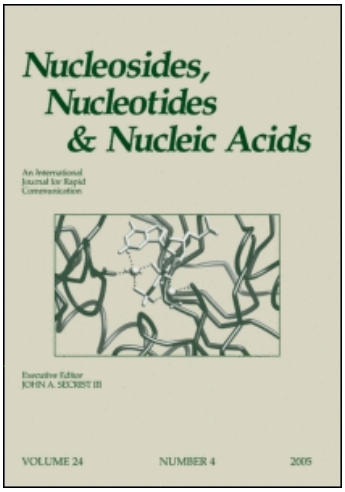


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Fast atom Bombardment Mass Spectrometry of Nucleosides, Nucleotides and Oligonucleotides

Debra L. Slowikowski[a]; Karl H. Schram[a]
[a] Department of Pharmaceutical, Sciences College of Pharmacy, University of Arizona, Tucson, Arizona

To cite this Article Slowikowski, Debra L. and Schram, Karl H.(1985) 'Fast atom Bombardment Mass Spectrometry of Nucleosides, Nucleotides and Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 4: 3, 309 — 345
To link to this Article: DOI: 10.1080/07328318508056166
URL: http://dx.doi.org/10.1080/07328318508056166

NUCLEOSIDES & NUCLEOTIDES, 4(3), 309-345 (1985)

FAST ATOM BOMBARDMENT MASS SPECTROMETRY OF NUCLEOSIDES, NUCLEOTIDES AND OLIGONUCLEOTIDES

Debra L. Slowikowski and Karl H. Schram*
Department of Pharmaceutical Sciences
College of Pharmacy, University of Arizona
Tucson, Arizona 85721

Abstract. Fast atom bombardment (FAB) mass spectrometry, a new ionization technique, has been applied to a variety of polar, non-volatile compounds with considerable success. Current literature regarding the analysis of nucleosides, nucleotides and oligonucleotides using FAB is reviewed.

I. Introduction

A powerful dimension was added to the field of mass spectrometry with the introduction of fast atom bombardment (FAB) ionization in 1981.[1,2] The FAB technique, in which fast atoms are impinged onto a liquid surface containing the dissolved analyte, is a desorption ionization method yielding not only a strong molecular weight related ion, MH^+ in positive and $(M-H)^-$ in negative ion detection modes, but also providing structurally diagnostic fragments for many compound

 0732-8311/85/0403-0309$3.50/0

classes. In particular, FAB allows the analysis of involatile and thermally labile compounds[3,4] intractable to the standard methods of electron impact (EI) and/or chemical ionization (CI) or that require prior derivatization for the production of molecular and other relevant ions.

The analysis of nucleic acid components by other "soft" ionization techniques such as field desorption (FD)[5-7], ^{252}Cf-plasma desorption (PD),[8-10] desorption chemical ionization (DCI)[11-13] and secondary ion mass spectrometry (SIMS)[14,15] has been successful, with significant contributions being made by each of these methods. However, FAB possesses a number of important advantages over these other techniques. Unlike the FD and PD methods, the accessibility of FAB is not severely limited since required modifications to existing mass spectrometers are relatively inexpensive and the equipment may be mounted on any mass spectrometer regardless of the type of analyzer.[16] In contrast to FD and DCI, FAB results have been shown to be generally reproducible between laboratories,[17] due partially to simple, although in some cases critical, sample preparation.[18] Finally, again in contrast to DCI and FD, long-lived ion currents in FAB permit time-consuming procedures, such as high resolution measurements[19] and metastable ion analysis, to be accomplished without repeated sample loading.

An important limitation of FAB and all "soft" or desorption ionization techniques is that only pure samples can be analyzed. Mixtures of compounds and biological samples necessitate the use of collisional activation (CA) and tandem mass spectrometry (MS/MS) to separate and selectively analyze components. The coupling of liquid chromatography with FAB ionization (LC/FAB MS), however, is the most novel approach to the separation of mixtures reported thus far, demonstrating outstanding results with peptides,[20] and promises to be of significant value in the future for the analysis of nucleic acid components.

Although the mechanism operating in FAB ionization is still speculative, current theories emphasize the importance of surface chemistry, solubility and other solute-solvent interactions[21-23] which affect not only the production but also the quality of the resulting spectrum. Consequently, sample preparation is a critical factor in

obtaining FAB mass spectra.[18] A variety of FAB solvents have been investigated ranging from glycerol[1], the original and usually first choice matrix, to the newly developed "magic bullet",[24] a mixture of dithioerythritol and dithiothreitol proposed to offer less background interference. Attempts to optimize experimental conditions, therefore, must take into consideration the concentration of the sample and the chemical properties of both the sample and the solvent matrix used in the experiment.

This review covers the literature throught mid-1984 describing the use of FAB for the analysis of nucleosides, nucleotides and oligonucleotides.

II. NUCLEOSIDES

A. General Characteristics

The mass spectra of simple purine and pyrimidine nucleosides have been examined and detailed fragmentation pathways proposed for the major ions present in both electron impact (EI)[25] and chemical ionization (CI)[26] mass spectra. However, some of the less volatile or thermally labile nucleosides do not produce mass spectra indicative of their structure by these two standard techniques. Derivatization, although of value in many instances,[27] requires an additional sample manipulation step and may produce unwanted or unsuspected side products.[28,29] Since volatility restrictions are not a consideration, FAB ionization offers a powerful alternative to derivatization and permits the analysis of free nucleosides. The application of FAB MS in the characterization of medicinally important nucleosides (and their metabolites), antibiotic nucleosides of natural or synthetic origin and nucleoside chemical-carcinogen adducts are just a few examples of the utility of this ionization method which have been reported in the literature.

Few reports have discussed the FAB mass spectra of nucleosides. The FAB mass spectra of the major ribo- and deoxyribonucleosides found in RNA and DNA have been examined and compared to data obtained using EI and CI[30] (See Tables 1 and 2). In an independent study, the FAB and FAB/collisionally activated decomposition (CAD) tandem mass spectrometry (MS/MS) analysis of a series of nucleosides are compared and related to EI and CI spectra.[31] The following discussion describes the findings of these two reports.

Table 1. Comparative intensities of the molecular or MH^+ ion and significant fragment ions using FAB, CI and EI techniques in the positive ion mode.[30]

		MH^+[a,b] m/z(%RI)	B+44[c] m/z(%RI)	B+30[d] m/z(%RI)	B+2H m/z(%RI)	S^+ m/z(%RI)	Other m/z(%RI)
Adenosine	FAB	268(92)	178(3)	164(7)	136(100)	133(3)	$(MH+G)^+$:360(28)
	CI	" (59)	" (3)	" (3)	" (100)	" (2)	
	EI	267(20)	" (35)	" (70)	" (78)	" (2)	BH^+:135(100)
Guanosine	FAB	284(62)	194(5)	180(3)	152(100)	133(2)	$(MH+G)^+$:386(12)
	CI	-	-	-	-	-	
	EI	-	-	-	-	-	
Uridine	FAB	245(58)	155(6)	141(1)	113(100)	133(11)	$(MH+G)^+$:337(13)
	CI	" (19)	" (3)	" (17)	" (100)	" (33)	
	EI	244(9)	" (6)	" (20)	" (100)	" (57)	BH^+:112(22)
Cytidine	FAB	244(63)	154(4)	140(4)	112(100)	-	$(MH+G)^+$:336(12)
	CI	" (2)	" (2)	" (2)	" (100)	-	
	EI	243(1)	" (6)	" (43)	" (100)	133(2)	BH^+:111(46) (B+41):151(26)
Deoxyadenosine	FAB	252(54)	162(7)	164(4)	136(100)	117(4)	$(MH+G)^+$:344(8)
	CI	" (31)	" (6)	" (23)	" (100)	" (5)	
	EI	251(10)	" (37)	" (12)	" (30)	" (5)	BH^+:135(100)
Deoxyguanosine	FAB	268(43)	178(4)	180(1)	152(100)	117(2)	$(MH+G)^+$:360(3)
	CI	-	-	-	-	-	
	EI	-	-	-	-	-	
Deoxycytidine	FAB	228(46)	138(6)	140(1)	112(100)	117(5)	$(MH+G)^+$:320(5)
	CI	-	" (2)	" (4)	" (100)	" (20)	
	EI	227(2)	" (15)	" (<1)	" (63)	" (15)	BH^+:111(100) 153(18)
Thymidine	FAB	243(42)	153(2)	155(<1)	127(100)	117(38)	$(MH+G)^+$:335(4)
	CI	" (19)	" (2)	" (24)	" (100)	" (49)	
	EI	242(8)	" (4)	" (<1)	" (27)	" (100)	BH^+: 126(32)
Pseudouridine	FAB	245(100)	155(29)	141(8)	113(15)	-	$(MH+G)^+$:337(17) $(MH-2H_2O)^+$:209(7) $(MH-3H_2O)^+$:191(2)
	CI	" (64)	" (100)	" (19)	" (28)	133(2)	$(MH-H_2O)^+$:227(5) $(MH-2H_2O)^+$:209(5) $(MH-3H_2O)^+$:191(4)
	EI	244(<1)	" (8)	" (100)	" (10)	-	(M^+-H_2O):226(4) (M^+-2H_2O):208(6)

Notes:
[a] Molecular ion species in EI is $M^{+\cdot}$.
[b] Methane used as reagent gas in CI mode unless noted otherwise.
[c] In the deoxy series, the ion corresponding to the B+44 ion is the B+28 ion.
[d] The relative contributions of $BH{\cdot}C_2H_5$ and $B{\cdot}CH_2O$ to the B+30 ion in the CI mode (methane) are not currently known.

The positive ion FAB mass spectra of nucleosides are characterized by the presence of a normally strong MH^+ ion and glycerol (G) or the thioglycerol (TG) adduct ions, depending on the matrix used in the analysis. In addition to providing confirmation of the molecular weight of the sample, the adduct ions may contain information concerning the type of sugar present.[30] A higher relative abundance is noted for the $(MH+G)^+$ ions in the ribose substituted

Table 2. Comparative intensities of the molecular or $(M-H)^-$ ion and significant fragment ions in the negative ion FAB and EI mass spectra.[a]

		$(M-H)^-$ m/z(%RI)	$(B+42)^-$ m/z(%RI)	B^- m/z(%RI)	Other m/z(%RI)
Adenosine	FAB	266(12)	-	134(100)	$(B-NH_3)^-$:117(10)
	EI	" (3)			" " (12)
Guanosine	FAB	282(37)	192(2)	150(100)	
	EI	-	-	-	
Uridine	FAB	243(45)	153(5)	111(100)	$(M-H-43)^-$:200(7)
	EI	" (9)	" (4)	" (100)	
Cytidine	FAB	242(72)	152(4)	110(100)	
	EI	" (15)	" (4)	" (100	
Deoxyguanosine	FAB	266(66)	-	150(100)	
	EI	-	-	-	
Thymidine	FAB	241(37)	167(<1)	125(100)	
	EI	" (13)	" (<1)	" (100)	

Note:
[a] EI intensity values from reference 37).

nucleosides relative to the deoxy series and may indicate the importance of the epimeric orientation of the cis-hydroxyl groups in the ribose ring for the formation of the nucleoside-glycerol complex.[30] Support for this hypothesis is gained from a recent report describing a similar observation in the formation of a 4-tolueneboronic acid-nucleoside complex where the same trend was observed.[32] The importance of stereochemistry has also been mentioned in reference to formation of a nucleoside-cis platinin complex.[33]

Other glycerol matrix ions of the form $(Gn+H)^+$, where n=2,3..., are normally considered to be interferences in a FAB spectrum and can be eliminated by at least two possible procedures. Matrix related and other interfering ions may be completely avoided by using FAB/CAD MS/MS in which only ions related to the sample are recorded.[31] If collisional activation MS/MS experiments are not within the capability of the available instrument, the use of more than one solvent matrix is a feasible alternative, particularly when sample ions are suspected to be coincident with the background glycerol peaks. Comparison of the spectra obtained in each solvent can then be made and sample related ions easily identified.

In addition to the production of glycerol adduct and cation exchange ions, e.g. Na, the formation of dimeric sample peaks has also

been observed using FAB. Thus, during the analysis of guanosine, deoxyguanosine and a number of nucleotides, ions corresponding to protonated dimers of the form $(M_2H)^+$ were noted in the positive ion FAB spectra of the nucleosides.[34] A similar observation has been made during the positive ion FAB analysis of natural products isolated from plant sources.[35]

The decomposition of nucleosides with FAB ionization leads to structurally diagnostic fragment ions, analogous to ions produced by EI[25] and CI[26], but with a closer correspondence to the CI spectra. The similarity between CI and FAB spectra arises for a number of reasons.[27] First, both techniques are "soft" ionization methods, in contrast to EI where a significant amount of excess energy is imparted to the sample. Second, fragmentations in CI and FAB proceed from even-electron ions while both ion and radical site initiated decompositions are possible in EI. The number of fragmentation pathways available in CI and FAB are thus more limited relative to EI and greatly simplified mass spectra are therefore produced with the soft ionization techniques.

Assuming initial protonation on the base, the major route of decomposition of the MH^+ ion in FAB is glycosidic bond cleavage, accompanied by transfer of an additional hydrogen from the sugar to the aglycone to form the $(BH_2)^+$ ion[36] (See Figure 1). Although the $(BH_2)^+$ ion was not the base peak in the positive ion FAB spectrum of all nucleosides reported using normal FAB conditions, this ion did appear as the base peak in all FAB/CAD MS/MS nucleoside spectra.[31] The $(BH_2)^+$ ion is, in all cases except one, the base peak in the positive ion spectra in the work from this laboratory.[30] The exception to the above statement is the C-nucleoside pseudouridine, where glycosidic bond cleavage is suppressed due to the greater strength of the C-C base-sugar bond relative to the C-N bond of normal nucleosides.

Two important fragments resulting from cleavage across the ribose ring appear at the same m/z values as the B+44 and B+30 ions described in EI[25] and CI[26]. These ions occur 90 and 104 amu, respectively, below the MH^+ ion in the positive ion FAB spectra (See Figure 1). The $(B+44)^+$ and $(B+30)^+$ ions in FAB, also referred to as S_1 and S_2 ions,[31] arise by proton abstraction from the sugar by the base. The mechanism of ion formation in FAB appears to be closely related to that of CI[26]

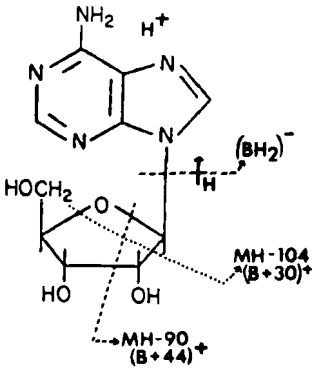


FIG. 1. Common fragments appearing in positive ion FAB mass spectra of nucleosides (adapted from Ref. 31). Adenosine is shown. No localization of the proton is intended in the structure.

where charge-site initiated cleavages lead to the formation of the $(B+44)^+$ and $(B+30)^+$ ions. In the 2'-deoxy series, an ion at $(B+28)^+$ is observed which corresponds to the $(B+44)^+$ ion of the riboside analogs. The intensity of the $(B+28)^+$ ion is approximately the same in all three ionization modes.[30]

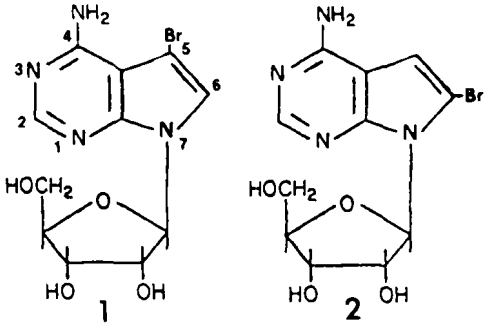
Sugar ions, S^+, resulting from retention of charge by the carbohydrate following glycosidic bond cleavage, are evident for most of the nucleosides examined in this laboratory.[30] The relative abundance of the S^+ ion is generally observed to be higher in the pyrimidine nucleosides relative to the purine analogs in the positive ion FAB spectra. This result corresponds to earlier work using EI and CI and is similar to observations published earlier on the FAB of nucleosides.[31] In the latter report, S^+ ions are observed in the FAB spectra of pyrimidine but not purine nucleosides recorded using either normal or CA MS/MS conditions. The presence of $(MH-H_2O)^+$ and $(MH-CH_2OH)^+$ ions is an additional feature in the spectra produced using the CA MS/MS technique but these ions are not detected in the spectra recorded in this laboratory using normal FAB conditions.[30]

An example of the structural information available in the spectra of nucleosides obtained using FAB is the ability to distinguish the site of methylation, base or sugar, in modified nucleosides. Examination of the $(B+44)^+$, $(B+30)^+$ and $(BH_2)^+$ ions permits the location of the methyl group to be assigned to the base or the 2'-

hydroxyl group. Although not unique to FAB ionization, the intensity of the MH^+ and BH_2^+ ions makes this assignment more straightforward in comparison with, for example EI, where a molecular ion may be weak or absent. Specific examples of modified nucleosides examined using FAB CA MS/MS whose site of methylation could easily be assigned include 2'-O-methylguanosine, 2'-O-methyluridine, and 1-methylguanosine. The advantage of using collisional activation in conjunction with FAB for these analyses is the clear demonstration of important structural fragment ions without the complications of matrix peaks or biological interferences.[31]

Negative ion detection is frequently used in conjunction with positive ion analysis since complementary information is generally obtained. The negative ion FAB spectra of all nucleosides examined[30,31] showed the presence of prominent $(M-H)^-$ ions two mass units lower than the MH^+ ion of the positive ion spectrum and served to establish unequivocal identification of the molecular weight of the sample. The negative ion FAB spectra also displayed significantly fewer fragments than were present in the positive ion spectra and, thus, correspond to observations made in comparing the positive vs negative ion EI generated spectra of nucleosides.[37] Although fragmentation pathways are decreased, the presence of a B^- ion as the base peak in the spectra of all nucleosides examined, except pseudouridine,[30,31] allows both identification of the aglycone and, by difference from the $(M-H)^-$ ion, assignment of a molecular weight to the carbohydrate portion of the molecule. A $(B+42)^-$ ion, analogous to the $(B+44)^+$ ion in the positive ion mode, is also present in the negative ion spectra[30,31] which provides information concerning modifications in the 1',2' or 4' (ring oxygen) positions of the sugar. Additional fragments arising from decomposition of the $(B+42)^-$ ion are only observed using the FAB CA MS/MS technique.[31]

The complementary use of positive and negative ion FAB spectra is also important in obtaining additional structural information since a fragmentation not observed in one mode may be present in the other. For example, differentiation of 5-bromo- (1) from 6-bromotubercidin (2) is possible using positive, but not negative, ion detection.[31] Expulsion of HBr is a facile process in the 5-bromo isomer as a result of the proximity of the Br group to the amino function at C4. The



1 2

loss of HBr is therefore not observed in 6-bromotubercidin or in the negative ion mode for 5-bromotubercidin since both protonation and proximity are necessary conditions for the loss of HBr. The greater number and intensity of the ions formed in the positive ion mode are also of value in the characterization of nucleosides substituted with alkyl side chains.[31]

Negative ion detection has been shown to provide structural information not available in the positive ion spectra in the analysis of pyrimidine nucleosides with FAB/CA MS/MS.[31] A major fragmentation of pyrimidine nucleosides observed using EI[25] and negative ion FAB, but not positive ion FAB, is the elimination of HNCO by a retro Diels-Alder mechanism to form the $(M-43)^{+\cdot}$ ion (EI) or $(M-H-43)^-$ ion (FAB) (See Figure 2).

In summary, the basic structure-fragmentation studies reported to date indicate:

1. both molecular weight and structurally significant fragment ions are observed in the FAB spectra of underivatized nucleosides. Many of the fragments present in FAB are similar to corresponding ions present in the EI and CI spectra. Mechanisms of fragmentation in FAB are very similar, if not identical, to those proposed for CI.
2. both positive and negative ion detection are important and the information obtained in each mode is complementary to the other. Differences in fragmentation may be observed in the positive vs negative ion modes. Both need to be examined.
3. the use of CA MS/MS in conjunction with FAB ionization has the advantages of eliminating background interferences and in

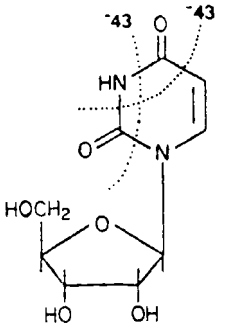
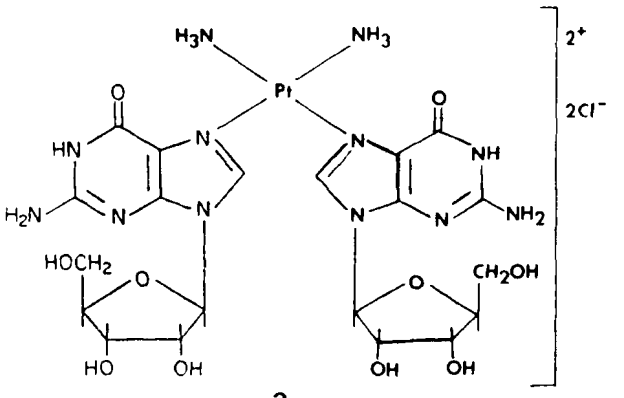


FIG. 2. Possible routes of formation of the $(M-H-43)^-$ ion in uridine via the retro-Diels-Alder reaction (adapted from Ref. 31).

providing additional structural information. Solvent "masking" of sample related fragment ions may also be revealed by use of a second solvent, e.g. glycerol-thioglycerol.

B. Applications

The major utility of FAB mass spectrometry is currently in the structure elucidation of modified nucleosides formed by reaction with anticancer drugs or chemical carcinogens. Because of the extreme nonvolatility and thermal lability of these nucleoside complexes, their mass spectral analysis has been, until recently, difficult and recourse had to be made to either derivatization[27] to form a volatile analog or the sample was submitted for analysis using one of the desorption ionization techniques, most commonly field desorption.[5] Although both of these approaches have been of value, they each suffer undesirable characteristics. Derivative formation may lead to the formation of artifacts[28,29] or result in incomplete reaction thus complicating the analysis. In addition, sample size is normally a limiting factor in the analysis of nucleoside adducts and sample losses, or incomplete utilization of sample, can not be tolerated. The difficulty in obtaining an FD spectrum on a small amount of sample is a well recognized limitation of the technique. The introduction of FAB has essentially eliminated the need for derivatization and, as previously mentioned, the experimental manipulations involved in the acquisition of FAB spectra are relatively simple. Thus, even though the technique is of relatively recent origin, a number of reports have described the successful application of FAB in the structure

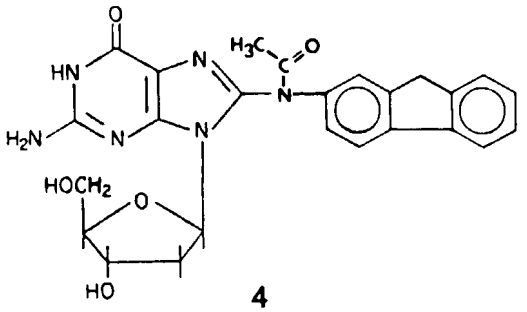


3

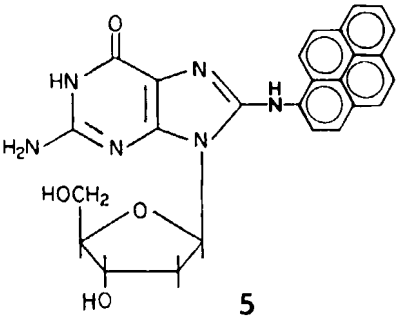
determination of a variety of nucleoside complexes with antitumor agents and carcinogens.

The antitumor agent cisplatin has been shown to undergo preferential binding to guanine rich DNA fragments[38,39] but the nature of the interaction and the precise structure of the guanine-cisplatin complex at the DNA level has not as yet been determined. The structure of a synthetic bis-guanosine adduct formed with cisplatin has been examined using both positive and negative ion FAB mass spectrometry in hopes of gaining insight into the structural features of the adduct.[33] The cisplatin-guanosine adduct (3), cis-$PT(NH_3)_2(Guo)_2 \cdot Cl_2$(MW864), displayed a number of ion clusters in the spectra obtained in both detection modes which were attributed to the stable isotope contributions of the platinum and chlorine atoms. A variety of fragmentation pathways were observed for the adduct and the observed isotopic distributions representing to the loss of HCl, NH_3 and guanosine corresponded closely to theoretical values. Ions consistent with expulsion of a ribosyl function and a guanosine residue were observed and confirmed by mass analyzed ion kinetic energy (MIKE) measurements. Direct indication of molecular weight was, however, only obtained in the negative ion mode where the molecular ion species $(M-H)^-$ for the intact cisplatin adduct is present.

Fast atom bombardment has also been used to characterize the adduct formed between 2'-deoxyguanosine and the chemical carcinogen N-acetyl-2-aminofluorene.[40] Attempts to obtain molecular weight and



4

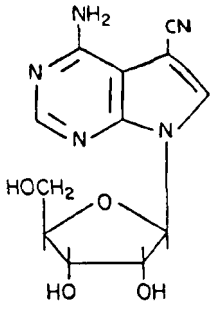


5

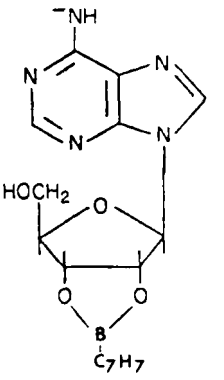
structural information using FD to analyze this sample proved unsuccessful. Positive FAB ionization gave an intense $(MH)^+$ ion at m/z 569 for 8-(N-fluoren-2-yl-acetamido)-2'-deoxyguanosine (4). Major fragments indicated decomposition of the MH^+ ion by cleavage of the glycosidic bond followed by loss of an acetyl group. In addition, formation of a fragment ion indicative of cleavage of the intact carcinogenic moiety was also noted. Similar results have been obtained in a study of the microsomal metabolites and DNA adducts of 1-nitropyrene formed in vitro.[41] In this report, the major adduct isolated was characterized as N-(2'-deoxyguanosin-8-yl)-1-aminopyrene (5) by both negative ion FAB and as the permethyl derivative by high resolution EI mass spectrometry.

A more recent paper describes the identification of isomeric substituted benzyl-guanosine adducts using FAB in combination with collisional activation mass spectrometry.[42] Thus, a series of N1, N^2, O^6, N7, and 8-(p-Y-benzyl)guanosines, where Y=H, CH_3, CH_3O, Cl and NO_2, were assigned their appropriate isomeric structures based on a combination of positive and negative ion FAB spectra and kinetic energy measurements of the metastable transition $(M-H)^-$ to B^-.

The production of the nucleoside antibiotic toyocamycin (6) in fermentation broth cultures has been monitored using FAB, high resolution mass measurements and MIKES.[43] The major ions present in the negative ion FAB spectrum of toyocamycin were the glycerol adduct ion $(M-H+G)^-$ (m/z 392), the $(M-H)^-$ ion (m/z 290), the B^- ion (m/z 158), representing the aglycone and a fragment resulting from the loss of HCN from the B^- ion at m/z 131.



6



7

Alluded to earlier in the basic structure-fragmentation section was the analysis of nucleoside boronate complexes with FAB mass spectrometry.[32] The reaction of boronic acids with polyfunctional compounds, such as nucleosides, carbohydrates, etc, results in formation of negatively charged complexes which are ideally suited to analysis with negative ion FAB. These boronate complexes may provide an elegant means of probing the structure of the carbohydrate moiety of nucleosides of unknown structure isolated from synthetic or natural sources. Thus, the negative ion FAB spectra of the products formed (on the probe in the mass spectrometer) between adenosine and 2'-deoxyadenosine with 4-tolueneboronic acid were examined. The spectrum of the adenosine product displayed a strong ion for an intact 2',3'-bidentate boronate complex (7) whereas the greater strain required for the formation of the 3',5'-boronate with 2'-deoxyadenosine afforded no ion for the boronate complex. This observation is similar to the appearance of a more intense ion for the glycerol adduct of the MH^+ ion in riboside spectra relative to the 2'-deoxy analogs.[30] Both of these phenomena may be of value in determining the presence of epimeric hydroxyl groups in the sugar ring, i.e., distinguishing ribo-, arabino-, xylo-, lyxo- and deoxyribosugars. The utility of both the glycerol adduct and the boronate complex in this regard are currently under investigation in this laboratory.

III. NUCLEOTIDES

The volatility characteristics of nucleotides are even less favorable than in the case of nucleosides. The added polarity of the

phosphate moiety, especially when in the salt form, renders the nucleotides intractable to mass spectral analysis using normal ionization methods. Although derivatization affords a sample amenable to analysis by direct probe introduction, the gas chromatographic separation of nucleotide derivatives is not a routine procedure. Therefore, the mass spectral analysis of nucleotides has been performed using one of the desorption methods, with FD[5] being the most commonly applied technique, although reports of the analysis of nucleotides using desorption chemical ionization (DCI)[11-13] and plasma desorption (PD)[8-10] have also appeared.

The introduction of FAB as an ionization method has truly revolutionized the mass spectral analysis of nucleotides, as is evidenced by the large number of reports describing results using this method in the analysis of heretofore intractable samples. Not only is molecular weight and structural information present in the FAB spectrum but, in the case of dinucleoside phosphates and oligonucleotides, sequence information is available for the first time. Thus, a long sought goal in the application of mass spectrometry to the analysis of this biologically and medically important class of compounds has, at least partially, been achieved.

A. MONONUCLEOTIDES

1. General Characteristics

The positive ion FAB mass spectra of a number of nucleotides have been reported and all of the spectra are dominated by an intense protonated or cationized molecular ion. The nature of the molecular ion species and the extent of proton replacement by counter ions, e.g., Na, K or Li, depends on the origin of the sample, the procedure used to prepare the sample for analysis and, in some cases, the base moiety of the nucleotide. For example, assuming M to represent the fully protonated, uncharged molecule corresponding to the free acid, commercially available mononucleotide disodium salts may exhibit the following series of ions: MH^+, $(M+Na)^+$, $(M-H+2Na)^+$ and $(M-2H+3Na)^+$. An examination of 10 commercially available nucleotide samples revealed the $(M-H+2Na)^+$ ion to be the predominent species, except in the case of UMP, with other protonated/cationized species being present in varying degrees.[34] Although the presence of numerous ions representing different molecular weight related species is at first

confusing, both advantages and disadvantages accrue from these multiple molecular ion species.[44] For instance, these ion patterns can be used not only as mass markers for the determination or confirmation of molecular weight but, in addition, the number of molecular ion species is indicative of the number of acidic sites in the sample. Theoretically, the number of titratable protons in a molecule is given by n+2,[44] where n is the number of molecular ion species observed, e.g., when n=1, MH^+, $(M+Na)^+$ and $(M-H+2Na)^+$ are generally seen. In some cases however, MH^+ may not be observed, possibly because of high salt content. For example, during the positive ion FAB MS analysis of a series of 2',5'-oligoadenylates, the number of cationized species observed was only one more than the number of phosphate hydroxyl groups in the molecule.[45]

A different approach to determining the total number of exchangeable hydrogens present in mononucleotides, and other compound classes, involved the use of deuterated glycerol as a FAB matrix to effect a hydrogen-deuterium exchange of all active hydrogens.[46] The deuterium exchange method has been used successfully for the determination of up to 29 hydrogens, whereas the cationization method is of more limited utility.

A disadvantage of the multiplicity of molecular ion species is a decrease in sensitivity for detection of a specific compound since the ion current normally carried by one ion, MH^+, is distributed among many ions, the cationized species. This decrease in sensitivity may be of consequence during quantitation studies where detection limits may be diminished by a factor dependent on the number of cationized species present.

Samples originating from a biological matrix may contain a variety of different cations, depending on the particular physiological buffers and/or purification procedures used in sample preparation. Consequently, the molecular ion region of the spectrum may be extremely complex. The spectrum may be simplified by acidification to exchange the cations and a more simple spectrum will thus be obtained. The number of active hydrogens may then be determined using the deuterated glycerol procedure or by the addition of a single inorganic salt, e.g., NaCl. The use of silver salts has been suggested for the identification of the $(M+Ag)^+$ species in

polyhydroxylated compounds.[47] Advantages of this method are that the molecular ion is easily distinguished due to the doublet produced by the Ag isotopes (52% and 48% for ^{107}Ag and ^{109}Ag), the absolute ion intensity is greater for the $(M+Ag)^+$ adduct than for the MH^+ ion, and the method can be used to unequivocally determine the molecular weight of the sample even in the presence of K^+ and Na^+ ions.

The most abundant fragment ion present in the positive ion FAB spectra of mononucleotides is the $(BH_2)^+$ ion[36] arising from cleavage of the glycosidic bond with transfer of hydrogen from the sugar to the aglycone. The presence of protonated and cationized glycerol adducts of M, dimers of M and of cationized aglycone $(BH+Na)^+$ ions aid in the assignment of molecular weight and identification of the heterocyclic base.[34] A number of inorganic phosphate ions, with varying degrees of sodium content, resulting from cleavage of the phosphate group are also observed, but corresponding ions indicative of charge retention by the nucleoside function are not present.[34]

The positive ion FAB spectra of a series of 7-alkylated guanosine monophosphates have been examined[44] and were shown to differ somewhat from non-mesoionic nucleotide analogs. Protonated molecular ions and BH_2^+, but not $(BH+Na)^+$, ions permitted identification of the intact molecule and the aglycone. In addition, the spectra of two of the five 7-alkylated GMP analogs examined (**8a** and **b**, see later) produced ions corresponding to the protonated nucleoside residues. A general conclusion of this work was that those nucleotides with more polar side chains produced a greater number of fragment ions.

Additional ions of structural importance have been observed in the positive ion FAB spectra of AMP and (7-deaza)-AMP in preliminary work involving 2',5'-oligonucleotides.[35] Following data system subtraction of peaks derived from the glycerol matrix, the presence of $(B+30)^+$ and $(B+44)^+$ ions at m/z 164 and 178 were observed in the spectrum of the adenosine containing sample. An ion corresponding to the loss of the phosphate group with a molecule of water was also present at m/z 250. The latter ion may represent N3,5'-cycloadenosine formed during sequential cleavage of the oligonucleotide chain, and efforts are underway to determine the origin of this ion. Although the identity and origin of these ions have not yet been established using high resolution or metastable ion analysis, an appropriate shift

of one mass unit in each of the ions was apparent in comparing the spectra of the monomer containing adenosine with the 7-deaza analog.

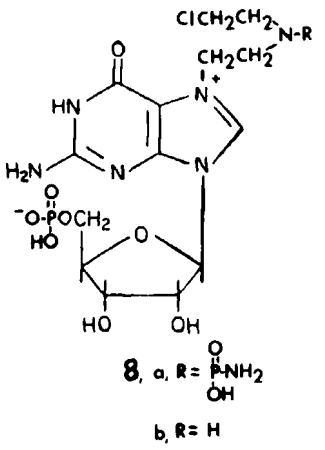
The negative ion FAB spectra of mononucleotides are characterized by a predominant molecular ion species generated either by loss of a hydrogen, $(M-H)^-$, or a cation, $(M-Na)^-$ for sodium. Unlike the complex appearance of the molecular ion region of the spectrum in the positive mode, the number of molecular ion species in the negative mode is equal to the number of titratable protons on the phosphate of the nucleotide.[44] The production of an intense $(M-H)^-$ ion, however, is exhibited even when the analysis is performed on the disodium salt[34] or, in the case of 7-methylguanosine-5'-monophosphate, where two molecular ion species are possible.[44] Although other molecular ion species are observed, e.g., $(M-2H+Na)^-$ or $(M-3H+2Na)^-$, their intensity is significantly reduced compared to the $(M-H)^-$ and to the corresponding species in the positive ion mode.

Other ions present in the negative ion FAB spectra of mononucleotides include the B^- ion, corresponding to cleavage of the glycosidic bond with the charge being retained by the nucleic acid base, and phosphate ions corresponding to the $(H_2PO_4)^-$ and $(PO_3)^-$ structures.[34] The intensity of the B^- ion is significantly reduced in comparison to the negative ion spectra of nucleosides and only in the case of 3'-AMP was this ion the base peak of the spectrum. The intensity of the B^- ion may thus be used to differentiate the 3'-isomer (RI=100%) from the 5'-analog (RI=29%).[34] However, since the 2'-monophosphates were not examined, the ability to distinguish 3'-from 2'-isomers needs to be explored, and the B^- intensity of a larger sample of isomers needs to be investigated to confirm the generality of this observation.

2. Applications

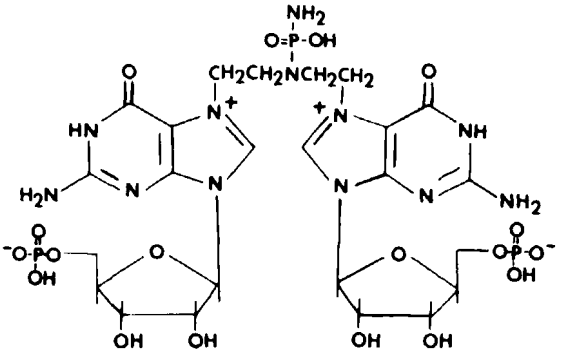
The successful analysis of mononucleotides with FAB ionization has led to the application of this technique to a number of problems of medical and biochemical interest which were previously beyond the capabilities of mass spectrometry. Particular emphasis has been placed on the structural characterization of nucleotide adducts formed with alkylating agents and in studying metabolic or enzymatic transformation of nucleotides, in some cases with the aid of stable isotope labels.

Phosphoramide mustard, a bifunctional alkylating agent of importance in cancer chemotherapy, is thought to form cross-links in cellular DNA, causing selective cell death in the tumor with resultant therapeutic activity. Although FD had previously been used to examine the identity of the adduct formed in the reaction of GMP with phosphoramide mustard,[48] a re-examination of the reaction products using FAB indicated the presence of three different adducts, all located in the 7 position of GMP.[49] Two of these N7-alkylated products had undergone reaction at only one of the two alkylating sites on the phosphoramide mustard, as indicated by the presence of chlorine isotopes in the MH^+ and selected fragment ions. These adducts, therefore, represented intact guanosine-5'-monophosphate-phosphoramide mustard **8a** and the corresponding nornitrogen mustard adduct **8b**. The positive ion FAB spectra of these two samples were very similar but did differ in the absence of fragments in the spectrum of **8b** associated with the loss of the phosphoramide group of the mustard, e.g., ions at $(MH-79)^+$, $(BH_2-79)^+$ and $(BH_2-79-HCl)^+$ were absent in the spectrum of **8b** but appeared in the spectrum of **8a**. Intense MH^+, protonated nucleoside and a base peak at m/z 221 were present in the spectra of both samples. In the case of **8a**, the m/z 221 ion was formed by expulsion of the phosphoramide group and HCl while simple loss of HCl from BH_2^+ led to the m/z 221 ion in the spectrum of **8b**. The spectrum of adduct **9**, however, indicated formation of a dimer in which both arms of the phosphoramide mustard



8, a. R= $-P(O)(OH)NH_2$

b. R= H



9

are joined to the 7-position of two different guanosine residues. Molecular weight was established on the basis of an ion at m/z 897 $(M+Na)^+$ and the presence of an ion at m/z 796 $(M+Na-101)^+$, corresponding to loss of the phosphoramide group and the associated sodium ion of the mustard. The only other ions in the spectrum were attributed to protonated GMP and 7-methyl-GMP.

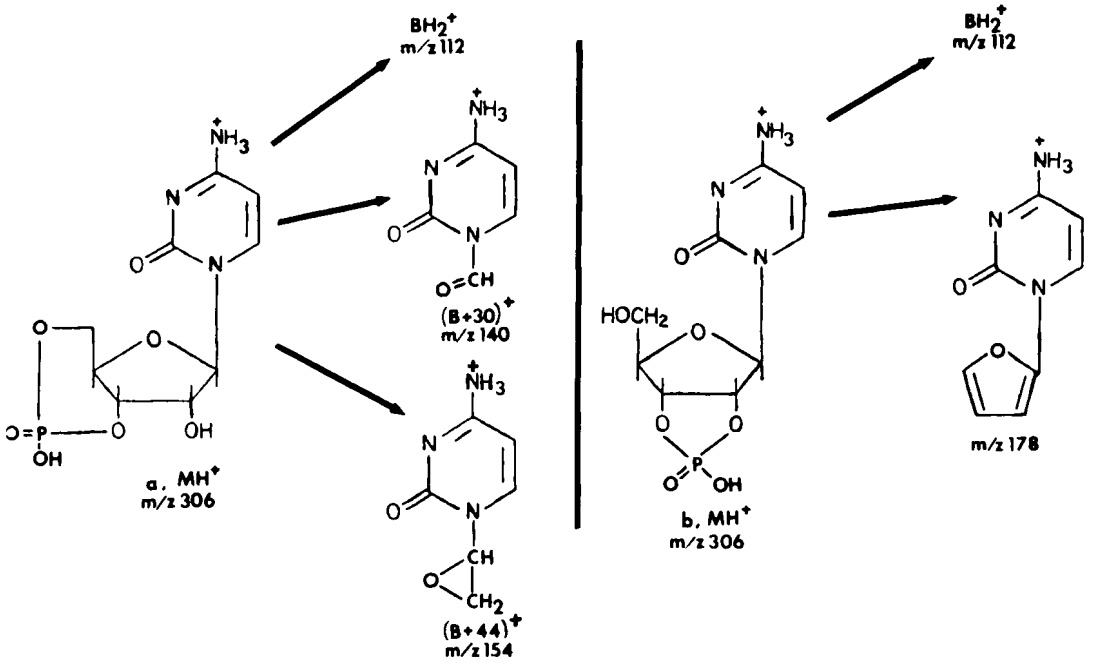
An extension of the 7-alkylated guanosine monophosphate studies has resulted in some interesting observations concerning the behaviour of zwitterions with FAB ionization.[49] Zwitterions are characterized by containing both a positive and a negative charge in the same molecule which balance and render the sample electrically neutral. The charges, however, may be localized on different atoms in certain preferred resonance forms. The detectability of zwitterions in either the positive or negative ion modes, therefore, depends on the propensity of the sample to undergo either protonation or deprotonation; protonation would favor positive ion detection while anion formation would make the negative ion mode the choice for detection. This behaviour of zwitterions is in contrast to organic sulfates or phosphates where either protonation or deprotonation may occur. The 7-alkylated guanosine monophosphates, which exist as zwitterions with the positive charge on the base and a negative charge on the phosphate, may be analyzed with either positive or negative ion detection while 7-methylguanosine, the nucleoside lacking the negative ion forming capability of the phosphate, is amenable only to positive ion detection. An additional observation of interest in this work is the formation of ion-radical species in the FAB spectrum of the phosphoramide mustard-GMP dimer. The appearance of ion-radicals in a FAB spectrum was rationalized on the basis of "higher energy decompositions".[44]

Cyclic nucleotides are of considerable biochemical interest due to their role as mediators of cell function and the analysis of this class of nucleotides has been investigated using FAB mass spectrometry.

Considerable controversy has surrounded the presence or absence of 3',5'-cyclic nucleotides in cellular extracts of plant materials. Spectral methods used to identify nucleic acids derived from plant materials have been unable to differentiate 2',3'-cyclic

monophosphates from the 3',5'-cyclic monophosphates and, therefore, unequivocal proof of the latter compounds in biological samples of either animal or plant origin has been difficult. A recent method using positive ion FAB has, however, been able to unambiguously establish the presence of cytidine-3',5'-cyclic monophosphate in plant materials.[50] The positive ion FAB mass spectra of the cyclic analogs of AMP, CMP and GMP were dominated by MH^+ ions but little additional fragmentation was observed. Addition of dilute HCl or KOH to the sample of a cyclic nucleotide has been reported[31] to enhance ionization in either the positive or negative ion modes respectively, and to also produce ions characteristic of the base moiety (BH_2^+ and B^-).

A method for differentiating 2',3'-cyclic monophosphates from the isomeric 3',5'-cyclic monophosphates utilizing FAB ionization with collision induced dissociation mass analyzed ion kinetic energy spectrometry (CID/MIKES) has resulted in the identification of distinct differences in fragmentation pathways indicative of the isomer being examined.[50] For example, although the spectra of both the 2',3'- and 3',5'-cyclic CMP exhibit the BH_2^+ ion as the base peak of the spectrum, only the 3',5'-isomer shows fragments arising from cleavages across the sugar ring to produce the $(B+30)^+$ (m/z 140) and $(B+44)^+$ (m/z 154) ions. These ions are weak or absent in the spectrum of the 2',3'-compound. On the other hand, the presence of a strong ion at m/z 178 in the 2',3'-CMP spectrum is unique to this sample and is thought to arise from loss of the phosphate group and a molecule of methanol from the MH^+ ion as illustrated in Scheme 1. The FAB CID/MIKE spectrum of the 2',3'- and 3',5'-cyclic adenosine pair showed a similar pattern. The corresponding guanosine cyclic monophosphates however, behaved somewhat differently. Thus, while 3',5'-GMP followed the pattern in producing the $(B+30)^+$ (m/z 180) and $(B+44)^+$ (m/z 194) ions, these ions being weak in the spectrum of the 2',3'-sample, the 2',3'-isomer contained three new, intense ions at m/z 195, 214 and 217. The ion corresponding to m/z 178 present in the spectra of the other 2',3'-isomers was not present but the three new ions, as yet uncharacterized, appear to be unique to the 2',3'-guanosine cyclic monophosphate system. Therefore, application of the FAB CID/MIKE



SCHEME 1. FAB CID/MIKE fragment ions used to differentiate a) 3',5'-cyclic CMP from b) 2',3'-cyclic CMP (adapted from Ref. 50).

method to the analysis of biological samples obtained from both plant and animal sources indicated the presence of 3',5'-cyclic but not 2',3'-cyclic nucleotides in the samples.

FAB has also been used in an attempt to develop a general method for the incorporation of stable isotopes into glucuronides by performing an ^{18}O exchange reaction on the enzyme cofactor uridine-5'-diphosphoglucuronic acid (UDPG).[51] This cofactor transfers a glucuronic acid group to an appropriate substrate catalyzed by the action of hepatic uridinediphosphoglucuronyl transferase. The stable isotope labeling of the cofactor in the glucuronic acid portion of the molecule would, therefore, provide a general method for the labeling of glucuronic acid metabolites. FAB analysis of the products isolated following an acid catalyzed ^{18}O exchange reaction with the cofactor thus permitted optimum experimental conditions for ^{18}O label incorporation to be developed. By comparing the negative ion FAB spectra of ^{18}O labeled and unlabeled cofactor, both the position and level of isotope incorporation could be determined. The $(M-H)^-$ ion

was the base peak in the spectra and peaks at $(M-H+2)^-$ and $(M-H+4)^-$ indicated the incorporation 44% mono-and 44% di-^{18}O label under optimum exchange conditions. Fragment ions corresponding to the $(UDP)^-$ and $(UDP\text{-}glucuronate)^-$ species permitted the site of label to be located in the glucuronic acid moiety (See Figure 3). An additional experiment was performed to determine the extent of stable isotope lost during the transglucosylation reaction. Thus, the labeled cofactor was incubated with p-nitrophenol substrate in the presence of enzyme and the enzymatic product, p-nitrophenol glucuronide, examined mass spectrometrically. Results of this study indicated that in the transfer of the labeled glucuronic acid group to the substrate less than 10% of the ^{18}O label was lost, and the procedure provided a general method for following transglucosylation reactions.

The ^{18}O labeling of adenosine phosphorothiate, ATPαS, a substrate for phe-tRNA synthetase used to examine the stereochemistry of reactions catalyzed by this enzyme, has also utilized FAB MS analysis.[52] The di- and triphosphate ions formed using negative ion FAB detection were key ions in distinguishing (α-^{18}O) from bridged (α,β-^{18}O)ATPαS as shown in Figure 4. Linked scanning techniques coupled with FAB ionization permitted differentiation of the two compounds on the basis of water losses from the appropriate di- and triphosphate sequence fragment ions, e.g., α-^{18}O-ATPαS lost $H_2{}^{18}O$ from the triphosphate while the diphosphate ion did not show this loss. The opposite situation was observed in the spectrum of the α,β-^{18}O-ATPαS sample.

A final example of the biochemical applications of FAB involves the mass spectral analysis of coenzyme-A(CoA) and acyl-CoA derivatives.[53] Certain metabolic disorders, termed organic acidurias, result from an enzyme deficiency associated with oxidative pathways dependent on CoA. In order to gain insight into these metabolic disorders and to evaluate new and potentially more effective therapies, methods for the direct analysis of metabolic intermediates in these biochemical pathways have been developed using FAB and linked scanning techniques. Both positive and negative ion FAB analysis of CoA and the acyl-CoA derivatives provided mass spectra with intense MH^+ and $(M-H)^-$ ions, along with a number of fragment ions diagnostic of each major portion of the molecule (see Figure 5) useful in

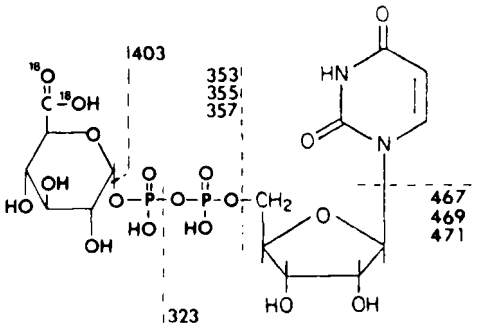


FIG. 3. Major fragment ions in the negative ion FAB mass spectrum of ^{18}O-labeled-UDPG used to assign site and level of stable isotope exchange (adapted from Ref. 51).

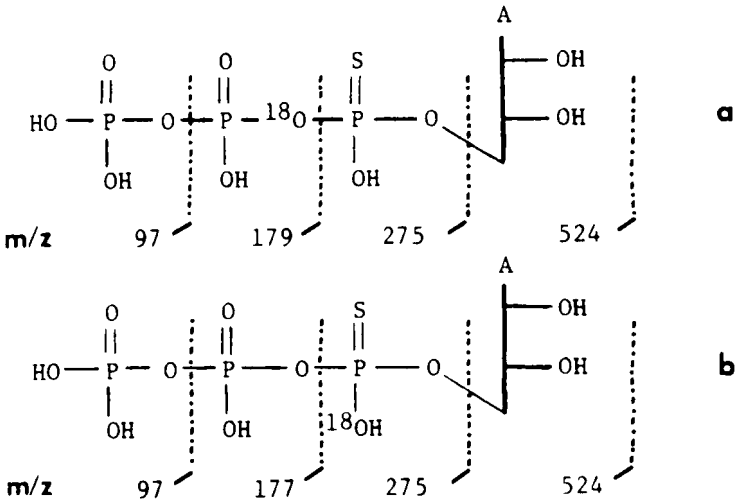


FIG. 4. Major fragment ions observed for a) α,β-^{18}O-ATPαS and b) α-^{18}O-ATPαS using negative ion FAB with linked B/E scanning (adapted from Ref. 52).

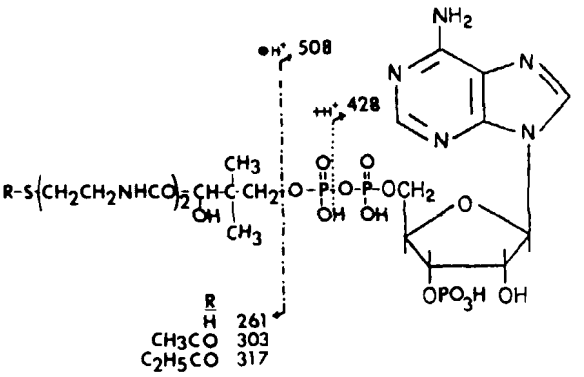


FIG. 5. Fragmentation observed for some acyl-CoA derivatives using positive ion FAB and linked B/E scanning (adapted from Ref. 53).

identifying sites of metabolic transformation. Interestingly, the negative ion spectrum provided a larger number of fragment ions, but with lower sensitivity, than did positive ion detection. For biological studies of the acyl-CoA analogs, linked scanning methods (constant B/E to monitor daughter ions of the MH^+ ion) were examined in anticipation of the presence of a number of artifacts contained in a biological sample which would interfere with the analysis using standard procedures. The resulting metastable ion spectra of the MH^+ ions were essentially the same as those obtained using normal FAB conditions. The base peak in the spectrum is characteristic of the acyl group which, in this study, is the portion of the molecule of greatest interest. The application of these FAB linked-scanning methods to the analysis of the biological samples is expected to provide information concerning the metabolic pathways, enzyme activities and structures of intermediary metabolites previously unavailable and to be of value in understanding the metabolic deficiencies leading to organic acidurias.

B. DINUCLEOTIDES

Dinucleotides are the smallest unit of a nucleotide chain to possess sequence information. A number of attempts have been made using other desorption techniques, e.g., FD and PD to obtain sequence information on this type of sample. The analysis of underivatized

dinucleoside phosphates by FD[5] have met with little success with regard in sequence specific fragment ions, but FD does provide molecular weight information. More positive results in obtaining sequence information on dinucleotides, and higher oligomers, has been achieved using PD,[8-10] especially in the negative ion mode. However, the analysis of nucleoside diphosphates with PD requires derivatization of the sample, adding substantially to the molecular weight of the sample. The consequence of derivatization is to restrict the number of residues in the chain which can be analysed within the operating mass range of the mass spectrometer. In the case of dinucleotides, this limitation is not normally a consideration but does become a factor in the analysis of larger oligonucleotides on a magnetic sector instrument. Analysis of dinucleoside phosphates with FAB ionization, however, avoids the problem of derivatization and has been shown to provide the sequence specific fragments needed for characterization of these samples.

The general observation that the positive ion spectra of nucleosides and mononucleotides are more complex than the negative ion spectra also applies to the dinucleotides. A number of factors have been suggested to account for this complexity, which becomes greater as the number of possible fragmentation pathways increases.[54] The presence of exchangable protons in the phosphate group(s) provides sites for cationization, as discussed previously, and the MH^+ and fragment ions containing the phosphate moiety will therefore exhibit a series of multiple ions.

A comparison has been made of the spectra of dinucleotides obtained using positive and negative ions.[34] The positive ion spectrum showed the presence of MH^+ ions, BH_2^+ ions and inorganic phosphate ions derived from fragmentation of the MH^+ ion, but no consistent indication of the presence of nucleoside or mononucleotide fragments was obtained. Analysis of the same dinucleotide samples using negative ion detection provided a greater number of structurally relevant fragment ions, including peaks attributed to the formation of deprotonated mononucleotide species, in addition to the $(M-H)^-$ and B^- ions.

Although the BH_2^+ and B^- ions are generally observed in the spectra of dinucleotides, the direct loss of these fragments from the

molecular ion related species is not commonly observed. An exception has been reported in the case of nicotinamide adenine dinucleotide (NAD) where the initial loss of nicotinamide base occurred from the MH^+, $(M+Na)^+$ and $(M-H+2Na)^+$ ions[55] (see Figure 6). Further decomposition of the $(MH\text{-nicotinamide})^+$ ion by expulsion of adenine permitted the direct identification of both of the bases present in this dinucleotide pyrophosphate. The negative ion spectrum of 3',5'-dithymidylate also exhibited the direct loss of a base from the $(M-H)^-$ ion and this ion was the most intense daughter ion in the FAB/MIKE spectrum of this compound.[54] In addition, deprotonated mononucleotide ion, $(TMP-H)^-$, fragmented with loss of the base to yield an ion corresponding to a $(TMP-H-B)^-$ species.[54]

Isomeric dinucleotides have been distinguished using FAB and at least two reports, both utilizing the negative ion mode, have appeared illustrating this capability. A fragmentation unique to 3',5'-linkages has been observed which requires cleavage of the ribose ring with retention of the 3' to 5' carbons and the phosphate moiety by the nucleotide on the free 5' side of molecule. The presence of this fragment has been used to differentiate A3'p5'C from C3'p5'A (Figure 7).[56] FAB coupled with the CA MS/MS technique has also been shown to distinguish A3'p5'C from A2'p5'C.[31] In the case of the A2'p5'C isomer (Figure 8), 3',5'-fragmentation is not possible and, instead, an ion corresponding to $(M-H-90)^-$ is observed. The $(M-H-90)^-$ ion of the 2',5'-linked dinucleotides cannot, however, be used to distinguish 2'Ap5'C from 2'Cp5'A since both bases are retained in this fragment.

The FAB analysis of isomeric trinucleotides has also been reported,[56] but the fragmentation characteristic of the 3',5'-link was absent from the spectrum and further analysis of the data was not presented.

The application of FAB to the characterization of a metabolite of the antitumor agent tiazofurin, a C-nucleoside analog of ribavirin, has been described.[57] Isolation of the metabolite from murine P388 tumor extracts and mass spectral analysis using negative ion FAB, produced a spectrum showing an intense ion at m/z 668, assumed to represent the $(M-H)^-$ ion indicating a molecular weight of 669 d. The appearance of two series of ions resulting from scission of phosphodiester bonds, shown in Figure 9, was indicative of the

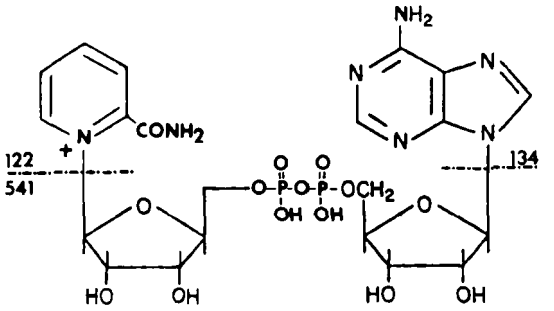


FIG. 6. Mode of formation of the major fragment ions in the positive ion FAB mass spectrum of nicotinamide adenine dinucleotide (adapted from Ref. 55).

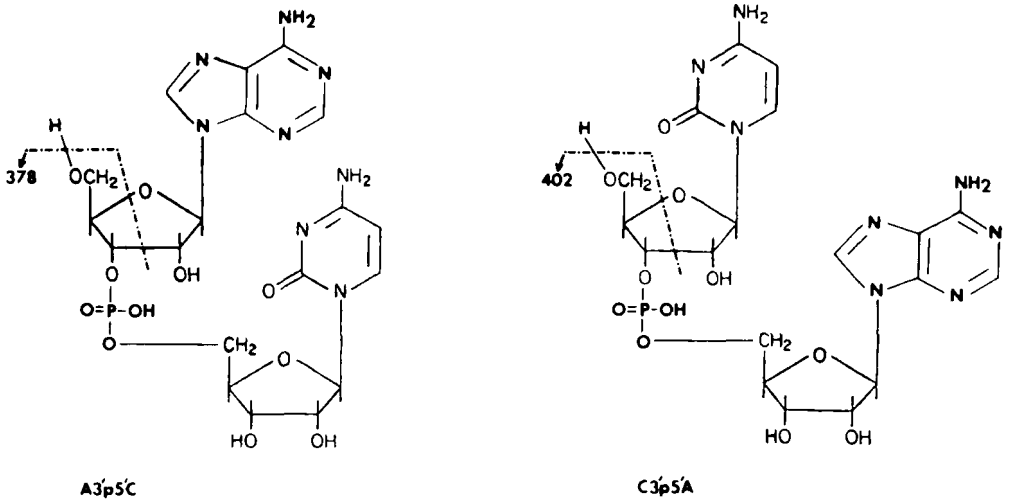
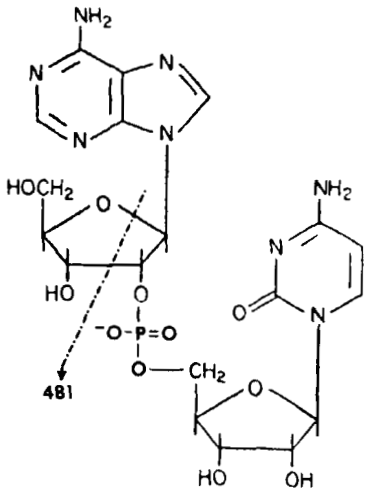


FIG. 7. Fragment ions used to differentiate isomeric dinucleotides, A3'p5'C and C3'p5'A, using negative ion FAB (adapted from Ref. 56).

presence of a dinucleotide resembling NAD, but differing in structure by replacement of the nicotinamide base by the thiazole-4-carboxamide structure present in tiazofurin.

C. OLIGONUCLEOTIDES

One of the ultimate goals in the mass spectral analysis of nucleic acids has been the direct determination of the sequence of an



A2'p5'C

FIG. 8. Formation of the (M-H-90)- ion for a 2',5'-linked dinucleotide, A2'p5'C, using negative ion FAB/CA MS/MS (adapted from Ref. 31).

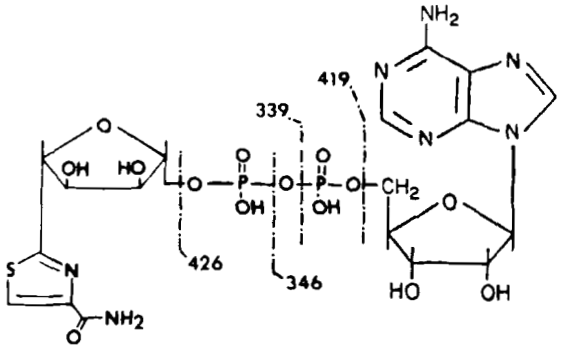


FIG. 9. Major fragment ions in the negative ion FAB mass spectrum of the tiazofurin metabolite (adapted from Ref. 57).

intact oligonucleotide chain. A number of limitations have prevented the realization of this goal, the most significant being the inability to vaporize these highly polar molecules and the limited mass range of most commercially available mass spectrometers. The introduction of FAB has effectively overcome the first limitation, and advances in magnet technology have extended the usable mass range of mass spectrometers to the 10,000 dalton, or higher, range. Although problems remain to be solved in high mass analysis, the sequencing of oligonucleotides containing up to 10 residues using mass spectrometry has been achieved.

The initial account of the sequencing of an underivatized oligodeoxynucleotide chain described a bidirectional fragmentation of the chain in the negative FAB mode.[58] The mass spectra obtained during the analysis of underivatized oligodeoxynucleotides, two octamers and a decamer of known sequence, displayed two ion series representing the formation of 5'- and 3'-phosphate anions. The 5'-phosphate ion series represented cleavage of the 3'carbon-5'phosphate oxygen bond with the negative charge remaining with the phosphate group. This series of fragment ions began from the free 5' end of the chain, the initial end, and proceeded in a stepwise, sequential manner the length of the chain producing readily recognizable ions indicating each cleavage. The 3'-series of ions arose in a similar manner beginning from the free 3' end of the DNA chain, the terminal end. Both of the ion series were present in an alternating fashion throughout the spectrum. Due to an apparent greater stability in ion formation, the 5'-ion series was observed to be more intense than the 3'-series of ions in the spectra of all of the samples examined. Identification of the ion series then permitted the sequence of the oligonucleotide chain to be determined (see Figure 10). A more recent report suggests that the intensity of the 3'-ion series is sometimes greater than the 5'-series, at least in 3 out of the 11 deoxynucleotide trimers examined.[55]

The coupling of negative ion FAB with CA MS/MS has produced abundant sequence information during the analysis of two tetraoligodeoxynucleotides.[59] The appearance of dimer and trimer fragment ions allowed deduction of the sequence of the tetramer. In addition, ions were observed for each of the monomeric anion species,

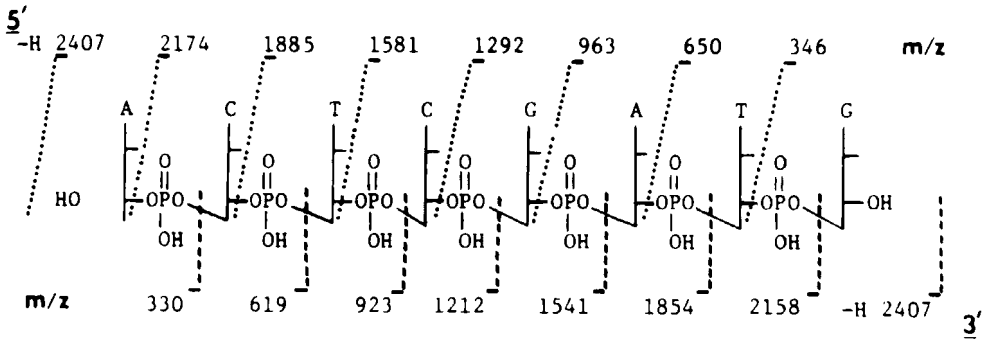


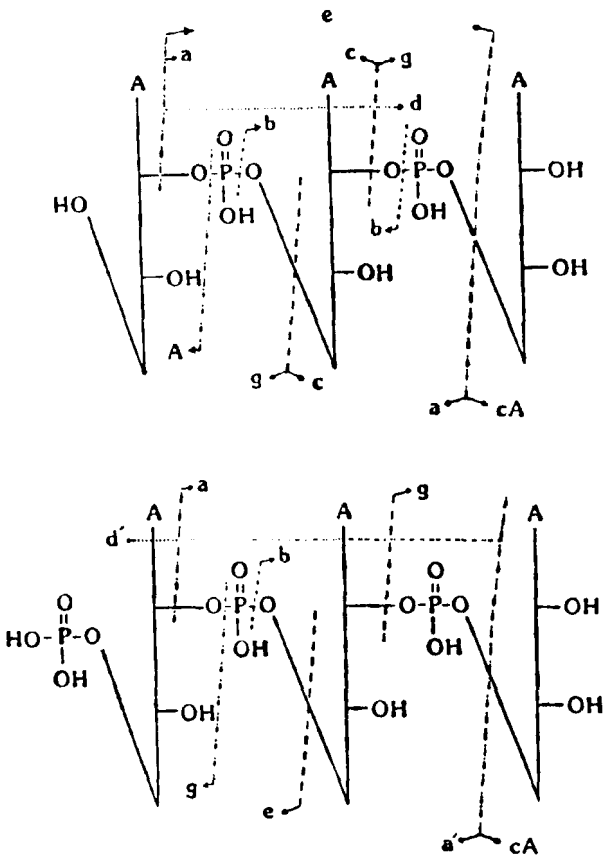
FIG. 10 Fragment ions (3' and 5' series) observed for an oligodeoxynucleotide (octamer) in negative ion FAB (adapted from Ref. 58).

i.e., $(dAMP-H)^-$, $(dGMP-H)^-$ and $(TMP-H)^-$, as well as the base anions, B^-, and 2- and 3-monophosphate anions of 2-hydroxymethyl-3-hydroxydihydrofuran, a decomposition product of the sugar phosphate group. A preference for specific base elimination was evidenced in the CA mass spectrum of d(TACC) in which cytosine and thymine losses were dominent. Since rearrangement reactions are diminished in CA experiments, the assumption was made that the cytosine and thymine bases in d(TACC) were sites of initial protonation. The structure of $d(TACC-H)^-$ was, therefore, postulated to contain 3 negatively charged phosphodiester functions and the 2 protonated bases. The intensity of the 3'-series of sequence ions relative to the 5'-series varied significantly in this study and again raises the question of the utility of ion intensity in distinguishing these two series. The analysis of the samples as triethylammonium salts should, however, be noted. Although one report has evaluated the FAB mass spectra of a number of inorganic salts,[60] the effect of various cations, present as a sample salt or as a component in a biological matrix, on the appearance of the FAB mass spectrum has not been studied in detail.

The direct analysis of protected oligonucleotides by negative ion FAB has also been described.[61] The advantage of using FAB mass spectrometry to characterize these intermediates used in the preparation of synthetic oligodeoxynucleotides of defined sequence is that time consuming deprotection steps, required by other methods of

analysis currently in use, are eliminated. The mass spectra produced using FAB ionization for the analysis of oligodeoxynucleotides containing monomethyoxytrityl and p-nitrophenylethyl protecting groups (dissolved in thioglycerol) provided molecular weight information and fragment ions permitting the assignment of structure to these compounds.[61] A comparison of the positive and negative ion spectra of oligodeoxynucleotides containing a variety of the protecting groups commonly used in the synthesis of longer chains has been made.[62] The oligonucleotides examined in this study were divided into three categories: fully protected (5'-dimethoxytrityl, 3'-cyanoethyl chlorophosphate); protected (5'-dimethoxytrityl) with 3'-chlorophenyl phosphate anion termination, as the triethylammonium salt; and protected 3'-terminating (5'-hydroxyl, 3'-O-benzoyl termination). The positive ion spectra of the fully protected samples gave weak MH^+ and $(M+Na)^+$ ions and limited sequence information. The negative ion analysis produced weak $(M+Cl)^-$ and very weak $(M-H)^-$ ions and a strong $(M-CH_2CH_2CN)^-$ ion but displayed sequence information. The presence of the chlorine isotope pattern was helpful in spectral interpretation. Phosphate ion fragments containing the 5'-protecting group were significantly more intense than the 3'-series. The second group of derivatives containing the 3'-chlorophenyl phosphate group showed excellent sensitivity in the negative ion mode with both 3'-and 5'-ions series being observed. Loss of the 5'-protecting group also produced prominent ions which complicated the identification of sequence ions. Negative ion analysis was therefore preferred in the last group of compounds. Both the 3'- and 5'- series of ions were present with the 3'-benzoyl containing series, in general, being dominant. By noting the presence of the 3'-and 5'-series of ions, the sequencing of a derivatized oligodeoxynucleotide may be performed by scanning only about half of the total molecular weight range, provided the total molecular weight is known.

Because of the simplicity of the spectrum and fragmentation, the negative ion mode has been the most commonly used method in the FAB analysis of oligonucleotides. However, a disadvantage of detecting negative ions is that the sensitivity is significantly lower, relative to the positive ion mode, on many mass spectrometers, including the instrument in this laboratory. For most applications, therefore, the



ION	PROPOSED IDENTITY
a	pApA +2H
a'	pApAp +2H
b	ApA +2H
c	b - H_2O
d	pApA +H -134
d'	pApAp+2H-2(134)
e	pAp +2H
f	e - H_2O
g	pA+2H or Ap+2H

FIG. 11. Fragmentation observed for 2',5'-oligoadenylates: trimer core (upper) and trimer monophosphate (lower) as the free acids in positive ion FAB (adapted from Ref. 45).

mass spectrometer in this laboratory has been operated in the positive ion mode. Although the spectra are definitely more complex than the negative ion spectra, the information content appears to be greater in the positive ion mode. An example is the analysis of a series of 2',5'-oligoadenylate trimers.[45] Interest in this class of compounds stems from their suspected role as interferon inducers and their potential utility as antitumor and antiviral agents. Positive ion FAB analysis of a number of these samples containing various modifications in the aglycone, sugar and phosphate groups showed the presence of extensive fragmentation (See Figure 11). Although complex, the mass

spectrum displayed clearly recognizable ions representing the important features of the structure. For example, differences in the observed fragmentation of the trimer core versus the trimer monophosphate reflected the presence of the additional 5'-phosphate in the latter. Ion series **a** and **d** in the trimer core are seen as **a'** and **d'** in the trimer monophosphate. In the case of the **d** and **d'** series, ion **d** represents the dimer fragment (pApA) with a loss of one adenine base whereas ion **d'** represents the dimer monophosphate fragment (pApAp) with a loss of two adenine bases.

IV. CONCLUSIONS

Fast atom bombardment ionization, although still in its infancy, has already been recognized as a powerful technique in the mass spectral analysis of nucleic acid components. The most striking features of FAB include the ability to analyze polar nonvolatile compounds, such as nucleotides, directly and to produce significant molecular ion species accompanied by important fragments providing sequence or structural information. The use of auxiliary techniques in conjunction with FAB, i.e., collisional activation or linked scanning, allows the effective separation of the solvent or biological matrix ions from sample ions and enhances the integrity of the spectral results. The sequencing of unprotected oligonucleotides, up to ten residues, with FAB has also been remarkable and has widened the domain of FAB utilization to the areas of DNA research and gene manipulation. Finally, recent advances in instrumental design should, in the near future, provide the means for the analysis of samples with molecular weights in the 5,000-10,000 dalton range using either on-line or off-line mass spectral procedures. The most dramatic of these developments has been the extension of the operating mass range of magnetic sector instruments and the use of FAB as an ionization technique in LCMS. The application of FAB mass spectrometry to solving chemical, biochemical and medical problems relating to the structure and function of nucleic acid components has just begun. Significant contributions by this new mass spectrometric tool may be expected to continue in the future.

ACKNOWLEDGEMENTS

This work supported in part by NCI Grant CA-24690.

REFERENCES

1) M. Barber, R.S. Bordoli, R.D. Sedgwick, A.N. Tyler and B.N. Green, J. Chem. Soc. Chem. Commun. 325 (1981).

2) D.J. Surman and J.C. Vickerman, J. Chem. Soc. Chem. Commun. 324 (1981).

3) D.H. Williams, C. Bradley, G. Bojesen, S. Santikarn and L.C. E. Taylor, J. Am. Chem. Soc. **103**, 5700 (1981).

4) K.L. Rinehart, Jr., Science **218**, 254 (1982)

5) H.R. Schulten, Int. J. Mass Spectrom. Ion Phys. **32**, 97 (1979).

6) H.R. Schulten and H.M. Schiebel, Z. Anal. Chem. **280**, 139 (1976).

7) H.R. Schulten and H.D. Beckey, Org. Mass Spectrom. **7**, 861 (1973).

8) C.J. McNeal, S.A. Narang, R.D. Macfarlane, H.M. Hsiung and R. Brousseau, Proc. Natl. Acad. Sci. USA, **77**, 735 (1980).

9) N. Otake, T. Ogita, Y. Miyazaki, H. Yonehara, R.D. Macfarlane and C.J. McNeal, J. Antibiot., **34**, 130 (1981).

10) C.J. McNeal, K.K. Ogilvie, N.Y. Theriault and M.J. Nemer, J. Am. Chem. Soc. **104**, 981 (1982). (See also the two preceding papers in this journal).

11) R.J. Cotter and C. Fenselau, Biomed. Mass Spectrom. **6**, 287 (1979).

12) E.L. Esmans, E.J. Freyne, J.H. Vanbroeckhoven and F.C. Alderweireldt, Biomed. Mass Spectrom. **7**, 377 (1980).

13) D.F. Hunt, J. Shabanowitz, F.K. Botz and D.A. Brent, Anal. Chem. **49**, 1160 (1977).

14) A. Eicke, W. Sichtermann and A. Benninghoven, Org. Mass Spectrom. **15**, 289 (1980).

15) S.E. Unger, A.E. Schoen, R.G. Cooks, D.J. Ashmorth, J.D. Gomes and C. Chang, J. Org. Chem. **46**, 4765 (1981).

16) R.A. McDowell, A. Dell, H.R. Morris and T. Redfern, Abstr. 29th Ann. Conf. Mass Spectrom. Allied Topics, Minneapolis, MN, 1981, p. 355.

17) K.L. Clay, L. Wahlin and R.C. Murphy, Biomed. Mass Spectrom. **10**, 490 (1983).

18) S.A. Martin, C.E. Costello and K. Biemann, Anal. Chem. **54**, 2362 (1982).

19) J.M. Gilliam, P.W. Landis and J.L. Occolowitz, Anal. Chem. **56**, 2285 (1984).

20) J.G. Stroh, J.C. Cook, K.L. Rinehart, Jr. and I.A.S. Lewis, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 119.

21) M. Barber, R.S. Bordoli, G.J. Elliott, R.D. Sedgwick and A.N. Tyler, J. Chem. Soc. Faraday Trans. **79**, 1249 (1983).

22) W.D. Lehmann, M. Kessler, W.A. Konig, Biomed. Mass Spectrom. **11**, 217 (1984).

23) Workshop: Quantitation Using Desorption Techniques/Quantitative Organic Analysis, Dan Knapp, Presiding. Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 908.

24) J.C. Cook and B.N. Green, in Abstr. 32nd Annu. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 835.

25) J.A. McCloskey in Basic Principles in Nucleic Acid Chemistry, Vol. 1, P.O.P. Ts'o, ed. Academic Press, New York, pp. 209-309 (1974).

26) M.S. Wilson and J.A. McCloskey, J. Am. Chem. Soc. **97**, 3436 (1975).

27) K.H. Schram and J.A. McCloskey in GLC and HPLC Determination of Therapeutic Agents, Part 3, K. Tsuji, ed., Marcel Dekker, Inc., New York, 1979, pp. 1149-1190.

28) D.L. von Minden, R.N. Stillwell, W.A. Koenig, K.J. Lyman and J.A. McCloskey, Anal. Biochem. **50**, 110 (1972).

29) R.P. Panzica, L.B. Townsend, D.L. von Minden, M.S. Wilson and J.A. McCloskey, Biochim. Biophys. Acta **331**, 147 (1973).

30) D.L. Slowikowski and K.H. Schram, Nucleosides and Nucleotides, in press.

31) F.W. Crow, K.B. Tomer, M.L. Gross, J.A. McCloskey and D.E. Bergstrom, Anal. Biochem. **139**, 243 (1984).

32) M.E. Rose, C. Longstaff and P.D.G. Dean, Biomed. Mass Spectrom. **10**, 512 (1983).

33) G. Puzo, J.C. Prome, J.P. Macquet and I.A. S. Lewis, Biomed. Mass. Spectrom. **9**, 552 (1982).

34) J. Eagles, C. Javanaud and R. Self, Biomed. Mass Spectrom. **11**, 41 (1984).

35) Unpublished results.

36) Nomenclature from reference 1). Ambiguities in the nomenclature of FAB generated ions is common. For the purpose of this review:

M refers to the uncharged molecule or the free acid in nucleotides. B is the base portion and S is the sugar portion after glycosidic bond cleavage, preceding any proton transfer. Thus, the molecular ion species is $(MH)^+$ in positive ion and $(M-H)^-$ in negative ion modes, and the base ions are BH_2^+, following proton transfer, and B^-. Cationized species are then labelled by exchanging protons. Sugar ring fragment ions, called S_1 and S_2 in reference 31, are referred to as the $(B+44)^+$ and the $(B+30)^+$ ions for purposes of comparison with other ion modes and uniformity in nomenclature.

37) D.L. Smith, K.H. Schram and J.A. McCloskey, Biomed. Mass Spectrom. **10**, 269 (1983).

38) P.J. Stone, A.D. Kelman and F.M. Sinek, Nature (London) **251**, 736 (1984).

39) P.J. Stone, A.D. Kelman, F.M. Sinek, M.M. Bhargava and H.P. Holvorson, J. Mol. Biol. **104**, 793 (1976).

40) R.K. Mitchum, F.E. Evans, J.P. Freeman, and D. Roach, Int. J. of Mass Spectrom. Ion Phys. **46**, 383 (1983).

41) J. Wang and A.L. Burlingame, Abstr. 31st Ann. Conf. Mass Spectrom. Allied Topics, Boston, MA, 1982, p. 564.

42) Y. Tondeus, R.C. Moschel, A. Dipple, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, Texas, 1984, p. 815.

43) Y. Tondeus, M. Shorter, M.E. Gustafson, and R.C. Pandey, Abstr. 31st Ann. Conf. Mass Spectrom. Allied Topics, Boston, MA, 1982, p. 899.

44) C. Fenselau, V.T. Vu, R.J. Cotter, G. Hansen, D. Heller, T. Chen and O.M. Colvin, Spectros. Int. J. **1**, 132 (1982).

45) D.L. Slowikowski, K.H. Schram, P.F. Torrence and J. Imai, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 817.

46) S.K. Sethi, D.L. Smith and J.A. McCloskey, Biochem. Biophys. Res. Commun. **112**, 126 (1983).

47) B.D. Musselman and J.T. Watson, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX., 1984, p. 244.

48) J.R. Mehta, M. Przybylski, D.B. Ludlum, Cancer Res. **40**, 4183 (1980).

49) V.T. Vu, C.C. Fenselau, and O.M. Colvin, J. Am. Chem. Soc. **103**, 7362 (1981).

50) E.E. Kingston, J.H. Beynon and R.P. Newton, Biomed. Mass Spectrom. **11**, 367 (1984).

51) C. Fenselau, P.C.C. Feng, T. Chen and L.P. Johnson, Drug Metab. Dispos. **10**, 316 (1982).

52) L. Grotjahn, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 396.

53) D.S. Millingston and D.A. Maltby, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 398.

54) G. Sindona, N. Uccella, K. Weclawek, J. Chem. Research (S) 184 (1982).

55) J.L. Aubagnac, F.M. Devienne, R. Combarieu, J.L. Barascut, J.L. Imbach and H.B. Lazrek, Org. Mass Spectrom. **18**, 361 (1983).

56) A.M. Hogg, J.G. Kelland and J.C. Vederas, Abstr. 31st Ann. Conf. Mass Spectrom. Allied Topics, Boston, MA, 1983, p. 693.

57) J.A. Kelley, G. Gebeyehn, P.N. Huguenin and V.E. Marquez, Abstr. 31st Ann. Conf. Mass Spectrom. Allied Topics, Boston, MA, 1983, p. 365.

58) L. Grotjahn, R. Frank and H. Blocker, Nucleic Acids Res. **10**, 4671 (1982)

59) M. Panico, G. Sindona and N. Uccella, J. Am. Chem. Soc. **105**, 5607 (1983).

60) C. Javanaud and J. Eagles, Org. Mass Spectrom. **18**, 93 (1983).

61) M.E. Hemling, J.C. Cook, K.L. Rinehart, Jr., R.I. Gumport and W. Pfleiderer, Abstr. 32nd Ann. Conf. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 402.

62) D.M. Hindenlang, R.D. Sedgwick, C.S. Aaron and P.D. Unger, Abstr. 32nd Ann. Cont. Mass Spectrom. Allied Topics, San Antonio, TX, 1984, p. 813.

Received October 23, 1984