The development of fast atom bombardment combined with tandem mass spectrometry for the determination of biomolecules

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I. INTRODUCTION

Since the initial report on fast atom bombardment (FAB) by Barber et al. in 1981 (1), FAB has fast become the method of choice for the mass spectral analysis of polar, nonvolatile compounds. The number of reported successful applications of FAB referenced in recent review articles attests to the continuing popularity of FAB/MS (2).

Rarely happy with the status quo, and certainly not content with just being able to determine the molecular weights of these compounds (which was an unrealized dream a few years ago), mass spectrometrists soon noted several deficiencies in the application of the technique. The major deficiency is that the structural information available from fragment ions is often lacking (3). This deficiency can be attributed to several factors.

1. Although the internal energies of the ion desorbed by FAB seem higher than those formed with some techniques, there is a considerably body of information that suggests that a major mechanism of ion formation is chemical ionization in the selvedge between the liquid matrix and the high vacuum region (4). Chemical ionization plus the thermalization which occurs in a high pressure gas, however, is a soft ionization process. Thus, as a result of these two steps, FAB spectra often exhibit little fragment ion intensity.
2. The chemical background due to the matrix often obscures any fragment ions that do occur.
3. Finally, many of the analytes of interest, especially in the realm of biochemistry, exist as mixtures. Therefore, even if significant daughter ions are produced, assignment of specific parent ion/daughter ion relationships may be impossible.

Another technique, tandem mass spectrometry (MS/MS), was undergoing development at the same time as FAB (5). The major applications of MS/MS lay in mixture analysis, in reduction of chemical noise, and especially when coupled with collisional activation, in structure elucidation as well as in studies of ion chemistry. Levsen and Beckey (6) had early on pointed out that coupling MS/MS with soft ionization techniques facilitates mixture analysis. Thus, the technique of MS/MS appeared to be ideally suited to alleviate the drawbacks sometimes encountered in FAB/MS. It is not surprising, therefore, that a number of groups began to explore the applicability of MS/MS in combination with FAB/MS shortly after Barber's initial report on FAB/MS (7–11).

This review will cover the major developments in MS/MS combined with FAB through circa 1988 with the exception of linear peptides. Linear peptides will be covered through circa 1986. This restriction largely omits applications of four-sector and hybrid MS/MS instruments to peptide analysis which occurred after this time. These applications have been reviewed recently by Biemann and Martin (12) and an update of this review is in preparation. The emphasis will be on instrumental approaches and the advantages of FAB combined with MS/MS, especially as applied to specific compound classes. To this end, the body of the review is divided into the four essential questions; Why? How? What? and Who (references)? Although a comprehensive discussion of the development of MS/MS is beyond the arbitrarily defined limits of the review, it should be noted that the combination of FAB and tandem mass spectrometry did not occur without antecedents, but owes a great deal to earlier experiments, such as the MS/MS studies of peptides by Levsen (13), Hunt (14), and Steinauer (15,16), and their co-workers, Cooks' complex mixtures analyses (17) and field desorption MS/MS studies by Gierlich et al. (18) and by Straub and Burlingame (19), to name only a few. Without this body of knowledge the field could not have progressed as rapidly as it did.

To adequately portray the rapid development of FAB/MS/MS, references to ASMS abstracts that are representative of the early developments in a given area are included, as well as formal publications.

II. WHY?

Gross and co-workers (20) have pointed out that four major motivations for combining FAB with MS/MS have emerged from the growing body of FAB/MS/MS studies. These are the ability to do mixture analysis, to enhance fragmentation, to induce new fragmentation, and to exploit the fragmentation of complex molecules into their constituent building blocks.

A. Mixture analysis

Many biological samples exist as mixtures because of the presence of contaminants such as phthalates and salts, or the presence of related compounds. In addition, the problem posed by interference due to matrix ions can be considered a subset of mixture analysis. One of the earliest reported examples of analysis of a naturally occurring mixture by FAB/MS/MS was that of an ornithine-containing

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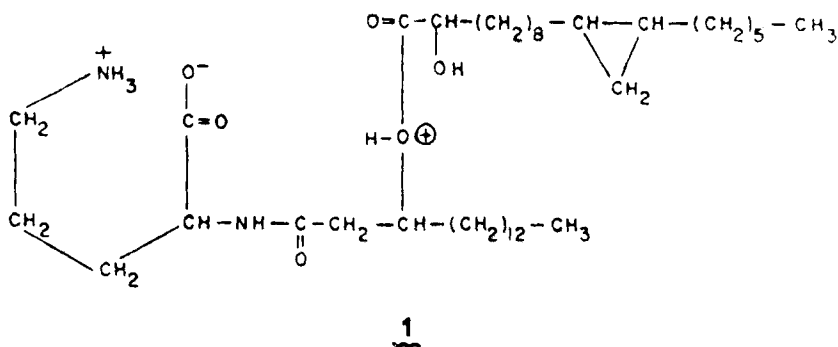
lipid [1] (9,21). This lipid consists of two homologous components whose positive ion MS/MS spectra are identical. This was interpreted to be the result of the loss of the α-hydroxy fatty acid, which, therefore, must contain the site of homology. Comparison of the negative ion FAB/MS/MS spectra of the two components verified this interpretation based on the appearance of an m/z 311 ion in the higher homolog and an m/z 297 ion in the lower homolog. These ions were interpreted as arising from formation of the 2-hydroxycarboxylate anion.

The approach of Biemann et al. (22) and of Raschdorf et al. (23) to protein analysis by determining the structures of the peptides formed by a tryptic digest with MS/MS is also, obviously, a subset of mixture analysis. This area was recently reviewed by Biemann and Martin (12) and will not be elaborated upon in this review.

B. Enhanced fragmentation

In the earliest reports of the FAB/MS/MS spectrum of a peptide, 5-ile-angiotensin, obtained by Barber et al., the MS/MS spectrum did not provide sequence information that was not available in the FAB/MS spectrum (8). Not unexpectedly, it soon became obvious that this observation was decidedly compound dependent. In the area of peptides, Katakuse and Desiderio (24) compared the FAB/MS/MS spectrum (B/E linked scan) of leu-enkephalin with the full-scan spectrum and observed a significantly more complete set of sequence ions in the MS/MS spectrum. In early reports (9,25) on the structure confirmation of ribosyl diphthamide [2], it was noted that the FAB/MS spectrum did not contain detectable fragment ions whereas in the FAB/MS/MS spectrum structurally significant fragment ions were evident (Fig. 1).

C. New fragmentation

In addition to enhancement of fragmentation processes normally observed, new fragmentations that have not been observed (or recognized) by FAB/MS alone have been observed with FAB/MS/MS. The most prominent example is that of charge-remote fragmentations. These fragmentations are most often observed for closed shell anions or cations that also contain long hydrocarbon chains. Fragmentation occurs at the end of the molecule remote from the site of the localized charge. The initial examples were long-chain fatty acids for which a series of
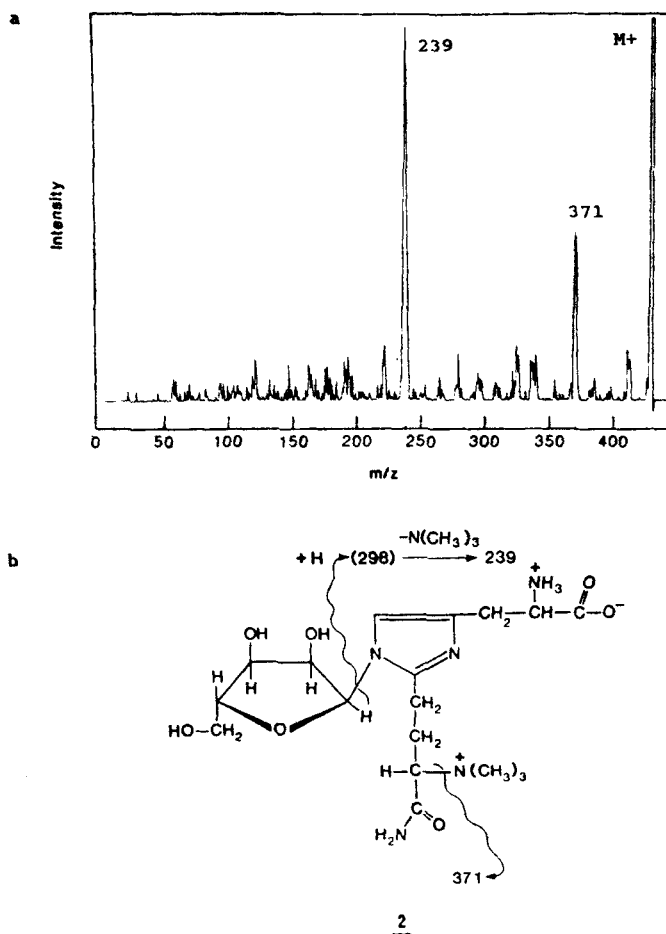
Figure 1 (a) MS/MS spectrum of ribosyl diphthamide. (b) MS/MS fragmentation of ribosyl diphthamide. From Ref. 25 with permission.

C_nH_{2n+2} losses were observed to be initiated at the ω-carbon (26). These fragmentations are very sensitive to structural variations within the hydrocarbon chain. Thus, for example, double bonds can be located (e.g., see Section IV.F.1).

D. Fragmentation into constituent building blocks

The most extensively exploited feature of FAB/MS/MS spectra has probably been the proclivity of biopolymers to dissociate into their constituent building blocks upon collisional activation. This feature is probably due to the fact that the bonds between the building blocks are the weakest chemically. This effect has been widely noted in MS/MS studies of peptides and has been widely applied in the structure determination of peptides (12). As we will see in more detail, this fragmentation tendency is not limited to peptides but is also observed in the MS/MS spectra of nucleotides, carbohydrates, and related glycosides and lipids.
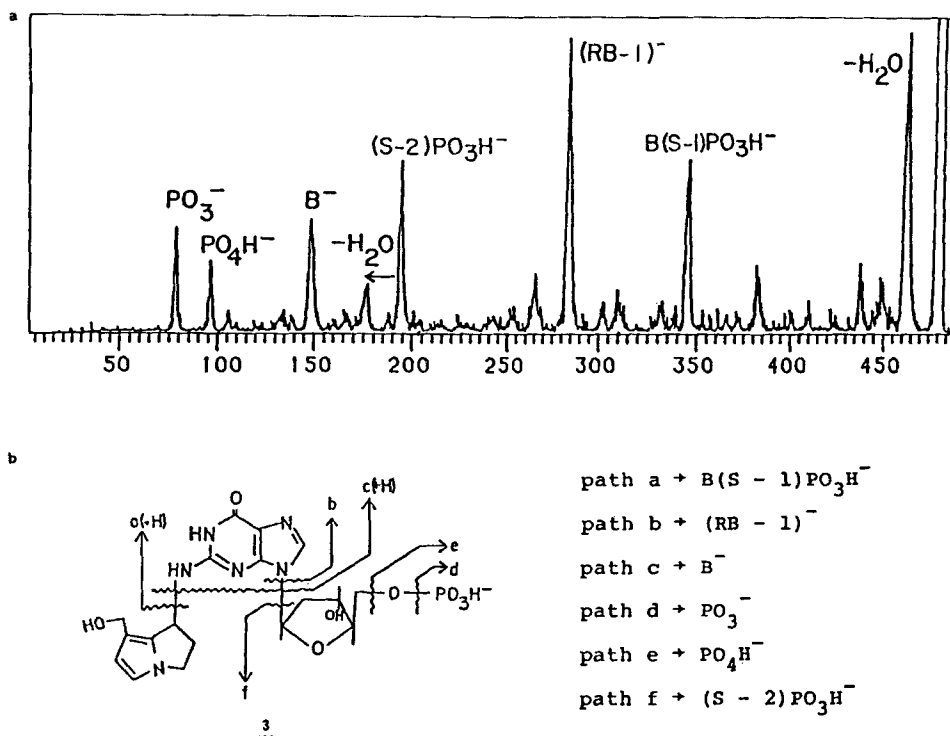
Figure 2 (a) MS/MS spectrum of the (M − H)⁻ anion of the dehydroretronecine deoxyguanosine-5'-monophospate adduct. (b) MS/MS fragmentation of the (M − H)⁻ anion of the dehydro retronecine deoxyguanosine-5'-monophosphate adduct. From Ref. 27 with permission.

It is also not limited to systems containing only similar building blocks such as the amino acid residues of peptides. An example of a more complex system is taken from a study of the FAB/MS/MS behavior of a series of nucleosides and nucleotides modified by pyrrolizidine alkaloids (27). As shown in Figure 2, under negative ion CAD conditions, the (M − H)⁻ anion of dehydroretronecine deoxyguanosine-5'-monophosphate dissociates into phosphate, base, modified base, sugar phosphate, and unmodified nucleotide anions. Thus, ions corresponding to and/or arising from loss of all subgroups of the molecule can be seen. These types of data can be used to reconstruct the original structure of complex biomolecules and to distinguish between potential sites of modification in modified biomolecules.

III. HOW?

Six instrumental approaches have been used to obtain MS/MS data from FAB-desorbed ions: (a) B/E-linked scan of EB or BE geometry instruments; (b) MIKES (mass-analyzed ion kinetic energy) scans of reversed geometry (BE) instruments or triple sector instruments (EBE or BEE); (c) B scans of triple-sector instruments with BEB or EEB geometry; (d) triple quadrupoles; (e) hybrid instruments (EBQQ

Table I. Characteristics of MS/MS instruments.

Instrument	Scan	Ultimate parent ion resolution	Ultimate daughter ion resolution	Energy of collisional activation
EB or BE	B/E	<200[a]	1000[a]	2–8 keV[b]
BE	E	5000[c]	200[a]	2–10 keV[b]
(EB,BE)E	E	100,000[c]	200[a]	2–10 keV[b]
(EB,BE)B	B	100,000[c]	1000[b]	2–10 keV[b]
(EB,BE)EB	B/E	100,000[c]	10,000[c]	2–10 keV[b]
QQQ	Q	Unit	Unit	0–500 eV
FT		>1000	>1000	0–30 eV
(EB,BE)Q	Q	100,000[c]	Unit	0–500 eV

[a]Dependent on kinetic energy release.
[b]Dependent on accelerating voltage and collision cell potential.
[c]Instrument dependent.

or BEQQ), and; (f) four-sector instruments. A brief comparison of techniques is presented in Table I. Those readers interested in a more detailed discussion of instrumental approaches and characteristics are referred to earlier reviews (5,23,28,29).

IV. WHAT?

This section will concentrate on categories of compounds with emphasis on the development and utility of MS/MS for the structural determination of members of the category.

A. Peptides

Undoubtedly, the first and most extensively studied category of compounds investigated by FAB/MS/MS is peptides. The determination of peptides has also been a major driving force behind the development of MS/MS instrumentation. Biemann and Martin (22) have comprehensively reviewed the mass spectrometric determination of peptides including FAB/MS/MS, and an update of that review for this journal is in preparation. This section, therefore, will paint the picture with broad strokes, and Biemann's review articles are recommended for a more detailed picture.

Shortly after the initial report on FAB/MS by Barber's group (1), Hunt and co-workers (7) reported on the MS/MS spectrum of several peptides, MPFA, RVYI, HPF (using the single letter nomenclature for amino acids) and the d_0- and d_3-N-acetylated analogs, obtained by CA in a triple quadrupole. Within the next year (1982) several other reports of FAB/MS/MS data by Barber et al. (8) and by Gross's group (9,11), using MIKES scans, and by Desiderio and Katakuse (24,30,31) and Kratos (10,32) using B/E linked scans, appeared. In a closely related work, Neu-
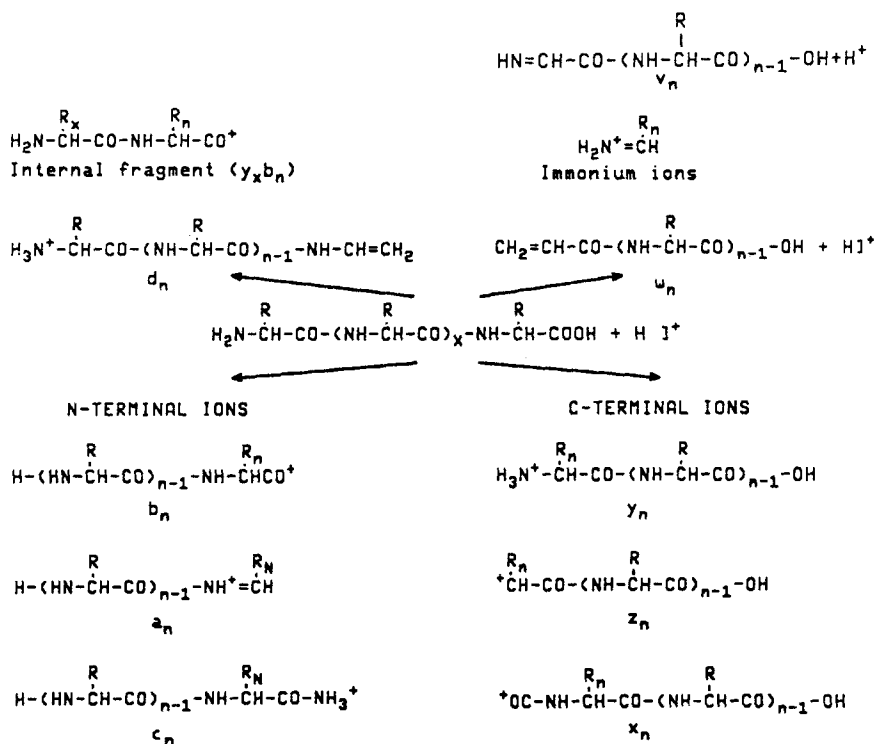
$$R$$
$$HN=CH-CO-(NH-CH-CO)_{n-1}-OH+H^+$$
$$v_n$$

$$R_x \qquad R_n$$
$$H_2N-CH-CO-NH-CH-CO^+$$
Internal fragment $(y_x b_n)$

$$R_n$$
$$H_2N^+=CH$$
Immonium ions

$$R \qquad\qquad R$$
$$H_3N^+-CH-CO-(NH-CH-CO)_{n-1}-NH-CH=CH_2$$
$$d_n$$

$$R$$
$$CH_2=CH-CO-(NH-CH-CO)_{n-1}-OH \;+\; H]^+$$
$$w_n$$

$$R \qquad\qquad R \qquad\qquad R$$
$$H_2N-CH-CO-(NH-CH-CO)_x-NH-CH-COOH \;+\; H \;]^+$$

N-TERMINAL IONS   C-TERMINAL IONS

$$R \qquad\qquad R_n$$
$$H-(HN-CH-CO)_{n-1}-NH-CHCO^+$$
$$b_n$$

$$R_n \qquad\qquad R$$
$$H_3N^+-CH-CO-(NH-CH-CO)_{n-1}-OH$$
$$y_n$$

$$R \qquad\qquad R_N$$
$$H-(HN-CH-CO)_{n-1}-NH^+=CH$$
$$a_n$$

$$R_n \qquad\qquad R$$
$$^+CH-CO-(NH-CH-CO)_{n-1}-OH$$
$$z_n$$

$$R \qquad\qquad R_N$$
$$H-(HN-CH-CO)_{n-1}-NH-CH-CO-NH_3^+$$
$$c_n$$

$$R_n \qquad\qquad R$$
$$^+OC-NH-CH-CO-(NH-CH-CO)_{n-1}-OH$$
$$x_n$$

Figure 3. Peptide fragmentation nomenclature.

mann and Derrick (33,34) utilized FD in conjunction with MIKES scans for the analysis of peptides. Although the three instrumental approaches gave similar results, there was sufficient differentiation in information and/or applications that the methods will be treated separately in this section (only).

In the following discussion, the Roepstroff and Fohlman nomenclature (35) for peptide fragments as modified by Biemann and Martin (12) will be followed (Fig. 3).

There are several ways to organize the large body of information relating to the development of FAB/MS/MS for the determination of peptides. This author has chosen to discuss separately the development of MS/MS techniques with a quadrupole of FT as MS-I, B/E linked-scans of two sector instruments, and MIKES and multisector (three or more) instruments. Within the latter section cyclic peptides and linear peptides have been treated separately.

1. Quadrupole and Fourier transform mass spectrometers as MS-I

In addition to publishing the first article on FAB/MS/MS, Hunt and co-workers (36–43) have applied FAB/MS/MS, using a tandem quadrupole instrument, to the structure elucidation of a number of peptides and proteins. The types of fragment ions produced from peptides are similar to those found in high energy collisions (b and y types). The authors noted that complete sequence information can be

obtained for many peptides. Thus, by digesting a protein into peptide fragments and obtaining the MS/MS data for the peptides, the structure of the parent protein can be ascertained. This approach was recently applied to a number of different peptides and proteins, including purple acid phosphatase (41) and uteroferrin (41). The latter two proteins have molecular weights of over 35 kD, and the FAB/MS/MS approach was successful at identifying over 90% of the residues. Raschdorf et al. (23) have also used tandem quadruple MS/MS for peptide structure determination but with thermospray ionization.

Hunt collaborated with several others (44,45) on the design of a tandem quadrupole-Fourier transform (QFT) mass spectrometer. In this design, the ions are formed by FAB in the quadrupole ion source and drifted into the FT cell where they are subsequently mass analyzed. The goal is to use the high mass resolution capabilities of the FT instrument to obtain unit-resolved spectra of high-molecular weight proteins. Improvements in the design have permitted the detection of proteins with molecular weights greater than 12,000 (46). To improve the structural information obtainable from this instrument, Hunt incorporated laser photodissociation of the ions trapped in the FT cell (47). The approach is based on Bower and McIver's laser photodissociation (LPD) studies of small peptides (48,49). The QFT/LPD-MS/MS technique was applied successfully to enzyme digests from unknown peptides including chemically modified horse cytochrome C and a benzyladenine binding-site peptide (42,43,50,51).

Krishnamurthy et al. (52) determined the structure of a toxic cyclic peptide isolated from blue-green algae using a tandem quadrupole. Cody et al. used FT with MS/MS for the determination of gramicidin S (53).

2. B/E linked scans

Although B/E linked scans suffer from low resolution in MS-I (especially troublesome when the ion of interest is not sufficiently separated from a matrix ion or from its own ^{13}C isotope peaks) and from artifact peaks, a considerable amount of information can be gained from this scan mode.

In 1982, Kratos Analytical published the B/E-linked scan metastable ion spectrum of antiamoebin I [AcPhe-(Aib)$_3$-Iva-Gly-Leu-(Aib)$_2$-Hyp-Gln-Iva-Hyp-Aib-Pro-Phol, m/z = 1670] and met-enkephalin (m/z = 574) (10,32). B/E-linked scans of a series of ions in the spectrum of antiamoebin I were used to establish the sequence ions. The authors stated that sufficient sequence ions were observed in the conventional FAB spectrum of met-enkephalin to elucidate the structure, but that the B/E linked scan data provided confirmation of daughter ion origins.

The published B/E metastable ion spectrum of met-enkephalin contains no fragment ions below the b$_3$ ion at m/z 278. Although this might be due to the use of unimolecular rather than CAD conditions, Katakuse and Desiderio (24) reported the CAD-B/E spectrum of the closely related leu-enkephalin under CAD conditions which contained no daughter ions of mass < 200 dalton. The absence of low mass ions may be due to loss of translational energy of the parent during the collision which can lead to daughter ions that no longer satisfy the B/E scan law. This phenomenon will be discussed in more detail in Section IV.B.1.

Desiderio along with Dass et al. (54,55) have investigated the fragmentation patterns of a number of neuropeptides, including Substance P and a variety of dynorphin fragments by using FAB/MS/MS and B/E-linked scans. Matsuo et al. (56) also investigated the utility of CAD-B/E scans for sequencing small peptides. In conjunction with a sequencing program, these data were used to determine the amino acid sequences.

B/E scans were also used as confirmation of the presence of the phosphate group in phosphorylated peptides (57). The authors noted that this approach is especially useful for the confirmation of the presence of phosphorylated peptides in mixtures and for peptides that do not undergo extensive fragmentation under conventional FAB conditions.

Witten, Gade, and co-workers (58–61) have also used B/E linked scans to help determine the structures of several neuropeptides with unknown structures. These included adipokinetic hormones isolated from cockroaches, crickets, and locusts and hypertrehalosaemic factor II from an Indian stick insect. The B/E scans were used to confirm the structures assigned to the daughter ions in the full-scan spectrum. Millington et al. (62) used B/E scans to help confirm the identities of acylcarnitines in the urine of children with metabolic disorders and Reye's syndrome.

In addition to structure determination, FAB-B/E linked scanning has also been applied to quantitative analysis. Desiderio's group (63,64) has pointed out that the reduction of chemical noise in the B/E scan is significantly greater than the reduction in signal intensity leading to an improved signal-to-noise ratio for quantification. They have utilized B/E scans to quantify peptides in a variety of tissues (64–67) (e.g., leu-enkephalin in canine caudate nuclei and leu-enkephalin and met-enkephalin in canine pituitary tissue and in tooth pulp). In these studies, a unique sequence ion arising from the parent ion of the peptide to be quantified was monitored by selected ion monitoring (SIM) at the appropriate B/E ratio. The $^{18}O_2$-labeled analog of the peptide to be quantified was used as an internal standard, and its appropriate fragmentation was monitored by selected ion monitoring at its B/E ratio. Analyte and internal standard transitions were monitored alternately.

In a comparison of FAB-B/E-SIM with radioimmunoassay (RIA), Desiderio et al. (67) noted that FAB-B/E-SIM has greater specificity than RIA and a competitive sensitivity. They then pointed out that, although the initial investment in a mass spectrometer is significantly higher, if the instrument is used for dedicated analyses, the cost of the instrument could be amortized within a year. This observation may be of great importance to those who are justifying new tandem instruments.

3. MIKES and multisector instruments

Soon after Hunt's (7) report on the tandem quadrupole MS/MS spectra of peptides and Barber's (8) report on the linked scan spectrum of Ala-Leu-Gly, Barber et al. (68) published the first MIKES scan of a FAB-desorbed peptide, 5-ile-angiotensin I, using a reverse geometry VG-ZAB. They noted that the majority of fragment ions can be accounted for on the basis of b, a, c, and y fragments.
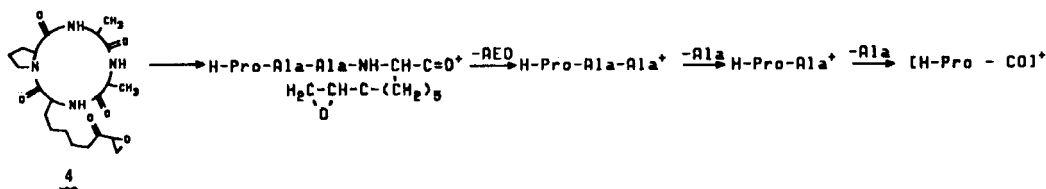
H—Pro—Ala—Ala—NH—CH—C≡O⁺ $\xrightarrow{-AEO}$ H—Pro—Ala—Ala⁺ $\xrightarrow{-Ala}$ H—Pro—Ala⁺ $\xrightarrow{-Ala}$ [H—Pro − CO]⁺

H₂C—CH—C—(CH₂)₅

Figure 4. Fragmentation of the (M + H)⁺ ion of HC toxin.

Neumann and Derrick (33) also reported the MS/MS spectrum of bradykinin by FD/MIKES in 1982.

Cyclic peptides. The first application of FAB/MS/MS to the structure determination of an unknown peptide, the cyclic peptide HC-toxin [4], was published by Tomer and co-workers at the University of Nebraska (21) with the Kratos MS-50 TA (EBE geometry) (69). Although HC toxin is a simple cyclic tetrapeptide, the correct structure had eluded investigators for 15 years. The difficulties encountered in the structure determination of HC toxin was typical of problems encountered in the structure elucidation of cyclic peptides in general. EI spectra were dominated by rearrangement ions, whereas hydrolysis in a preliminary step was difficult to control and is time consuming.

The Nebraska group recognized that the MS/MS spectrum can be interpreted in a straightforward manner. Protonation of this cyclic peptide occurs preferentially on the most basic amide nitrogen. This leads to ring opening with formation of a linear acylium ion followed by loss of amino acid residues (Fig. 4). This process was confirmed in an MS/MS/MS experiment in which the MS/MS spectrum of the m/z 169 ion produced uniquely from m/z 240 in the first field-free region was obtained. The same strategy was later employed to ascertain the structure of a naturally occurring analog of HC toxin in which an alanine replaced the glycine adjacent to proline (70).

The methodology applied to the structure determination of HC toxin was shown to be valid as a general approach for these types of cyclic peptides in a study of the MS/MS behavior of a series of nine synthetic cyclic peptides (71). Protonation of an amide nitrogen leads to ring opening to form a linear acylium ion, which then loses amino acid residues. As the cyclic molecule increases in size and as the number of competitive sites of protonation increases, the MS/MS spectrum becomes increasingly complicated. Added to this difficulty are the occurrences of overlapping ions due to the low resolution of MS-II in MIKES instruments, and also the uncertainty of mass assignment in daughter ions arising from higher mass parent ions due to loss of translational energy in the collision process (sometimes referred to as Derrick shifts; see Section IV.C.1).

To overcome the problems with interpretation encountered in the more complex peptides, Eckart and Schwarz in collaboration with Tomer and Gross (72) recommended that the amino acid doublets (e.g., AB⁺, BC⁺, CD⁺, and DA⁺) that arise from cyclo[ABCD] or are lost as neutrals, be determined. This information, along with the fragmentation observed in the MS/MS spectra of the amino acid doublets and their decarbonylated species provided the data essential for structure assignment. This approach was successfully applied to at least nine other cyclic
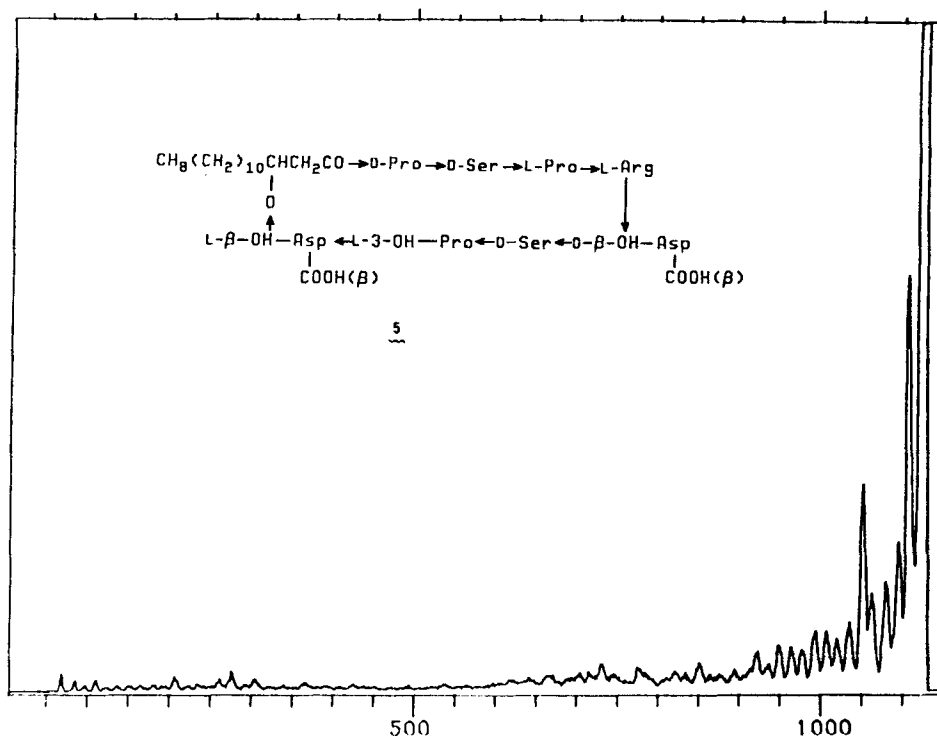
Figure 5. MS/MS (EBE) spectrum of empedopeptin. (Sample kindly provided by M. Konishi, Bristol-Myers Research Institute-Tokyo.)

peptides (72–74) and is applicable to cyclic peptides containing up to at least 8–10 amino acids.

The analysis of cyclic peptides by FAB/MS/MS has also attracted attention from other groups (see Sect. IV.A.1). Aubagnac et al. (75) also published the MS/MS spectrum of tentoxin and gramicidin S.

The problems caused by overlapping masses and mass shift become increasingly more apparent for MIKES as the mass of the cyclic peptide increases (as well as for linear peptides, Sect. IV.A.3). This makes structural assignments of unknowns solely on the basis of the FAB/MS/MS data extremely difficult, if not impossible. An example is the MS/MS spectrum of the $(M + H)^+$ ion (m/z 1126) of empedopeptin [5] (Fig. 5). In such cases, MS/MS data of hydrolytic fractions can be used in the structure elucidation process as in the determination of scytonemin A, c[5-(N-Ac-Ala)Ahda-Ser-Gly-HyMePro-HyMePro-Leu-Hse-Phe-Gly-HyLeu-MePro] (76).

At this time, MS/MS spectra of cyclic peptides using the new four-sector instruments with a high-resolution MS-II have not been published. It is expected, however, that these instruments will improve the ease of structure determination of higher molecular weight cyclic peptides.

Linear peptides. Research into the application of FAB/MS/MS for the determination of linear peptides was even more active during this time period than for the determination of cyclic peptides.

In a 1982 study, Heerma et al. (77) investigated the MS/MS spectra (metastable ion and collisionally activated) of the heptapeptide ALW(For)NFRA, m/z 927. They observed a complete set of y ions and nearly complete sets of b, z, and x ions. In comparison a number of these ions were missing in the conventional FAB mass spectrum.

Amster and co-workers (78) reported the CAD spectra of 15-Met-gastrin, bradykinin and Lys-bradykinin in 1983. This report noted that the daughter ion yield for the heptadecapeptide 15-Met-gastrin was 1.6% (metastable yield subtracted out) which compared favorably with the 2.3% daughter ion yield from the tripeptide Met-Asp-Phe-NH$_2$. Equally important was the observation that, while the metastable ion yield from the heptadecapeptide was large (12%) the CAD spectrum was dominated by sequence ions rather than by side chain cleavages which dominated the unimolecular fragmentations. In addition, the collisionally activated MS/MS spectrum of $Cs_{35}I_{44}{}^+$ (m/z 8966) was reported (78), indicating that CAD MS/MS might be useful for high mass ions.

In 1984, Barber et al. (79) published the MS/MS spectrum of bovine insulin under both positive ion (m/z 5734) and negative ion (m/z 5732) conditions. Fragment ions due to the B chain (positive and negative ion) and A chain (negative ion) were observed. Although mass resolution was poor and the number of assignable ions small, these spectra did show that McLafferty's observation that CAD-MS/MS spectra could be obtained for high mass compounds was not strictly limited to inorganic salts but that some MS/MS data for fairly high-molecular weight peptides can be obtained.

The basic fragmentation pathways observed in the FAB/MS spectra of peptides have been explored in a number of reports. The development of the understanding of the fragmentation processes has been reviewed by Biemann and Martin (12). The same type of fragment ions have also been observed in the FAB/MS/MS spectra. In addition to studies in which these fragment ions are noted implicitly, there have been several studies in which the types and abundances of various fragment ions found in MS/MS spectra were examined. Lippstreu and Gross (80) and Tomer et al. (81) identified the major fragment ion types, b, a, c, y, and z, and their relative amounts in a series of eight tri- to hexapeptides and in a series of six endorphin and ACTH peptides 12 to 17 residues in length. They observed that the type of fragment ion (N- or C-terminal) that predominates is dependent on the composition of the peptide. These studies as well as the other MIKES studies discussed above also demonstrated that many of the fragment ions observed in FAB/MS spectra originate from the protonated molecular ion.

A major advantage of FAB/MS over conventional peptide sequencing techniques is the ability to sequence blocked and modified peptides. FAB/MS/MS shares this advantage and, because of the low chemical background, has been shown to offer advantages over FAB/MS.

Gibson et al. (57) investigated phosphorylated peptides by using both B/E and MIKES scans. The FAB/MS and FAB/MS/MS spectra of phosphorylated and non-phosphorylated kemptide, LRRASLG, were compared. They noted that the FAB spectrum does not contain significantly abundant ions due to loss of HPO$_3$ or

CH₃
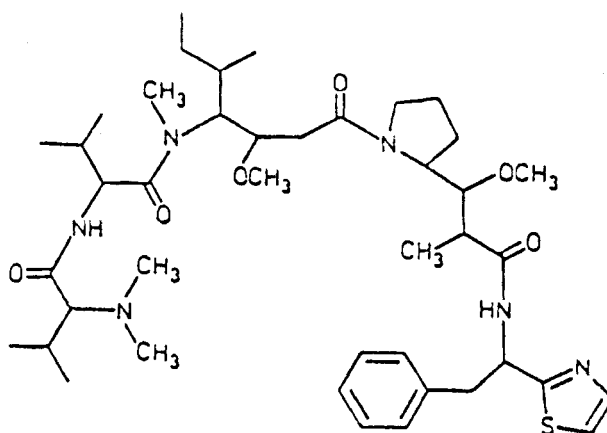
DOLASTATIN 10

6

HPO₄ from phosphorylated kemptide although, in the MS/MS spectrum (obtained on a four-sector ZAB), losses of HPO₃ and HPO₄ are established to be major processes.

Schwarz's group at the Technical University of Berlin has also made extensive use of FAB/MS/MS for the determination of blocked peptides and modified peptides. This work includes structural determination of adipokinetic Hormone I (82), adipokinetic hormone from *Maduca serta* (83), eighteen N- and C-terminal-blocked peptides, and peptides containing N-alkylated amino acids (84). In the study of blocked and N-alkylated peptides, 19 [<GLU][6] analogs of substance P(6-11) were studied using a VG ZAB 3F of EBB geometry. A detailed methodology was developed in this article for the sequence determination of these types of peptides using MS/MS spectra.

Bathelt and Heerma (85) examined the FAB/MS/MS spectra of six *t*-BOC protected peptides and their unprotected analogs. They concluded that better sequence information is contained in the spectra of the underivatized peptides than in those of the derivatives. In some cases, the sequence ions are absent for the derivative, and, in others, the relative abundances of the sequence ions are greatly reduced because of competition from sequence ions formed by losses of 56 or 100 dalton units from the derivatizing group.

Pettit and collaborators (86) were able to deduce the structure of the structurally unusual pentapeptide, dolastatin 10,[6], on the basis of FAB/MS/MS and NMR data.

B. Peptide-related substances

FAB/MS/MS has also been used for studies of compounds related to peptides and to peptide investigations not directly aimed at structure elucidation.

Mallis, Russell et al. (87,88) utilized FAB/MS/MS to investigate the interaction

of alkali metal ions with small peptides. The site of binding of the alkali metal to the peptide can be determined from the MS/MS data. He observed that sodium bound preferentially to the most basic part of the peptide such as an arginine or a histidine. A comparison of the MS/MS spectra of the $(M + H)^+$ and $(M + Metal)^+$ ions can also provide information as to the energetics of fragmentation processes because only those processes that have an appearance energy lower than the energy needed to break the peptide-metal bond will be observed. For the small peptides he studied, a cleavages (see Fig. 3) were favored as the lowest energy process.

Bodley et al. (25) used FAB/MS/MS in conjunction with HRFAB/MS to confirm the proposed structure of ribosyl diphthamide [3] (Fig. 1). Diphtheria toxin in-actives protein synthesis elongation factor 2 by attaching ADP-ribose to diphtham-ide, an unusual posttranslationally modified amino acid derivative found in the factor. The MS/MS spectrum is dominated by ions arising from loss of the tri-methylamine group and subsequent loss of the ribose moiety (i.e., fragment ions or neutral losses characteristic of the building blocks of the molecule).

FAB/MS/MS has also proven useful for the differentiation of isomeric amino acid residues (89,90). Aubagnac et al. (89) differentiated leucine and isoleucine on the basis of the MS/MS spectra of the immonium ions appearing in the FAB/MS spectrum. Leucine was observed to lose C_3H_6 as a major process whereas isoleu-cine undergoes predominant loss of NH_3. These observations were extended by Heerma and Bathelt (91) to include differentiation of norleucine as well and to identify which isomeric amino acid was present in peptides containing one of the isomers. Johnson and co-workers (92), using a four-sector instrument, found that leucine and isoleucine could be differentiated within the peptide chain on the basis of loss of C_3H_6 from the a ion formed at leucine and loss of C_2H_4 from the a ion at isoleucine. The authors also recognized the presence of w ions (Fig. 4) which can be used to differentiate leucine and isoleucine as well as to characterize other amino acids (93).

Eckart and Schwarz (94) used FAB/MS/MS to differentiate C- versus N-alkylated amino acids such as N-isobutyl glycine and its isomer N-methyl valine. The MS/MS spectrum of the immonium ion from N-isobutyl glycine is evidence that formation of $C_4H_9^+$ predominates whereas losses of CH_3 and CH_4 occur from the isomeric immonium ion (94). They concluded that MS/MS may prove to be the method of choice to characterize N-alkylated amino acids.

C. Ion activation studies

In addition to the numerous studies related to sequencing peptides, a number of reports have addressed the more physical aspects relating to the acquisition of MS/MS spectra of biomolecules. Because these studies relied primarily on pep-tides as model compounds, they are included here.

1. Energy effects in MIKES scans

When they undergo collisional activation, a small amount of the translational energy of ions is transformed into internal energy. This internal energy becomes

available to initiate the fragmentations observed in the CAD process. The implications of this translational energy loss have been studied extensively by Neumann, Derrick, and Sheil (95–97) using field desorption. These workers observed that the translational energy lost by peptide ions increased with increasing mass of the peptide. This energy loss can be in the range of 20–30 eV for peptides of mass >1000. At an incident ion energy of 8 keV for a peptide of mass 1000 and a translational energy loss of 24 eV, the energy loss translates into a mass shift of 3 dalton. As the peptide increased in mass to m/z 1620 (bombesin), the translational energy loss, ΔE, increased to 50 eV (± 7 eV) and an observed average mass shift of 10 dalton. This variable mass shift implies that inaccurate mass assignments can be made in the spectra of unknowns, leading to improper identifications. The group has also noted that ΔE and, thus, the observed mass shift, is not identical for all fragment ions. Guevremont and Boyd (98) studied these apparent mass shifts on a BEQQ tandem instrument. They showed that the apparent mass shifts are real and not due to overlapping multiple fragment ions or the presence of further losses of hydrogen from the daughter ions under CAD conditions.

The conclusion to be drawn from these experiments is that the mass of daughter ions formed from higher mass peptides in a MIKES-based MS/MS instrument cannot be accurately defined. Obviously one can use mass assignments based on the FAB spectrum and/or on the structure of a known or analogous compound. When faced with an unknown or a compound that gives negligible fragmentation, these "tricks" no longer work. One experimentally valid approach is to increase the mass of the collision gas. Neumann et al. (95) noted that using argon in place of helium for the CAD MS/MS of bombesin decreases the average translational energy loss from 50 to 19 eV. Gross and co-workers (20), comparing helium and xenon as collision gases, observed that the apparent mass of the [H-RPPKPQQF-CO]$^+$ fragment (854.5 dalton calc.) from Substance P-free acid shifted from 851 D with helium to 854 D with xenon. An additional advantage of the heavier collision gas is that the proportion of collision energy converted to internal energy will increase, leading to increased energy available to form fragment ions (higher daughter ion yields). It is obvious, however, that, as the masses of compounds being investigated increases, the increasing uncertainty in mass assignment due to energy shifts and overlapping fragment ions detracts from the utility of the MIKES approach to MS/MS at high mass.

These problems associated with high mass MIKES in conjunction with the increasing importance of the MS/MS technique for structure determination of biomolecules and in the field of biotechnology were of sufficient motivating effect that instrument manufacturers took the next logical step [based on McLafferty's pioneering work (99)] and are building tandem instruments with high-resolution MS-IIs, usually of EB design (29,100–102). These instruments use a B/E-linked scan of MS-II to obtain the daughter ion spectra.

One additional point should be made at this time, however. Energy shifts can still affect the MS/MS spectra obtained with four sector instruments. Derrick surmised that the scan law for a B/E scan of MS-II should try to take into account the average translational energy loss. Because this will not be identical for all

fragments, the transmission of all fragments will not be identical. This will lead to variations in relative abundances of fragment ions, depending on the scan law used (97). The author has experimentally observed this to be the case with four-sector instruments (103). Boyd and co-workers (104,105) have employed a floated collision cell to help alleviate this problem. These workers also noted that the use of a floated collision cell involves a significantly more complex scan law and the presence of artifact signals near the precursor ion.

2. Sensitivity

Sensitivity in the context of FAB/MS/MS relates to at least two parameters: (a) parent ion abundance; and (b) daughter ion abundance.

As the molecular weight of an analyte increases, the abundance of the molecular ion can be expected to decrease because of several factors. One is that the monoisotopic molecule-ion species becomes less abundant relative to other ions in the molecule–ion envelope (i.e., ^{13}C-containing ions). Another is that FAB desorption usually becomes less efficient at higher analyte mass. Gross et al. (20) showed that this expected decrease in molecule–ion abundance does occur, and, while compound dependent, it is not severe enough to preclude the occurrence of reasonably intense parent ion currents for compounds to mass 3000–4000 D (20).

Daughter ion yield is a function of parent ion mass, collision gas mass, and collision energy (95–97). Gross, Tomer, and co-workers (20,81) showed that total daughter ion yield can increase with increasing mass at least to about mass 2000 and certainly Biemann's peptide sequencing work has shown that good MS/MS spectra can be obtained up to at least mass 3000 (12,22). As the molecule becomes heavier, however, the number of different daughter ions that are formed also increases. The absolute abundance of any one daughter ion, therefore, may become quite small. Combining the effect of decreased parent ion abundance and decreased daughter ion abundance, one can see that the sensitivity problem can rapidly become a major handicap.

One approach that has been proposed to deal with the problem of decreased daughter ion sensitivity is the use of an instrument that provides for simultaneous ion detection. As described above (Sect. IV.A.1), Hunt's solution (44,45) to this problem is to use FTMS for simultaneous detection both in the parent ion mode and the daughter ion mode. The use of FTMS also permits accumulation of ions prior to detection.

Another approach to this problem (106–111) is to utilize a channel-plate detector to detect simultaneously all daughter ions. This approach has been incorporated into the Kratos four-sector concept (102,112) and by Biemann (113) into his four-sector JEOL instrument. Improvements in sensitivity by factors of 10 to 100 have been noted. Due to limitations on size of the array detector, simultaneous detection is limited so far to less than the full mass range of the spectrum of daughter ions. Nevertheless, the development and application of array detectors offers a means of significantly increasing sensitivity in MS/MS.

Since this review will not cover FAB/MS/MS of peptides and proteins obtained

on four-sector instruments or the recently introduced hybrid instruments, the reader is directed to Biemann's review articles (12) for further information on this subject.

D. Nucleosides, nucleotides, and oligonucleotides

Nucleic acid analysis is another area that has benefitted greatly by the advent of FAB/MS. Schram pointed out that, to his knowledge, no nucleoside, not even highly polar mesoionic nucleosides, fails to provide a useful FAB spectrum (114). It is not surprising, therefore, to find that considerable effort has been expended in applying FAB/MS/MS to these compounds. Even though these compounds are no less important to biochemistry than peptides, fewer reports have dealt with the FAB/MS of members of the nucleic acid family than have been published on peptides. This is due, in part, to the importance of MS in biotechnology where it is used to verify the identity of the peptide and protein products. It is also to some extent due to the greater difficulties encountered in the determination of larger oligonucleotides relative to the determination of larger peptides. As a corollary, there have been fewer papers dealing with the FAB/MS/MS behavior of these compounds. Nonetheless, applications of FAB/MS/MS to nucleic acid chemistry began to appear by 1983 (29,115–117).

As with the MS/MS studies of peptides, the MS/MS studies of the nucleic acid family are based on the firm foundation of a large body of existing knowledge. Included in this foundation are the works of, for example, McCloskey (118), Wilson (119), Linscheid and Burlingame (120), and others (121).

1. Nucleosides

Crow and co-workers (29) published the first MS/MS spectra of FAB-desorbed simple nucleosides (e.g., guanosine, uridine, and dihydrouridine) in 1983. The spectra were dominated by BH_2^+ (protonated base) ions and sugar ions. This work was expanded upon in 1984 (122). This report contains the positive and negative ion MS/MS spectra of 30 nucleosides and related compounds such as tubercidins and nebularines. In addition to formation of sugar$^+$ and BH_2^+ ions, other general fragmentations of nucleosides, S_1 and S_2, and fragmentations specific to a given base were noted (Fig. 6). Similar fragmentations were observed for negative ions (Fig. 7). The FAB/MS/MS spectra, thus, provide a considerable amount of the structural data of the type usually found in EI spectra but lacking in FAB spectra. Also in 1983, Glish et al. (123) and Tondeur et al. (124) published MS/MS spectra of the nucleoside-related compounds thiamine and toyocamycin, respectively.

Included in the reports of the Nebraska group on nucleosides are descriptions of CAD spectra of a number of modified nucleosides. From a comparison of the MS/MS spectra of three methylated guanosines, 1-methyl, 2'-O-methyl, and N^2-methylguanosine, methylation on the sugar or base can be distinguished, the site of methylation on the sugar can be distinguished and, in the case of the N^2-
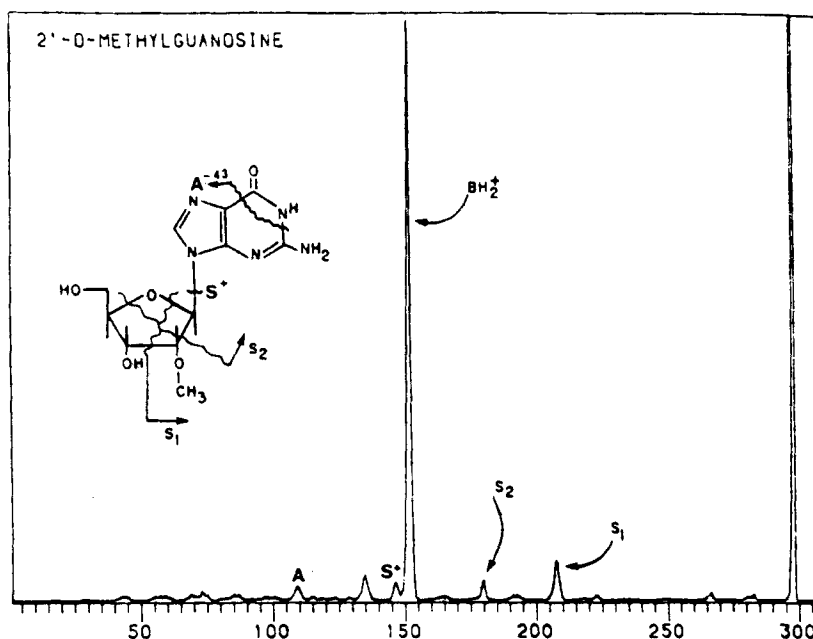
Figure 6. MS/MS fragmentation of the $(M + H)^+$ ion of 2'-O-methylguanosine. From Ref. 122 with permission.

methyl, the location of the methyl group on the base can be ascertained (Table II). Ashworth et al. (125) noted similar results in a study of CI-produced methylated nucleosides. Kralj et al. (126), in a study of the utility of FAB/MS/MS for structure confirmation of synthetic modified nucleosides, also observed similar behavior. Thus, MS/MS is a realistic tool for the elucidation of the structure of modified nucleosides.

Modification of nucleosides by exogenous substances is a major mechanism leading to toxicity and/or carcinogenicity. The structure elucidation of these adducts is, therefore, of fundamental importance for understanding the processes by which carcinogenicity and/or toxicity occur. Considering the potential for structural elucidation of modified nucleosides based on their FAB/MS/MS spectra, it is not surprising that a number of studies of such compounds have been undertaken.

Wickramanayake and co-workers (127) in a report on the FAB spectra of dehydroretronecine (DHR)-nucleoside adducts included MS/MS data on a methylated dehydroretronecine-guanosine adduct. The MS/MS data indicate that methylation occurs on the DHR residue. Tomer et al. (27) then undertook a detailed study of the utility of FAB/MS/MS for the structure elucidation of these alkaloid-nucleoside adducts. In this work, they observed that the modified nucleosides and nucleotides, either as $(M + H)^+$ or as $(M + K)^+$, fall apart into ions representative of the subunits comprising the parent (e.g., the sugar, the modified base, the modifying group and the parent base, or by loss of subunits). This is exemplified in Figure 8. The site of covalent bonding between the alkaloid and the nucleoside can also be verified from the MS/MS data for some of the examples.
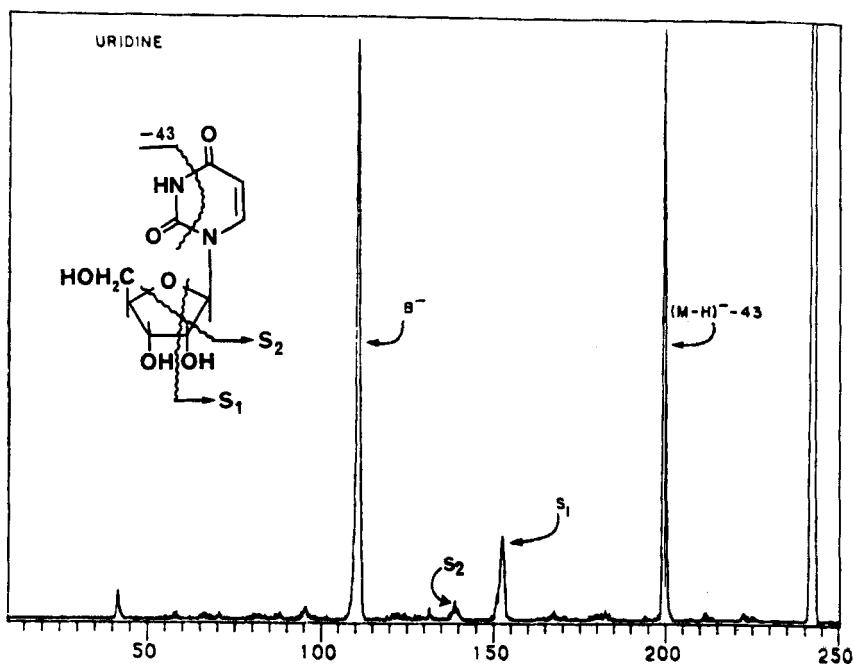
Figure 7. MS/MS fragmentation of the (M − H)⁻ anion of uridine. From 122 with permission.

Tondeur and co-workers (128) studied the FAB/MS and FAB/MS/MS behavior of 25 benzylated guanosines (five attachment isomers and four benzyl substituents). In this study, the authors were able to differentiate positional isomers on the basis of consistent differences in their MS/MS spectra under both positive and negative ion conditions.

DNA adducts of polynuclear aromatic hydrocarbons and related compounds have also received attention by FAB/MS/MS. The Nebraska group in collaboration with the Eppley Institute for Cancer Research investigated the products formed by the electrochemically induced reaction between the benzo[a]pyrene radical cation and guanosine or deoxyguanosine, and the horseradish peroxidase-mediated reaction of benzo[a]pyrene and DNA (129). The FAB/MS/MS data can be used to distinguish clearly between the C-8 and N-7 adducts. Adduct formation at N-7 leads to loss of the sugar. This process is less prominent when adduct formation occurs at C-8. Thus, the supernatant of the DNA reaction, which con-

Table II. Positive ion MS/MS spectra of methylated guanosines.

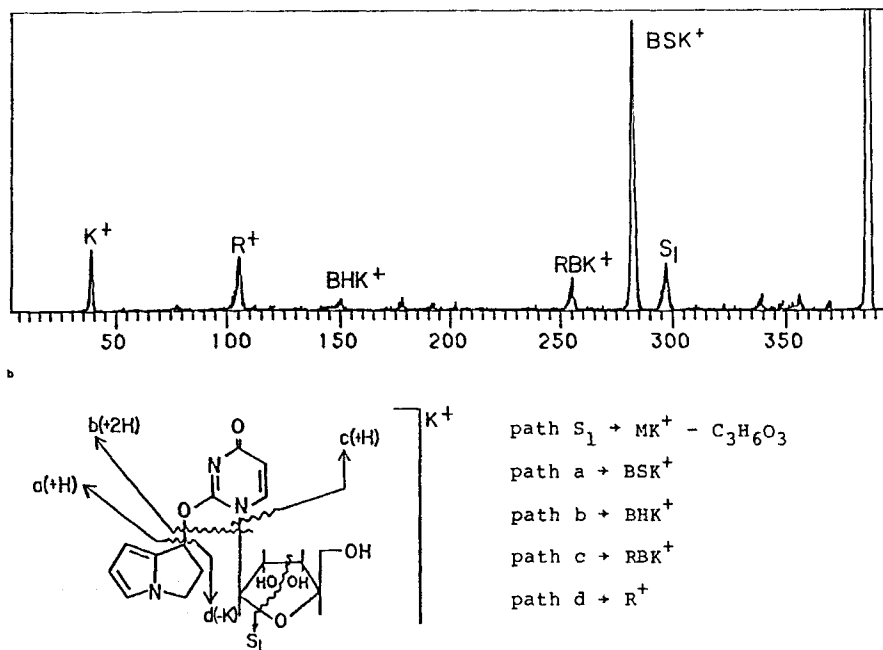| Compound	Fragment ion masses			
	S_1	S_2	BH_2	Other
Guanosine	194	180	152	
1-Methylguanosine	208	194	166	
2'-O-Methylguanosine	208	180	150	
N^2-Methylguanosine	208	180	166	(BH_2—NHCH₃)

Figure 8 (a) MS/MS spectrum of the (M + K)$^+$ ion of the dihydropyrrolizinyl uridine adduct. (b) MS/MS fragmentations of the (M + K)$^+$ ion of the dihydropyrrolizinyl uridine adduct. From Ref. 27 with permission.

tained products formed by depurination, contained 95% of the N-7 adduct. The major adduct in the DNA digest is the C-8 adduct. Dietrich et al. (130) observed that the MS/MS spectrum of the major adduct between 3-nitrofluoranthene and DNA, N-(deoxyguanosin-8-yl)-3-aminofluoranthene, displays a pattern similar to that of the benzpyrene adduct.

In summary, FAB/MS/MS techniques not only have proven useful in the structural confirmation of known nucleosides but also have proven invaluable in the structure elucidation of unknown nucleoside adducts. The utility is based on the decreased background and ability to work with mixtures and on the tendency of these compounds to dissociate into their stable constituent building blocks in a recognizable, and often predictable, manner.

2. Nucleotides

The FAB/MS spectra of mononucleotides are usually considerably more complex than those of the nucleosides because metal ions, typically Na$^+$, are usually associated with the phosphate residue. This leads to formation of a series of cationized species in the positive ion mode. Even under negative ion conditions, cationized species such as (M − 2H + Na)$^-$ are observed along with the (M − H)$^-$ anion. As a result, FAB/MS/MS is an obvious solution to the problem of

sorting out parent ion-daughter ion relationships, especially if a mixture of nu-
cleotides is under study.

The earliest reports on the FAB/MS/MS behavior of mononucleotides were from
a study of cyclic monophosphates carried out by Kingston and co-workers (131,132).
Under positive ion conditions the FAB/MS/MS spectra of the 3',5'- and the 2',3'-
cyclic cytidine monophosphate (cCMP) can be distinguished. The S_1 and S_2 frag-
ments (see Fig. 6) were observed for the 3',5'-isomer, but these ions were very
weak or nonexistent for the 2',3'-isomer. A comparison of the MS/MS data for
the cyclic CMP isolated from rat liver and the known CMPs identified the un-
known as the 3',5'-isomer. Similar differences in behavior were observed for 3',5'-
and 2',3'-cyclic guanosine monophosphate (cGMP). These differences were used
to identify the cGMP isolated from *Phaseolus* leaves as the 3',5'-isomer. Similar
differences in abundances of S_1 and S_2 fragments were obtained for other cyclic
nucleoside MP isomer pairs.

An extensive study of mononucleotides was reported by Cerny et al. (133).
They examined the FAB/MS/MS spectra of eight 2'-deoxymononucleotides and
eight ribomononucleotides under negative ion conditions. These compounds frag-
ment into their constituent building blocks: loss of BH, and formation of B⁻, PO_3^-,
and $H_2PO_4^-$. Additional ions due to loss of CONH from C and T nucleotides
were observed. Loss of BH was always observed to be more facile when the
phosphate group is at the 3'-position than when it is at the 5'-position.

As with modified nucleosides, considerable interest has been generated in
applying FAB/MS/MS to the study of modified nucleotides. In a report on the
mass spectrometric identification of DNA adducts formed by carcinogenic amines,
Mitchum et al. (134) included positive and negative ion FAB/MS/MS data for the
adduct of 2-acetylaminofluorene (AAF) and dGMP. The most abundant ion under
positive ion conditions is the modified base and, under negative ion conditions,
the modified base less ketene.

As part of the study of the MS/MS behavior of the adducts of pyrrolizidine
alkaloids and nucleosides (27), two monophosphates were also investigated. Un-
der negative ion conditions, in addition to the ions reported for the modified
nucleosides, the corresponding nucleotides fragment to give ions characteristic
of the phosphate group (PO_3^- and HPO_4^-), and undergo loss of the modifying
group, and loss of the modified base. Under positive ion conditions, the major
species observed in the FAB spectrum is the $(M + K)^+$ ion. The MS/MS spectrum
of this ion also contains ions due to loss of the phosphate group and due to the
formation of an ion containing the sugar and the phosphate. The identity of the
alkali metal was confirmed by the observation of a K^+ ion.

The Nebraska group also studied the interaction of the cancer therapeutic drug
cis-diammine-dichloroplatinum(II) by FAB/MS/MS (135). In this study adducts
were observed to be formed by the drug with adjacent bases and with DNA-
interstrand crosslinks. The adjacent base adduct undergoes loss of the two sugars
and the connecting phosphate whereas the interstrand adduct undergoes suc-
cessive loss of the two sugar units.

The detection of covalently modified nucleotides by ³²P postlabeling (136) has

become an important tool in the study of carcinogenesis. This technique, however, can only detect the presence of modified nucleotides, not determine the structure. Because mass spectrometry provides the best combination of structural information and sensitivity, it will become increasingly important to this area. The studies mentioned thus far have only scratched the surface of this important area, and one can predict that many more examples will be reported in the future. With the advent of increasingly more sensitive detectors, such as the array detector, analysis of biopsy samples by FAB/MS/MS may become a reality.

FAB/MS/MS also proved invaluable in the identification of ADP-ribosylated *p*-nitrobenzylidine aminoguanidine, the end product of the cholera toxin-catalyzed reaction between ADP-ribose and *p*-nitrobenzaldehyde (137). Quantification of this adduct was then used to measure ADP-ribosyltransferase activity in animal tissues.

Mallis et al. (138) used FAB/MS/MS to determine the positional isomers of isotopically (oxygen) labeled uridine triphosphate. Studies of this type are useful in the structure elucidation of the reaction products of positional isotope exchange experiments that are used in elucidating mechanistic details of many enzymatically mediated reactions.

3. Oligonucleotides

The first report of the FAB/MS/MS spectrum of an oligonucleotide was by Panico et al. in 1983 (115). As part of a study of the sequencing of GGTA and TACC by FAB, they utilized MS/MS data of the $(M - H)^-$ anions to identify those processes occurring in the gas phase as opposed to those that result from condensed phase chemistry.

Neri et al. (116) then examined the isomeric dinucleotides, 3'-5'-TpT and 5'-5'-TpT. These isomers can be readily distinguished on the basis of their MS/MS spectra. In their study of nucleosides, Crow et al. compared the MS/MS spectra of 3',5'-ApC and 2',5'-ApC under both positive and negative ion conditions (122). Whereas the positive ion spectra were nearly identical, the negative ion spectra contained significant differences arising from cleavages across the adenosine sugar ring.

Gross' group in collaboration with Grotjahn (133,139) expanded this study to include all possible 3',5'-ribodinucleotides and 3',5'-2'-deoxyribonucleotides, two deoxyribotrinucleotides, five ribotrinucleotides, a deoxytetranucleotide, a deoxypentanucleotide, and two deoxyhexanucleotides. Under negative ion MS/MS conditions, the dinucleotides eliminate the 3' base as BH in preference to elimination of the 5' base (Fig. 9). (The 5' base was defined as the base attached to the terminal sugar that is bound to the adjacent sugar through a phosphate group a C-5, B_n in Fig. 10.) Liguori et al. (140) also confirmed this behavior for four dinucleotides. Preferential loss of the 3'-terminal base, however, becomes less significant for the higher oligonucleotides and cannot be used to assign the 3' base. Sequence ions indicative of the structure of the oligonucleotide are formed, and those ions arising from the 3' end were observed to be more abundant than those arising from the 5' terminus (Fig. 10) (B_n defined as the 5'-terminus). The
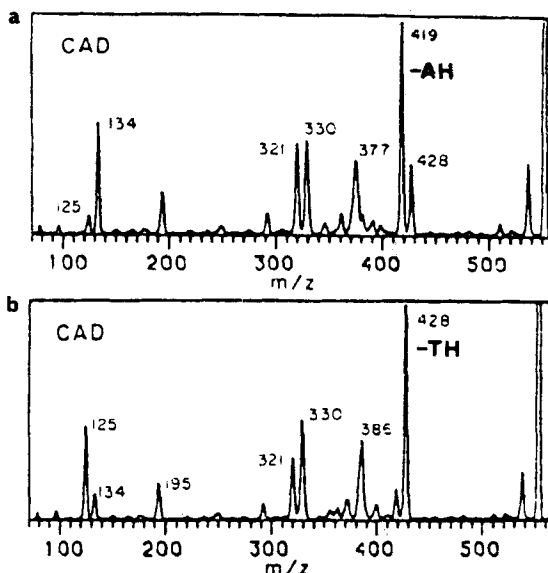
Figure 9. Comparison of the MS/MS spectra of the $(M - H)^-$ anions of (a) d(AT) and b) d(TA) showing preferential 3' base loss. From Ref. 133 with permission.

authors noted, however, that lack of resolution in MS-II and mass shifts, due to loss of translational energy, made assignment of sequence ions problematic for the higher oligonucleotides. The variety of fragment ions was also observed to increase with increasing chain length, which adds to the problems encountered in sequencing an unknown oligonucleotide.

Oligonucleotides modified covalently with xenobiotics have also been investigated by FAB/MS/MS. Lay, in collaboration with staff at the Midwest Center for Mass Spectrometry (141), examined the negative and positive ion MS/MS behavior of the acetylaminofluorenyl adduct with (3',5')-2'-dCdG. The negative ion MS/MS
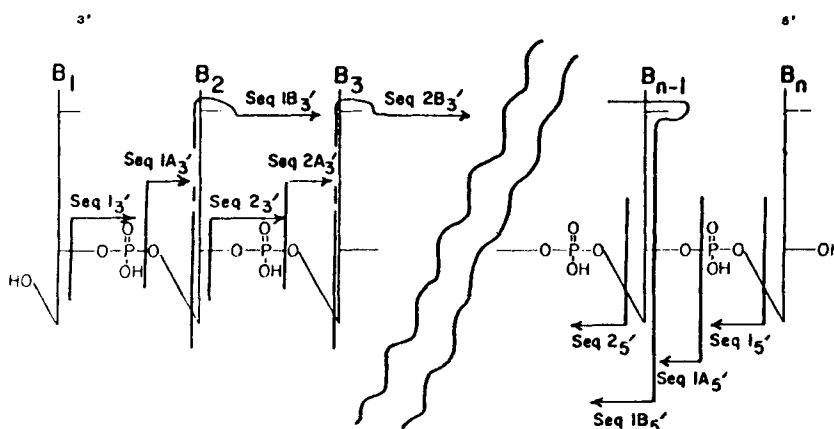

Figure 10. MS/MS fragmentation scheme of $(M - H)^-$ ions of oligonucleotides. From Ref. 139 with permission.

spectrum contains fragment ions such as a modified guanine and ions resulting from loss of the modified guanine, from loss of a neutral species of 43 dalton from the parent anion and from the modified base anion, and from loss of C. These data, therefore, support modification occurring preferentially on G.

The MS/MS behavior of several modified tetranucleotides were also investigated by Lay, Dino et al., and others (141–144). As with the unmodified oligonucleotides, the MS/MS spectrum becomes increasingly difficult to interpret. Sufficient sequence ions were observed in the negative ion spectra to confirm the location proposed for the site of bonding of the carcinogen. Few fragment ions were observed in the positive ion spectra, but the ions that were observed were highly informative. For example, the MS/MS spectrum of the $(M + 4Na - 3H)^+$ ion of benzo[a]pyrenediolepoxide-modified pd(CTCT) contained an ion originating by loss of the elements of BPDE and NH_3, which indicates that reaction has occurred with the exocyclic amine group of C (144).

From these reported applications of FAB/MS/MS in the analysis of nucleic acid constituents and their xenobiotic adducts, we see that the techniques may play an important future role in the analysis of these compounds. The complexity of the MS/MS spectra of higher molecular weight oligomers, the difficulty in obtaining salt-free samples, and the relatively lower sensitivity compared to peptides combine to make such applications more difficult than with some other classes of compounds. Given the importance of this class of compounds to toxicity and carcinogenicity, however, we can expect to see continued developments in the field.

E. Carbohydrates

Due to their low polarity relative to zwitterionic compounds and to their relatively high hydrophilicity, carbohydrates have proven to be one of the more difficult and less sensitive compound classes to be analyzed by FAB. Even given these problems, FAB/MS is still one of the most useful techniques for analyzing carbohydrates.

1. Mono- and dissacharides

The earliest FAB/MS/MS studies of simple carbohydrates were directed at understanding the behavior of saccharide-matrix adducts. Suzuki et al. reported the MS/MS spectrum of the proton-bound stachyose-N-2-hydroxyethylfomamide adduct in 1983 (145). In 1984, Puzo and Prome (146) compared the MS/MS spectra of the disaccharide trehalose in the matrices glycerol, diethanolamine, and triethanolamine. They observed that these complexes decompose to disaccharide and matrix ions, the ratio of which is dependent on the relative proton affinities.

In a study that was simultaneous with that of Puzo and co-workers, Tondeur et al. (147) also observed the same phenomenon occurring with monosaccharides. They were also able to distinguish four isomeric hexoses on the basis of the MS/MS spectra of the hexose-glycerol adducts in the positive ion mode.

Puzo and co-workers (148–150) observed that differentiation of eight hexoses, four anomeric methyl glycosides, and N-acetylhexosamines can be accomplished

by using the ratio of (glycerol + alkali metal) cation to (hexose + alkali metal) cation as a function of alkali metal (Li vs. Na). Puzo et al. (151–152) extended this work to disaccharides, but was unable to distinguish isomers because of fluctuations in ion abundances.

Guevremont and Wright (153) showed that the MS/MS spectra of peracetylated glucose and galactose could be used to differentiate the isomers in a manner similar to that used by Richter and Blum under DCI conditions. This approach was extended successfully to three stereoisomeric aminohexoses (154). Laine et al. (155) were also able to differentiate isomeric fucosyl-lactosamines that differed in the position of the saccharide residue linkages, for example (1 → 3) vs. (1 → 4), on the basis of differences in the MS/MS spectra of the $(M + H)^+$ ions produced under CI conditions. The observed differences should also be valid for FAB produced $(M + H)^+$ ions.

2. Oligosaccharides

Carr and co-workers (156) first investigated the utility of FAB/MS/MS for the analysis of a number of higher oligosaccharides. In contrast to a FAB spectrum, which contains ions formed by loss of the nonreducing terminal sugar, the FAB/MS/MS spectrum of two isomeric hexasaccharides obtained on a four-sector instrument showed that abundant sequence-related fragment ions form (cf. Figs. 11a, 11b). The authors (156) also observed that isomeric linear and branched oligosaccharides undergo unique fragmentations that can be related to structure. In addition, in a comparison of synthetic and natural oligosaccharides, the FAB spectra may show significant differences because of differences in salts and other impurities. The FAB/MS/MS spectra are, however, identical.

Harada and co-workers used B/E-linked scans in a series of reports to obtain structurally significant ions from oligosaccharides (157) and aminoglycosides (158) and to confirm the structure of viridopentaoses isolated from sporaviridin (159).

FAB/MS/MS also has been successfully applied to the structure elucidation of unknown oligosaccharide derivatives. Trimble, in collaboration with the Nebraska group (160), identified the product of glycerol transfer to oligosaccharides formed during chitobiose core cleavage. The MS/MS data were used to identify the nonreducing oligosaccharide as containing glucose at the reducing end of $Man_5GlcNAc$. A complete series of sequence ions were observed for both the glycerol-containing and the unmodified oligosaccharide.

Heerma et al. (161) identified a new oligosaccharide found in the urine of two patients with β-mannosidase deficiency. The new compound was identified on the basis of comparisons of the MS/MS spectra of the unknown and known related compounds to contain a mannosyl (1→4)-D-N-acetylglucosaminyl(1→N)urea compound.

Heerma's (162) work included examination of the MS/MS spectra of four isomeric methyl α-D-galactopyranoside sulfates under both positive and negative ion conditions. The spectra of the isomers differ significantly, allowing for facile isomer identification.
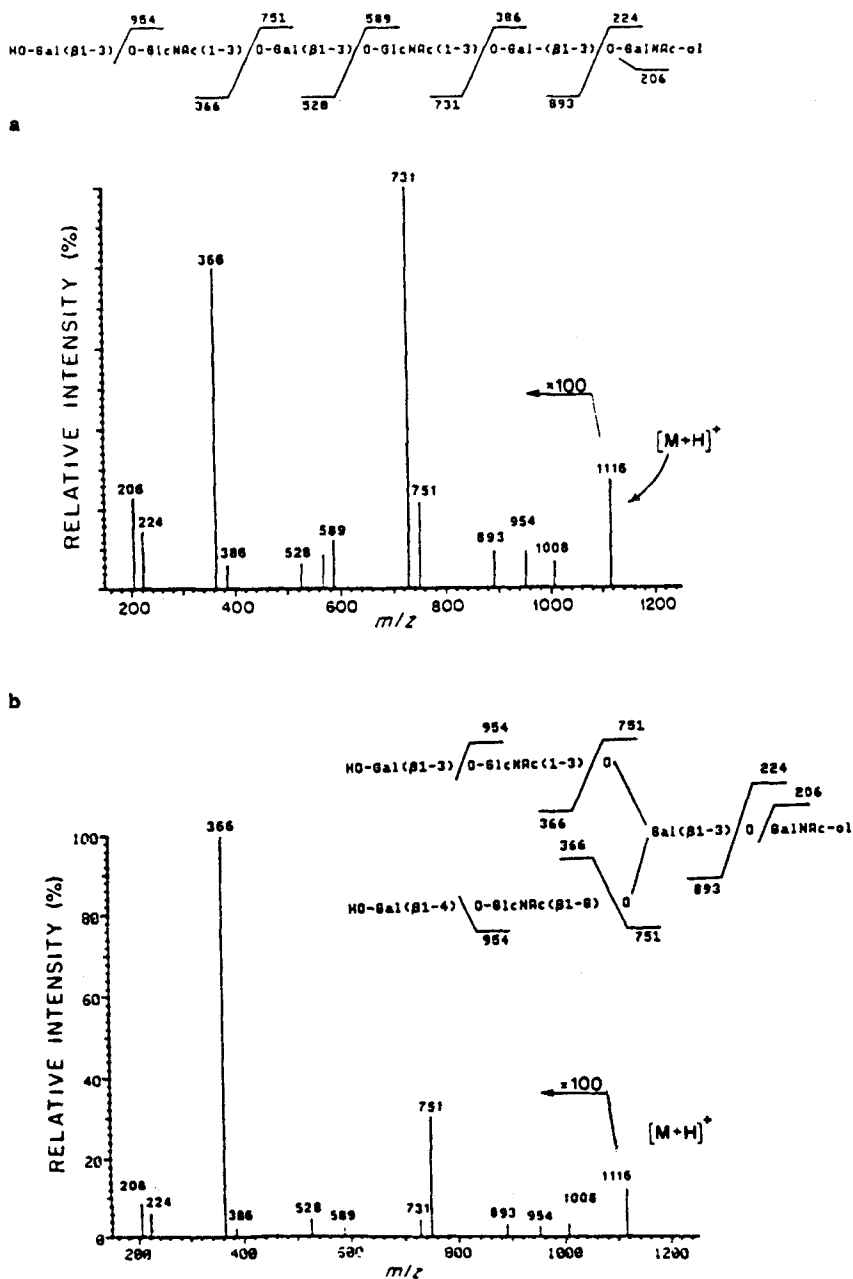
Figure 11. (a) MS/MS spectrum of a linear hexasaccharide. (b) MS/MS spectrum of an isomeric branched hexasaccharide. From Ref. 156 with permission.

3. Glycosides

Many natural products such as flavonoids and steroid glycosides exist in nature as conjugates with carbohydrates. In this section we will emphasize nonsteroidal compounds. Steroidal glycosides will be treated in Section IV.F.2.

FAB/MS/MS has proven to be particularly suited to the analysis of flavonoid glycosides. The major efforts in this area were reported by the staff at the Midwest Center for Mass Spectrometry (163,164) and by de Koster's group (165). Both groups observed that differences in substitution between the rings of the aglycone could be distinguished (e.g., between quercitin and robinetin).

For the flavonoid glycosides, the major processes observed under both positive and negative ion FAB/MS/MS is sequential losses of sugar moieties ($F_1 \ldots F_n$ ions) leading to formation of the aglycone (AH^+ or A^-). When the sugar moieties in flavanoid polysaccharide differ in molecular weight, the sequence ions reveal the order of the sugars in the molecule. When the sugars are identical in molecular weight but differ in structure, the MS/MS data are inadequate to differentiate the possibilities. Under negative ion conditions, however, differences in bonding such as 1-6 vs. 1-2 can be ascertained. This information is provided in the ions formed by sugar ring cleavages, S_1 [$(M-H)^- - 90$] and S_2 [$(M-H)^- - 104$]. These sugar ring cleavages are identical to those occurring for nucleosides.

F. Lipids

The lipid category covers a multitude of compound types including fatty acids, steroids and their conjugates, phospholipids, and neutral complex lipids such as gangliosides. FAB/MS/MS has been successfully applied to the determination of all of these subclasses. To simplify the discussion, each subcategory will be presented separately.

1. Fatty acids

The first reported application of FAB/MS/MS to fatty acid analysis was that of Tomer et al. in 1983 (166). In this article, it was reported that the location of the double bond in a series of fatty acids can be readily located on the basis of its MS/MS spectrum under negative ion conditions. Figure 12(a) shows a typical MS/MS spectrum of an unsaturated acid. Peaks are visible corresponding to ions formed by cleavage at successive methylene groups, except few ions occur for cleavages of vinyl bonds or double bonds. Enhanced fragmentation was observed at the allylic carbons.

In this first report, it was noted that fragmentation seems to be occurring preferentially at the end of the carbon chain remote from the charge, which can be considered as localized on the carboxylate group (26). The MS/MS spectrum of the same compound acquired on a four-sector instrument [Fig. 12(b)] shows that the major losses are of C_nH_{2n+2} except for the occurrence of some radical (distonic?) anions formed by cleavage at the positions allylic to the double bond.
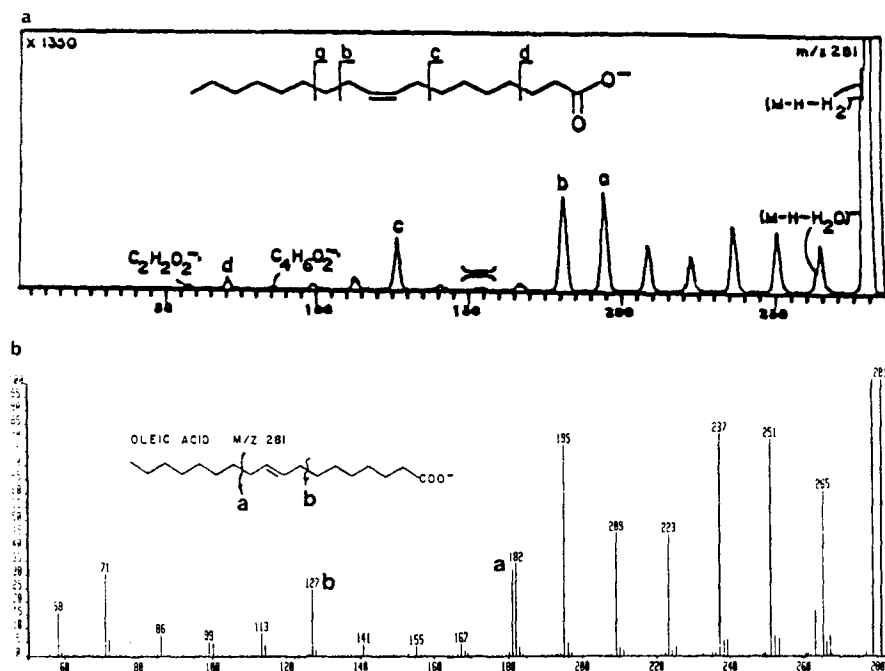
Figure 12. (a) MS/MS (EBE) spectrum of the (M − H)⁻ anion of oleic acid. From Ref. 26 with permission. (b) MS/MS (BEEB) spectrum of the (M − H)⁻ anion of oleic acid.

In contrast, the negative ion FAB/MS spectrum did not contain any fragment ions. This is an example, therefore, where FAB/MS/MS reveals a new fragmentation that is not observed in the conventional FAB mass spectrum.

The type of fragmentation uncovered in the MS/MS spectra of the fatty acid anions, termed charge-remote fragmentations (26), has been the subject of a number of studies for a variety of compound classes. A review on the subject will be forthcoming, so in this review, we will restrict ourselves to the analytical applications within the FAB/MS/MS framework.

Several groups (166-171) extended the investigation of the MS/MS behavior of fatty acid anions to include a number of substituted fatty acids, including hydroxy, epoxy, cyclopropane, cyclopropene, acetylenic, branched, and fatty acids containing heterocyclic rings in the chain. For all of these cases, the substituent interrupts the successive C_nH_{2n+2} losses in a fashion characteristic of the substituent. The perturbed chemistry permits identification and location of the substituent. The FAB/MS/MS spectra are also sufficiently sensitive to structural variations such that the presence and identity of isomers can be ascertained.

The charge-remote fragmentations were also observed to occur in the positive ion MS/MS spectra of compounds containing long-chain alkyl groups. Examples include quaternary ammonium ions, phosphonium ions, and picolinyl esters of fatty acids (172-175), and even an example of odd electron species (176).

Under positive ion conditions, the major fragment ions from fatty acids occur at low mass and give little or no structural information. Riviere et al. (177), how-

ever, analyzed mycolic acids, α-alkyl β-hydroxylated fatty acids, under positive ion conditions. Double bonds were derivatized by addition of OH and $N(CH_3)_2$ moieties across the double bonds (178). The positive ion FAB/MS/MS spectra showed ions due to cleavage at the location of the double bond.

2. Steroids

The earliest report of a FAB/MS/MS spectrum of a steroid was in a report by Gaskell et al. in 1982 (179) and a full report in 1983 (180). Gaskell showed that the isomeric dehydroepiandrosterone sulfate (DHAS) and testosterone sulfate can be differentiated on the basis of fragmentation occurring in the D-ring of the DHA sulfate and in the A-ring of testosterone sulfate. These reactions are early examples of charge-remote fragmentations although they were not identified as such by the authors.

Kingston, Liehr, and co-workers (132,181) investigated the positive ion MS/MS spectra of a number of sodium salts of bile acid tauro- and glycoconjugates. The authors noted that isomeric salts, differing in location of a hydroxyl group, can be differentiated on the basis of their MS/MS spectra.

Tomer and co-workers (173,182,183) also investigated a series of bile salts, bile conjugates, and steroid conjugates such as sulfates and glucoronides, but under negative ion conditions. The spectra are dominated by charge-remote fragmentations, and are very sensitive to structural variations, including stereochemistry, in parts of the molecule remote from the charge-bearing group.

Guenat, Cole, and co-workers (184,185) examined experimental conditions that affected the MS/MS spectra of isomeric steroidal glucoronides. In addition to the expected influence of collision energy and average number of collisions (percent attenuation of the parent ion), analyte concentration also could affect the MS/MS spectra. Two possible explanations for this phenomenon were postulated: (a) if the parent is desorbed as a solvated ion, $[(M + H)·Gly_n]^+$, desolvation of differing numbers of matrix molecules may cause differing amounts of internal energy to be lost by the parent ion, or (b) the protonated steroid molecules in the surface monolayer may be structurally different from steroid ions formed within the matrix. The conclusion for structure differentiation is that, even though there are distinct differences in the positive ion spectra, these differences are not necessarily reliable for structure elucidation.

Gaskell (186) has also investigated the utility of FAB/MS/MS for quantification of DHAS in serum by using a hybrid system. In this study, the $(M - H)^-$ to HSO_4^- ion transition was monitored. A deuterated analog was used as an internal standard. Low nanogram detection limits were reported, and the results compared well with those obtained by a much more complex GC/MS procedure.

In their study of flavanoid glycosides, Crow et al. (164) included three steroidal glycosides: digoxin, digitoxin, and cymarin. The fragmentation patterns for these compounds closely resemble those of the flavanoid glycosides in that loss of carbohydrate residues predominate. Chen et al. (187) examined five steroidal glycosides with 2–4 sugar units. Loss of sequential sugar units was observed,

and branched and linear sugar units can be differentiated. In addition, the isomeric aglycones are distinguishable on the basis of the MS/MS spectra.

3. Phospholipids

Phospholipids are an important class of biological compounds that serve a number of functions in the cell. Typically these compounds contain glycerol that has been diesterified by fatty acids. Thus, there are a number of building blocks within the molecule that are of interest to the analytical chemist. In 1983, Sherman et al. (188) observed that the MS/MS spectrum of the $(M - H)^-$ anion of a soybean phosphatidylinositol contains fragments due to the carboxylate anions of the fatty acids contained in the phosphatidylinositol. Later Sherman and others (189) showed that phosphatidylinositol-4-phosphate and phosphatidyl-4,5-biphosphate also give MS/MS spectra containing carboxylate anions indicative of the ratio of fatty acids in the parent compound. Other ions were observed that were consistent with the presence of a phosphatidylinositol headgroup.

Some groups (163,190,191) observed that the structures of the fatty acids in complex lipids such as the phospholipids and glucosamine phospholipids can be determined by obtaining the MS/MS spectrum of the carboxylate anions. These anions behave identically to the free carboxylate anions.

Jensen et al. (192) later extended their work to include the FAB/MS/MS behavior of other phospholipids such as phosphatidyl-ethanolamine, phosphatidylglycerol, cardiolipin, phosphatidic acid, and platelet-activating factor. They showed that the structures of the carboxylate residues could be elucidated from their MS/MS spectra. In addition, ions representative of the other parts of the molecule were observed, as is shown for cardiolipin (Fig. 13). Easton et al. (193) demonstrated that the MS/MS spectra of the ions corresponding to the headgroups of mono- and dimethyl phosphatidylethanolamine and phosphatidyl choline can be used to identify the nitrogenous base. Recently Roberts and co-workers (194) utilized negative ion FAB/MS/MS in the structure characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase [7]. As in the MS/MS spectra of the phospholipids described above, ions resulting from loss of fatty acids, loss of acylated sugar and formation of carboxylate anion and phosphoglycerol anions were observed.

The elucidation of the structure of unusual fatty acids in marine sponge phos-
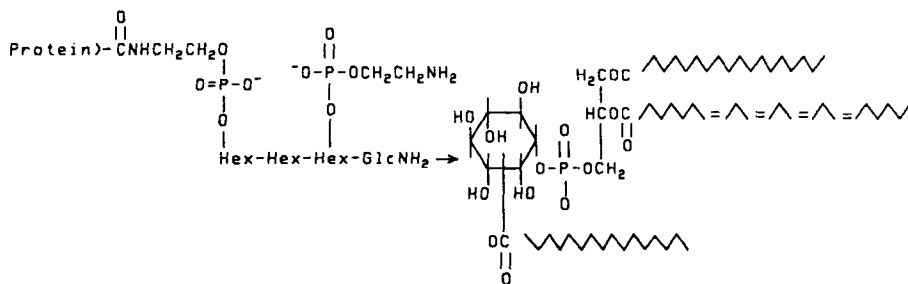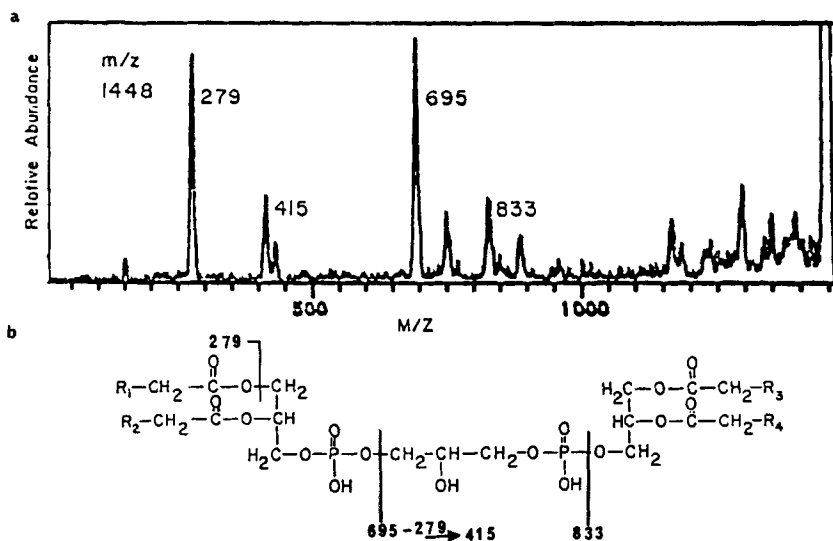
Figure 13 (a) MS/MS spectrum of the (M − H)⁻ cardiolipin. (b) Origin of fragments in the MS/MS spectrum of the (M − H)⁻ anion of cardiolipin. From Ref. 192 with permission.

pholipids validates this approach for determining the structure of the fatty acid constituents of phospholipids (195,196). Fatty acids identified in this manner include 3,7,11,15-tetramethylhexadecanoic acid, 5,9,19-octacosatrienoic acid, 5,9,23-triacontatrienoic acid, and 5,9-hexacosadienoic acid.

In a study that can be considered to be related to those of the phospholipids, Clifford and co-workers (197) used negative ion FAB/MS/MS to confirm the structure of retinyl phosphate mannose isolated from cells. This compound had been postulated as an intermediate in retinoid metabolism, a process that is implicated in glycoprotein biosynthesis.
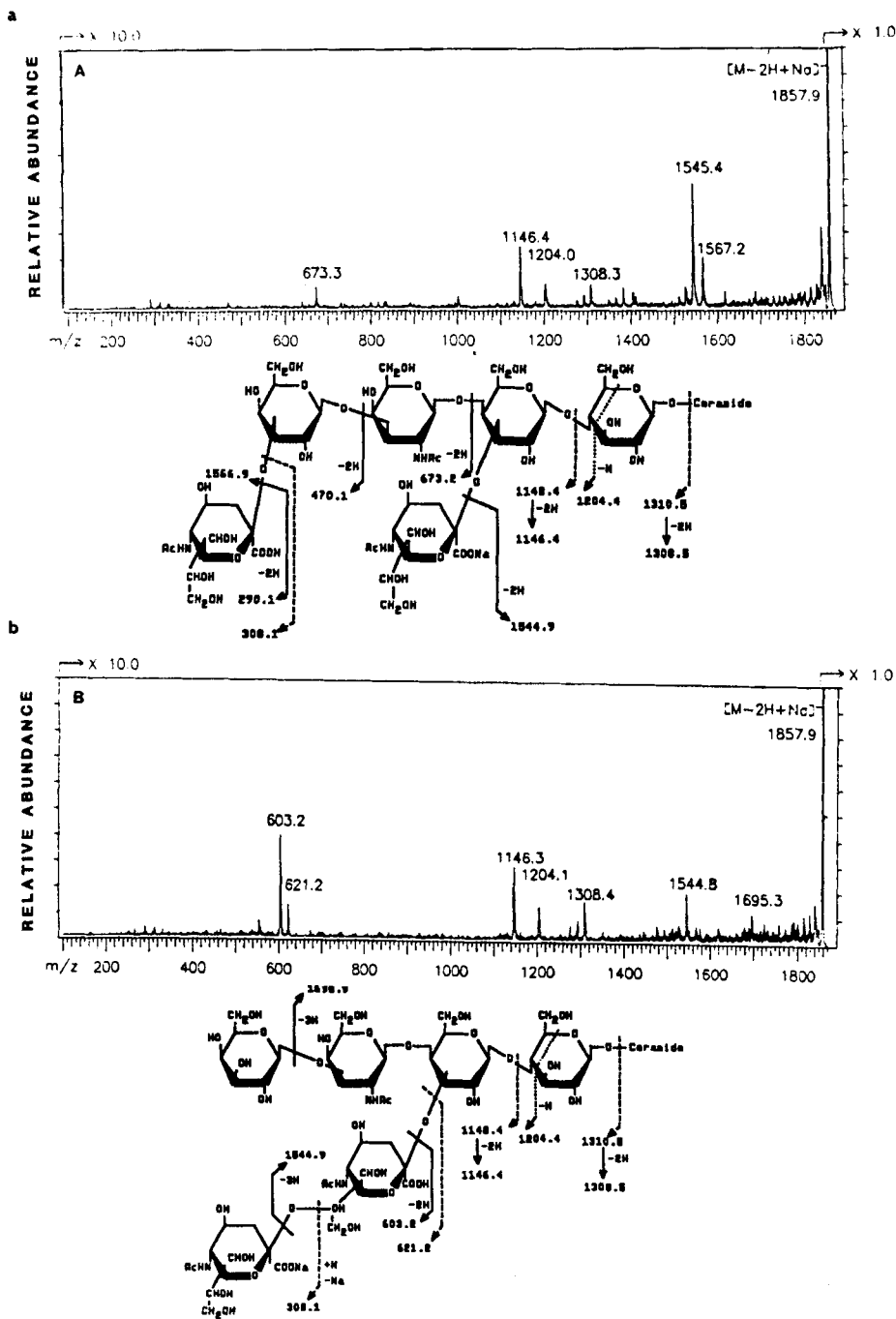
FAB/MS/MS data were also the key to the confirmation of glycerol-1,2-cyclic phosphate as a metabolite in centric diatoms (the biological, not chemical type) (198).

4. Glycolipids

Glycolipids are sugar-containing lipids. In animal cells, sphingosine forms the backbone of the molecule. Cerebrosides contain one sugar group whereas gangliosides contain more complex carbohydrate residues.

Higuchi et al. reported the FD/MS/MS (B/E) spectrum of N-acetylpsychosine in 1983 (199). Loss of the hexose and part of the C_{18} chain were observed.

Domon and Costello (200) have published the most thorough examination of the utility of FAB/MS/MS for the structure elucidation of glycosphingolipids and gangliosides. These compounds contain ceramide and oligosaccharide moieties, both of which exhibit significant heterogeneity. As with other complex biomolecules, these compounds fragment to give ions that provide information about the basic constituents of the molecule. Under positive ion conditions, ion rep-

Figure 14.   (a) MS/MS spectrum of ganglioside GD$_{Ia}$. (b) MS/MS spectrum of ganglioside GD$_{Ib}$. From Ref. 200 with permission.

resenting the sugar residues are not observed, whereas sugar-related ions are in the negative ion MS/MS spectrum. The negative ion MS/MS spectra of gangliosides isomeric in the location of the position of the sialic acid residue(s) contain information as to the location of the residue(s) (Fig. 14). Also of interest (to this author at least) is the observation of alkane elimination occurring from the aglycone ion in the positive ion mode and from the parent anion in the negative ion mode. These losses can also be used to characterize the acyl moiety.

V. CONCLUSION

Because the development of FAB/MS/MS instrumentation and the applications of the technique to compounds of biochemical importance are ongoing processes, there can be no conclusion—only a summary.

FAB/MS/MS has been shown to be applicable to a wide variety of biochemical problems and is rapidly becoming the method of choice for peptide determination where the N-terminus is blocked or where unusual or modified amino acids are present and a critical component in protein sequencing. Its utility for mixture analysis, low chemical noise, new fragmentation processes and the propensity of complex molecules to fragment into their building blocks guarantee its increasing utility in the years to come. Current instrument developments to increase sensitivity and mass range in MS/MS experiments will undoubtedly increase the popularity and utility of the technique. The past has been exciting, but the best of times may be yet to come.

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