ester



spectra of two penicilloic acid dimethyl esters.

The CI mass spectra of the penicilloic acid dimethyl esters show more abundant protonated molecule ion peaks than those observed in the penicillin methyl ester spectra. For example, the only significant fragment ion in the isobutane CI mass spectrum of benzyl penicilloic acid dimethyl ester (Fig. 5) is a relatively low-abundance thiazolidine ion (*m/e* 174). Two explanations can be offered for the relative stability of protonated molecule ions of penicilloic acid derivatives. First, release of the strain in the fused β-lactam ring system presumably contributes to the driving force for fragmentation of the protonated penicillin molecule ions. This ring strain is absent in the penicilloic acids. Second, opening of the lactam ring generates a highly basic secondary amine. Consequently, protonation of the penicilloic acid diester should occur preferentially at the amine, giving a relatively stable ammonium ion.

The ease with which these spectra may be obtained and interpreted encourages us in the belief that chemical-ionization mass spectrometry will play a significant role in the future of

β-lactam chemistry. For analytical and identification studies, the methyl esters are preferable. For structural analysis, the greater fragmentation occurring with the free acids offers some potential advantage in that more structural information can be extracted from the spectra, although at the cost of greater variation in reproducibility.

Acknowledgement

This work was supported in part by the National Institute of Health, Division of Research Resources (Grant RR0065). H. D. H. S. thanks the American Foundation for Pharmaceutical Education for stipend support.

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