Simple Peptides. IV.[1-3] B/E Linked Scan Sputtered Ion Mass Spectrometry (SIMS) Technique Useful to Distinguish the Mode of Linkage of an N-Terminal Acidic α-Amino Acid in Oligopeptides

Ko NAKAMURA, Kunihiko HIGASHIURA and Kazuharu IENAGA*

Department of Natural Products Chemistry, Institute of Bio-Active Science (IBAS), Nippon Zoki Pharmaceutical Co., Ltd. Kinashi, Yashiro-cho, Kato-gun, Hyogo 673-14, Japan. *Received July 25, 1988*

If oligopeptides contain a free and acidic α-amino acid at the N-terminal, the B/E linked scan sputtered ion mass spectrometry (SIMS) technique is useful, without derivatization, as a general method not only to distinguish each pair of α- and ω-isomers but also to elucidate some of their structures. Ten pairs of such glutamyl oligopeptides (1—10, 11—20) and five pairs of aspartyl dipeptides (28—37) were examined in order to prove the usefulness and generality of the technique. γ-Linkage of the terminal acidic amino acid in naturally occurring norophthalmic acid (17) and glutathione (21) could be identified easily by using this method. Spectra of a pair of acetylated glutamyl dipeptides (21, 23), and two pairs of esters (24—27) of glutamic acid were also measured for comparison.

Keywords oligopeptide containing acidic amino acid; acidic amino acid linkage mode; B/E linked scan technique; SIMS; B/E linked scan SIMS; mass spectrometry

Introduction

A large number of γ-glutamyl[4-10] and a lesser number of β-aspartyl[4,5] oligopeptides are widely distributed in nature. However, it has always been difficult to determine how terminal acidic amino acids, such as Glu and Asp, are linked in such molecules. Classical techniques,[11-13] such as measurement of the rate(s) of chemical/biochemical bond hydrolysis or direct nuclear magnetic resonance (NMR) or infrared (IR) spectral analysis, are simply not practical in the microanalytical investigation of natural peptides, which are usually isolated only in minute amounts. About ten years ago, mass spectral techniques, *e.g.* chemical impact mass spectrometry (CI-MS),[14] were reported to be useful for the above purpose. However, they suffered from several practical difficulties: for example, CI-MS does not yield quasi-molecular ions and it cannot be applied to most peptides because of their low volatility. Recently, we reported[3,15] that the B/E (B, magnetic field; E, electric field) linked scan sputtered ion mass spectrometry (SIMS) technique could indeed distinguish the mode of linkage of a terminal acidic amino acid in some peptides (without the help of other physical methods or even the availability of authentic samples) but the examples quoted were confined to rather specialized taurine dipeptides containing a free acidic α-amino acid moiety. In the present paper, we evaluate this new technique as a general method applicable to ordinary oligopeptides with an N-terminal acidic α-amino acid: it proved remarkably successful.

Results and Discussion

The spectra from positive liquid SIMS for isomeric α- and γ-glutamylalanines (**1**, **11**) are shown in Fig. 1. Both isomers gave a clear $[MH]^+$ ion peak at *m/z* 219 without derivatization. However, the isomers could not be distinguished by their fragment ions, because both spectra were almost identical at a glance owing to concealment of the genuine peaks by prominent ions from the glycerol matrix. This interference in the lower mass unit region can be overcome only if a relatively large amount of sample can be used for obtaining the mass spectrum of an oligopeptide by the SIMS technique.

In contrast, as shown in Fig. 2, the B/E linked scan SIMS method gave completely different spectra for the same isomeric pair of dipeptides, H–Glu–Ala–OH (**1**, **11**), just as we previously observed[3,15] in the case of taurine derivatives. Since the linked scan technique[16] can selectively recognize metastable ions (MI) for the special precursor ion, if SIMS and linked scan techniques are combined and

H–αGlu–X–OH
X: Ala (**1**), Tyr (**2**), Glu (**3**), Lys (**4**), εAcp (**5**), Tau (**6**), Ala–Gly (**7**), Ser–Tau (**8**), Phe–Ala–Gly (**9**), Ala–Gly–Gly (**10**)
H–γGlu–X–OH
X: Ala (**11**), Tyr (**12**), Glu (**13**), Lys (**14**), εAcp (**15**), Tau (**16**), Ala–Gly [norophthalmic acid] (**17**), Ser–Tau (**18**), Phe–Ala–Gly (**19**), Ala–Gly–Gly (**20**)
H–γGlu–Cys–Gly–OH [glutathione] (**21**)
Ac–αGlu–Ala–OH: (**22**) Ac–γGlu–Ala–OH: (**23**)
H–αGlu–OR R: Me (**24**), Bn (**25**)
H–γGlu–OR R: Me (**26**), Bn (**27**)
H–αAsp–X–OH
X: Gly (**28**), Ala (**29**), Leu (**30**), Lys (**31**), Tau (**32**)
H–βAsp–X–OH
X: Gly (**33**), Ala (**34**), Leu (**35**), Lys (**36**), Tau (**37**)

Chart 1. Structures of Specimens

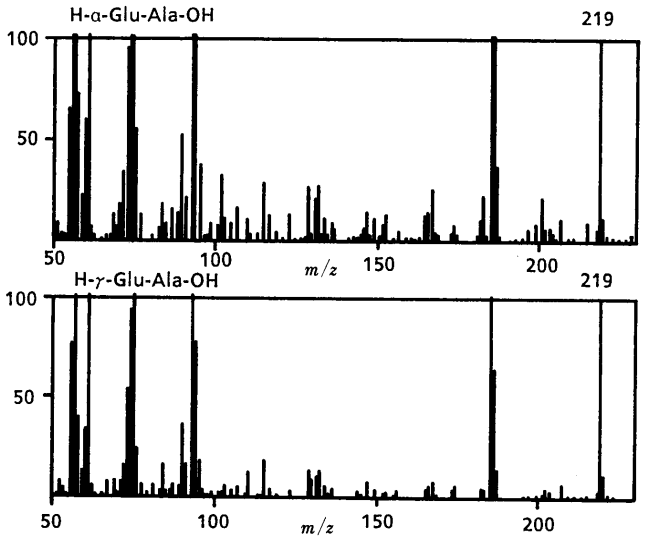

Fig. 1. SIMS (5 μg)

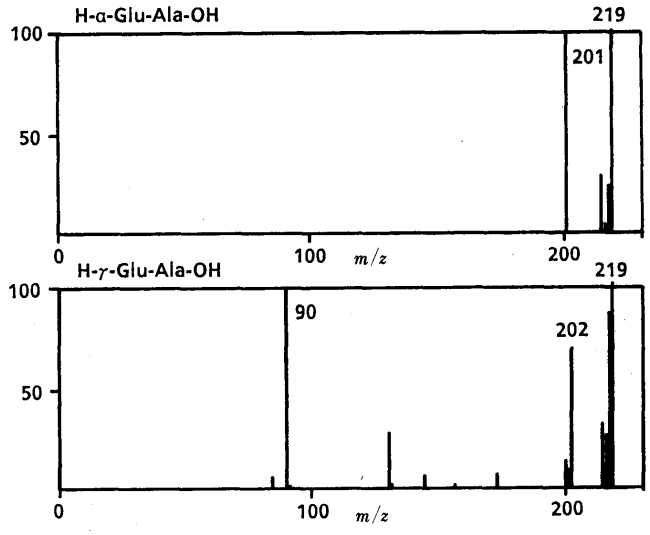

Fig. 2. B/E Linked Scan SIMS (5 μg)

TABLE I. Comparison of α- with γ-Glutamyl Oligopeptides [H–Glu–X–OH] in Terms of Major Fragment Ions[a]

A) Comp. –X–	$(MH)^+$	α-Isomer [m/z (%)]		γ-Isomer [m/z (%)]			
		$(MH-H_2O)^+$	$(N)^+$	$(C'+2)^+$	$(MH-NH_3)^+$	$(N')^+$	$(MH-H_2O)^+$
–Ala–	219	201 (100)		90 (100)	202 (70)	130 (28)	
–Tyr–	311	293 (100)		182 (100)	294 (23)		
–Glu–	277	259 (100)		148 (100)	260 (23)		
–Lys–	276	258 (100)		147 (100)		130 (15)	258 (20)
–εAcp–	261	243 (100)		132 (74)	244 (100)		
–Tau–	255	237 (100)		126 (85)	238 (100)	130 (45)	
Ac–Glu–Ala–OH	261	243 (5)	172 (100)	90 (58)		172 (60)	243 (100)

B) Comp. –X–	$(MH)^+$	α-Isomer [m/z (%)]				γ-Isomer [m/z (%)]		
		$(MH-H_2O)^+$	$(N^2)^+$	$(N^3)^+$	$(C^2+2)^+$	$(C'+2)^+$	$(N^2)^+$	$(N^3)^+$
–Ala–Gly–	276	258 (100)	201 (22)			147 (100)	201 (70)	
–Ser–Tau–	342	324 (100)				213 (100)		
–Cys–Gly–	308		[b]			179 (100)	233 (48)	
–Phe–Ala–Gly–	423	405 (45)	277 (100)	348 (53)		294 (100)	277 (95)	348 (45)
–Ala–Gly–	333	315 (100)	201 (82)		133 (45)	204 (90)	201 (100)	258 (21)

a) This table indicates the relative ion intensity as a percentage of the most intense peak among the fragment ions in each spectrum. *b*) Not yet measured. A) Dipeptides. B) Tri- and tetrapeptides.

the quasi-molecular ion $[MH]^+$ is chosen as a precursor, each MI derived therefrom can be recorded as a clear peak without interference from those of the matrix; quasi-molecular ions in SIMS are sufficiently stable to exist during B/E linked scan measurement, which requires no additional instrument as does the alternative MS-MS procedure.

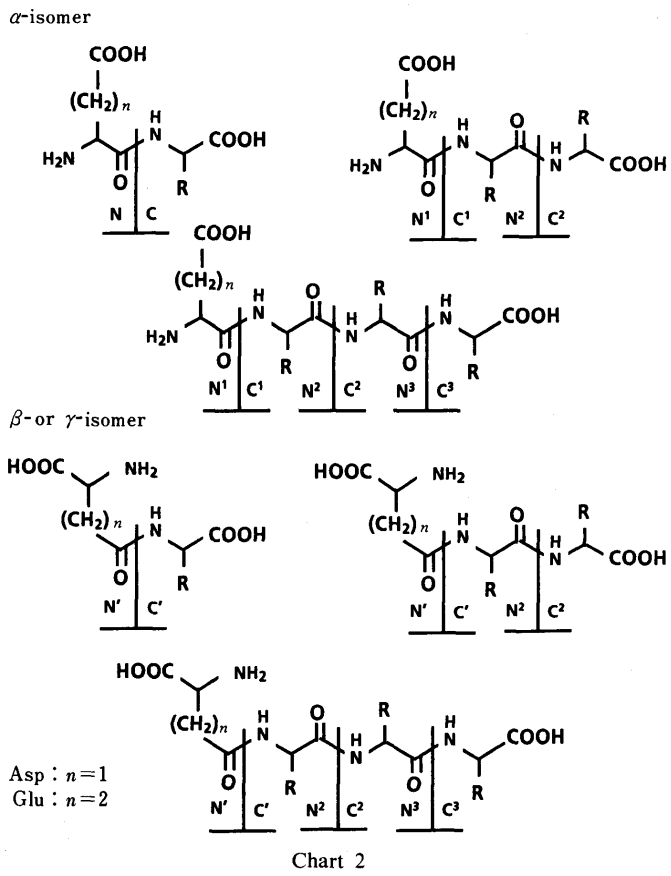

Chart 2

A characteristic fragment peak for the α-isomer was observed at m/z 201 $[MH-H_2O]^+$ while one for the γ-isomer (**11**) occurred at m/z 90 $[Ala+H: C'+2]^+$ (as a C-terminal side ion due to the cleavage of the amide bond: Chart 2). Other additional fragment ions at m/z 202 $[MH-NH_3]^+$ and 130 $[N']^+$ (Chart 2) were also observed for the latter isomer (**11**). These results were closely similar to those for the taurine dipeptides (**6**, **16**).[3]

Furthermore, detailed analyses of main metastable ions (Table I), observed in the spectra of six pairs of di- (**1**—**6**, **11**—**16**), two pairs of tri- (**7**, **8**, **17**, **18**) and two pairs of tetrapeptides (**9**, **10**, **19**, **20**) revealed that all the α-isomers in these peptides gave a characteristic dehydration peak, $[MH-H_2O]^+$, while the γ-isomer gave a $[C'+2]^+$ peak. For example, the two naturally occurring peptides, norophthalmic acid (**17**) and glutathione (**21**), gave the corresponding $[C'+2]^+$ peaks, which might not be formed by the respective α-isomers. Hence, α- and γ-isomers of glutamyl oligopeptides can be easily distinguished without authentic specimens, simply by means of the B/E linked scan SIMS. This generalization can be explained with the help of a neighboring group effect involving the N-terminal amino group as shown in Chart 3 (major fragmentation pathways for glutamyl oligopeptides). Blocking of the N-terminal amino group causes a crucial change (as we reported previously)[3,15] and α- and γ-isomers of Ac–Glu–Ala (**22**, **23**) were no longer distinguishable without the use of authentic specimens: thus, the neighboring group effect involving the free amino group is essential.

There are two possible pathways from the α-isomers of

dipeptides to dehydrated fragment ions, $[MH-H_2O]^+$; path (a) gives pyroglutamyl derivatives while path (b) gives diketopiperazine derivatives. Path (a) is especially important for taurinedipeptides such as (**16**) which can give $[MH-H_2O]^+$ peak only in this way; however, path (b) cannot be neglected because the larger α-oligopeptides seem to give $[N^2]^+$ peaks thereby. The γ-isomers give rise to $[C^2+2]^+$ ions by loss of pyroglutamic acid at the same time.

Table II shows MI of isomeric pairs of glutamic acid esters. The α-glutamyl methyl ester showed a predominant peak at *m/z* 144 $[MH-H_2O]^+$; in contrast, the γ-glutamyl methyl ester gave a predominant peak at *m/z* 130 $[MH-MeOH]^+$. Comparison of the two MI spectra enables to distinguish these isomers. It seems that their fragmentation pathways, as shown in Chart 4, are analogous to those of glutamyl peptides. However, it should be noted that those of the α- and γ-glutamyl benzyl esters are exceptional in that both compounds give the same MI spectra, because the tropilium cation at *m/z* 91 is by far the most stable ion formed.

Comparison of the MI spectra of isomeric pairs of H–Asp–Ala–OH (**29**, **34**), shown in Fig. 3 and Table III, can easily distinguish both compounds; the α-isomer (**29**) showed the base peak at *m/z* 187 $[MH-H_2O]^+$, whereas the β-

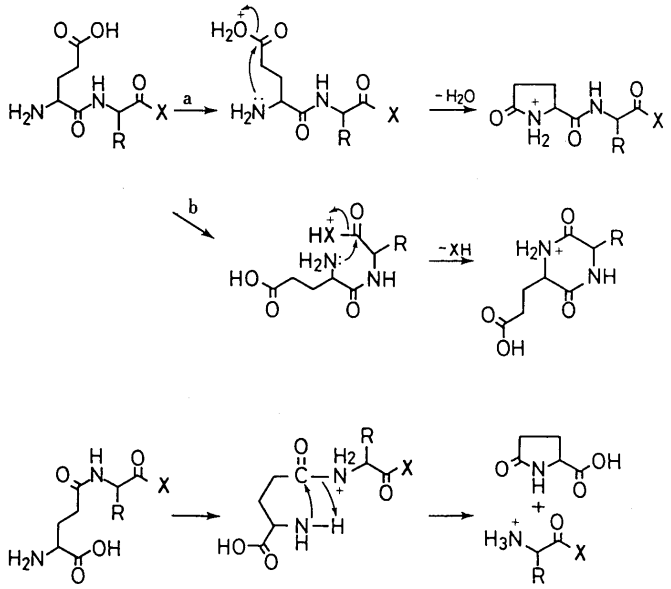

Chart 3

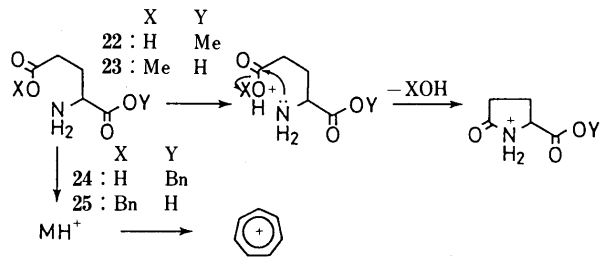

Chart 4

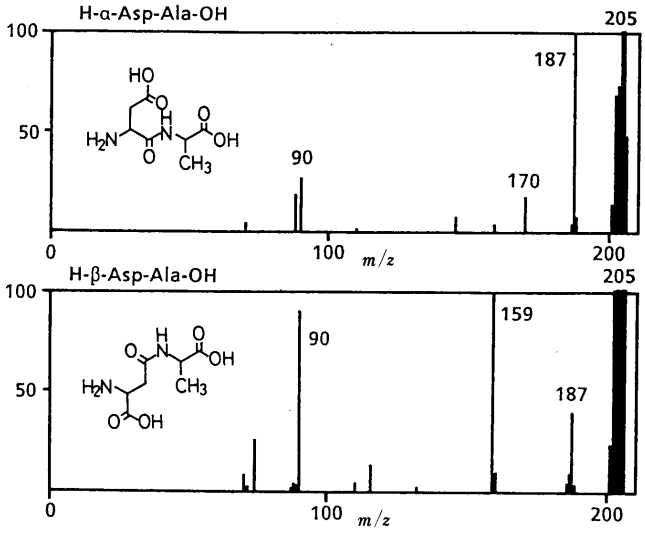

Fig. 3

TABLE II. Comparison of α- with γ-Glutamyl Esters in Terms of Major Fragment Ions[a]

Comp.	$(MH)^+$	α-Isomer [*m/z* (%)]			γ-Isomer [*m/z* (%)]		
		$(MH-H_2O)^+$	$(MH-147^{b})^+$	$(MH-MeOH)^+$	$(MH-HCO_2H)^+$	$(MH-NH_3)^+$	$(MH-147^{b})^+$
Glu–OMe	162	144 (100)		130 (100)	116 (33)	145 (27)	
Glu–OBn	237		91 (100)				91 (100)

a) This table indicates the relative ion intensity as a percentage of the most intense peak among the fragment ions in each spectrum. *b*) H–Glu–OH: 147.

TABLE III. Comparison of α- with β-Aspartyl Dipeptides in Terms of Major Fragment Ions[a]

Comp.	$(MH)^+$	α-Isomer [*m/z* (%)]				β-Isomer [*m/z* (%)]				
		$(MH-H_2O)^+$	$(C+2)^+$	$(MH-HCO_2H)^+$	$(MH-NH_3)^+$	$(MH-H_2O)^+$	$(C'+2)^+$	$(MH-HCO_2H)^+$	$(MH-NH_3)^+$	$(N')^+$
Asp–Tau	241	223 (100)					126 (100)	195 (44)		
Asp–Gly	191	173 (100)				173 (35)	76 (90)	145 (100)		
Asp–Ala	205	187 (100)	90 (28)			187 (38)	90 (90)	159 (100)		
Asp–Leu	247	229 (100)	132 (97)	201 (92)			132 (60)	201 (100)		
Asp–Lys	262	244 (36)	147 (100)		245 (50)	244 (45)	147 (58)		245 (100)	130 (15)

a) This table indicates the relative ion intensity as a percentage of the most intense peak among the fragment ions in each spectrum.

isomer (**34**) exhibited a peak due to the $[MH-HCOOH]^+$ ion as the base peak at m/z 159 accompanied with a $[C+2]^+$ peak at m/z 90. The metastable ions for other isomeric pairs of aspartyl dipeptides (**28**—**37**) are summarized in Table III; most pairs of compounds can be similarly distinguished by analyzing the MI patterns, except for H–Asp–Leu–OH (**30**, **35**) and H–Asp–Lys–OH (**31**, **36**). β-Aspartyltaurine cannot give an $[MH-H_2O]^+$ ion but normal β-aspartyl dipeptides do show such an ion as a weak peak. Therefore, differentiation of aspartyl isomers is less clear than in the case of glutamyl isomers and such analyses are better done with the aid of authentic specimens.

On the basis of results mentioned above, the B/E linked scan SIMS method appears to be well suited for determining the linkage of the terminal acidic amino acid in peptides and esters. In addition, our method has the following advantages. (a) Impurities in the sample do not interfere with measurement of the MI spectrum; (b) even mixtures can sometimes be analyzed by selecting MI ions one by one; and (c) the MI spectrum can give considerable information on the amino acid sequence and hence the method can often be used to confirm the whole structure as well as to determine the mode of acidic amino acid linkage.

Three conclusions emerge from this work. (1) The α- and γ-glutamyl isomers give characteristic fragmentation patterns in B/E linked scan SIMS: hence the structure of each such glutamyl derivative can be determined by simply analyzing the spectrum without any need for other instrumental analyses or authentic samples. (2) Similarly, the structures of α- and β-aspartyl isomers can usually be determined by analyzing their analogous spectra, although in some cases it is better to employ authentic specimens. (3) The B/E linked scan SIMS requires no derivatization, much less sample and a much simpler technique than conventional methods; moreover, the fragmentation pattern is usually informative for overall structural elucidation. Accordingly, this technique should find considerable use in wider fields of organic chemistry, especially within the area of natural products.

Experimental

Materials Four pairs of glutamyl dipeptides (**1**—**4**, **11**—**14**) and four pairs of aspartyl dipeptides (**28**—**31**, **33**—**36**) were purchased from Kokusan Chemical Works (Japan) and BACHEM Finechemical (Switzerland), respectively. Glutathione (**21**) was obtained from Sigma Chemical Company and other peptides were synthesized in our laboratory by conventional liquid-phase methods as analytically pure materials.

Mass Spectrometry The SIMS spectra were obtained through the use of a Hitachi M-80B double-focussing mass spectrometer with its standard SIMS source. The mass scale was calibrated by using PFK with electron impact ionization. The primary ions were Xe^+, and the accelerating voltages of primary and secondary ions were 8 and 3 kV, respectively. Structurally informative metastable ions were obtained by the linked scan (B/E) technique using the $(MH)^+$ ion as we previously reported. Peptide samples were dissolved in water at a concentration of 2 μg/μl.

Each sample solution (1.0—2.0 μl) was loaded on a stainless steel substrate, and about 0.5 μl of glycerol was added to the sample on the substrate.

References and Notes

1) This work was presented at the 108th Annual Meeting of Pharmaceutical Society of Japan, Hiroshima, April 1988.
2) Amino acids, peptides and their derivatives are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972).
3) Part III: K. Ienaga, K. Nakamura, K. Higashiura, Y. Toyomaki and H. Kimura, *Chem. Pharm. Bull.*, **36**, 2796 (1988).
4) D. L. Buchanan, E. E. Haley and R. T. Markiw, *Biochemistry*, **1**, 612 (1962).
5) M. F. Lou, *Biochemistry*, **14**, 3503 (1975).
6) Y. Kakimoto, T. Nakajima, A. Kanazawa, M. Takesada and I. Sano, *Biochim. Biophys. Acta*, **93**, 333 (1967).
7) K. L. Reichelt, *J. Neurochem.*, **17**, 19 (1970).
8) K.-M. Marnela and P. Lähdesmäki, *Neurochem. Res.*, **7**, 933 (1983).
9) K.-M. Marnela, M. Timonen and P. Lähdesmäki, *J. Neurochem.*, **43**, 1650 (1984).
10) H. J. Klosterman, G. L. Lamoureux and J. L. Parsons, *Biochemistry*, **6**, 170 (1967).
11) T. Kasai and S. Sakamura, *Agric. Biol, Chem.*, **38**, 1257 (1974).
12) T. Kasai and S. Sakamura, *Nippon Nogei Kagaku Kaishi*, **48**, 521 (1974).
13) J. van Heijenoort, E. Bricas, B. C. Das, E. Leaderer and W. A. Wolstenhoime, *Tetrahedron*, **23**, 3403 (1967).
14) H. T. Nagasawa and S. D. J. Magnan, *Biomed. Mass Spectrom.*, **9**, 252 (1982).
15) K. Ienaga, K. Nakamura, K. Higashiura and H. Kimura, "Peptide Chemistry 1987," ed. by T. Shiba and S. Sakakibara, Protein Research Foundation, Osaka, 1988, pp. 93—96; *idem*, Abstracts of Papers, Japan Symposium on Peptide Chemistry, Kobe, 1987, p. 124.
16) A. P. Bruins, K. R. Jennings and S. Evans, *Int. J. Mass Spectrom. Ion Phys.*, **26**, 395 (1978).