chemical dimerization of acrylonitrile to adiponitrile using surface-active ammonium salts.[36] Fluid surfactant coatings designed with specific properties should be a promising route to progress in this area. Appropriate coatings could in the future enhance rates and control selectivity on demand. Present work is directed toward surfactant films that incorporate and protect mediators and, at the same time, preconcentrate substrates.

Electrosynthesis requires feeding reactants to electrodes and removing products. Conductive microemulsions dissolve significant amounts of polar and nonpolar solutes and can bring together reactants of unlike solubilities. They may prove especially suitable for synthetic electrochemical catalysis. Even when the reaction occurs in surfactant aggregates in solution (cf. Figure 3), respectable rate enhancements are possible from local concentration increases. Selectivity may be influenced by reactant binding properties.[22]

Components of microemulsions may destabilize surfactant coatings on electrodes. A challenge for the future will be to design coatings to be compatible with microemulsions yet perform with high catalytic efficiency.

(36) Adiponitrile is the precursor to hexamethylenediamine in the manufacture of Nylon 66. Acrylonitrile is electrochemically dimerized to adiponitrile in aqueous solutions of tetraethylammonium (TEA) salts in a very successful industrial process[14b] discovered in 1963 at Monsanto by M. Baizer. TEA^+ forms an adsorbed hydrophobic layer at Hg or Pb cathodes, facilitating dimerization. In solutions of alkali salts, propionitrile, but no adiponitrile, is formed by reduction of acrylonitrile.

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Structural Characterization of Natural Nucleosides by Mass Spectrometry

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Introduction

Mass spectrometry plays a major role in the structure determination of compounds of biological importance,[1-5] due largely to its high sensitivity and the complementary nature of information it conveys with respect to other structural techniques. Applications of mass spectrometry to nucleic acid constituents[6] have long represented a notable experimental challenge, due to the high intrinsic polarity of nucleosides and nucleotides and the problems associated with their conversion into gaseous ions. Despite these difficulties, mass spectrometry has played a dominant role in the structure determination of new nucleosides from nucleic acids and other sources.[7] For example, of the 82 known nucleosides in RNA (including the four major nucleosides), nearly all of those discovered in the last 20 years have relied on mass spectrometry as a major, and sometimes the sole, method of structural characterization.[8,9] The key elements of structural studies of nucleosides mirror those in other areas in which mass spectrometry has found successful applications: (1) the development of microscale (microgram level and below) chemical derivatization procedures to increase the information content of mass spectra, or to test for selected structural features; (2) a detailed understanding of gaseous ion chemistry of model compounds; and (3) the design of experimental protocols that permit direct examination of a targeted component in biological mixtures. In the latter case, two methods for the analysis of mixtures can be used for rapid screening of crude biological isolates to gain preliminary structural information. One is directly combined liquid chromatography–mass spectrometry (LC/MS); the other is tandem mass spectrometry (MS/MS)[10] in which ions from the substance of interest are selected in one mass spectrometer and transmitted to a gas cell, where they

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(1) *Biochemical Applications of Mass Spectrometry*; Waller, G. R., Ed.; Wiley-Interscience: New York, 1972.
(2) *Biochemical Applications of Mass Spectrometry*, first suppl. vol.; Waller, G. R., Dermer, O. C., Eds.; Wiley-Interscience: New York, 1980.
(3) *Mass Spectrometry in Biomedical Research*; Gaskell, S. J., Ed.; Wiley: New York, 1986.
(4) *Mass Spectrometry*; Lawson, A. M., Ed.; Walter de Gruyter: New York, 1989.
(5) *Mass Spectrometry* (*Methods Enzymol., Vol. 193*); McCloskey, J. A., Ed.; Academic Press: New York, 1990.
(6) Crain, P. F. *Mass Spectrom. Rev.* **1990**, *9*, 505.
(7) Schram, K. H. In *Mass Spectrometry*; Lawson, A. M., Ed.; Walter de Gruyter: New York, 1989; p 507.
(8) McCloskey, J. A.; Nishimura, S. *Acc. Chem. Res.* **1977**, *10*, 403.
(9) Crain, P. F.; Hashizume, T.; Nelson, C. C.; Pomerantz, S. C.; McCloskey, J. A. In *Biological Mass Spectrometry*; Burlingame, A. L., McCloskey, J. A., Eds.; Elsevier: Amsterdam, 1990; p 509.
(10) For reviews, see refs 6 and 11.
(11) Nelson, C. C.; McCloskey, J. A. *Adv. Mass Spectrom.* **1989**, *11B*, 1296.

0001-4842/91/0124-0081$02.50/0

undergo collision-induced dissociation (CID) and product ion analysis by a second mass spectrometer.

In addition to their fundamental role as the central constituents of RNA and DNA, nucleosides are also of interest in other areas in which their structural characterization is important. These include nucleoside antibiotics, the cytokinin plant growth hormones, urinary excretion products as disease markers, products of xenobiotic DNA damage, and a variety of synthetic nucleoside antiviral agents (and their metabolites), including those used against HIV (AIDS). This *Account* focuses on the development of mass spectrometric techniques that have been used in all of these areas[7] and emphasizes experimental approaches in which sample quantities are limited, as exemplified in the detection and characterization of posttranscriptionally modified nucleosides from RNA. In many instances these methods are generally applicable to structural studies of other classes of compounds. The reader is referred to earlier articles and reviews of fundamental studies of the mass spectra of nucleosides[6,12-15] and of applications to their structural characterization.[6,7,9]

Microscale Chemical Conversions

The conversion of polar molecules to volatile derivatives has long been a hallmark of electron-ionization (EI) mass spectrometry, in which ions are formed by electron bombardment of the sample in the vapor phase. Derivatization not only serves to prevent thermal degradation but also, in favorable cases, may result in a more structurally informative mass spectrum.[16] Whether or not chemical derivatization is required depends on the method of ionization chosen (discussed below), sample purity, and similar issues that influence the overall experimental strategy.[17,18]

Trimethylsilylation[19] and, to a lesser extent, permethylation[20] have proven most useful for derivatization of nucleosides of unknown structure.[21] Although trimethylsilyl (TMS) derivatives suffer from the modest disadvantage of lability of *N*-TMS groups (from traces of water), both derivatives exhibit three principal features required for structure studies: (1) The reactions can be carried out in generally high yield on a microscale (<10 μg), with readily available reagents. (2) The

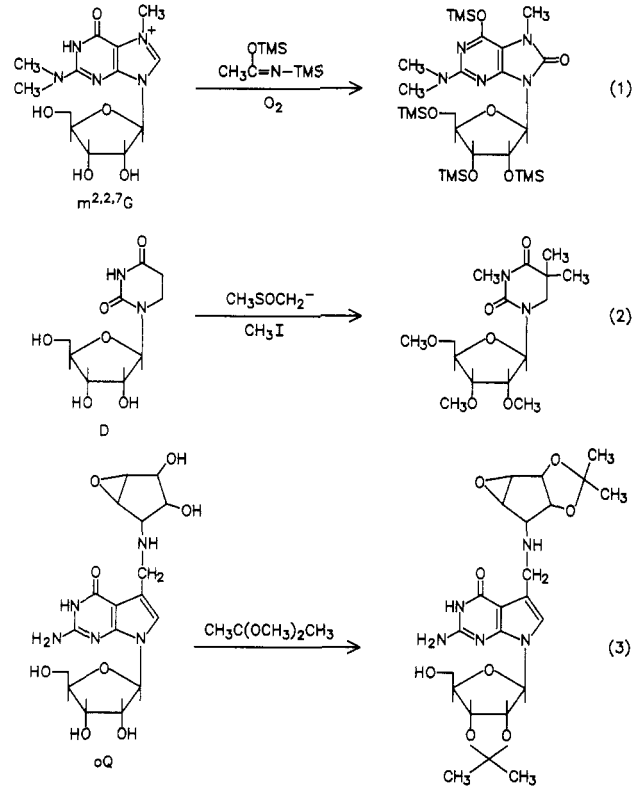

Figure 1. Microscale chemical reactions used to test for structural features common to nucleosides. $m^{2,2,7}G$: $N^2,N^2,7$-trimethylguanosine. D: 5,6-dihydrouridine. oQ: epoxyqueuosine.

extent and sites of reactions in the molecule can generally be predicted. (3) The resulting mass spectra of either derivative are structurally informative, as a result of extensive model studies.[13,20,22-24] Both derivatives offer the additional advantage that commercially available deuterated reagents can be used to introduce labeled blocking groups ($Si(CD_3)_3$ or CD_3), whose number can be determined from mass shifts. This procedure aids interpretation of the resulting mass spectrum and reduces the number of computer-generated structure candidates derived from exact mass data by defining the number of Si atoms or CD_3 groups present.[25]

Likewise, the direct exchange of deuterium for heteroatom-bound (active) hydrogen may be used to determine the number of active hydrogens in a molecule or ion.[26] This technique was first demonstrated in early experiments involving thermal vaporization and EI mass spectrometry of simple nucleosides,[27] but can also be used with so-called desorption ionization methods, such as those involving bombardment of the sample by an energetic beam of atoms (fast atom bombardment, or FAB) or ions (secondary ion mass spectrometry, or SIMS), by use of labeled matrices such as glycerol-O,O,O-d_3,[28] and in LC/MS through use of deuterated

(12) McCloskey, J. A. In *Basic Principles in Nucleic Acid Chemistry*; Ts'o, P. O. P., Ed.; Academic Press: New York, 1974; Vol. I, Chapter 3.
(13) Pang, H.; Schram, K. H.; Smith, D. L.; Gupta, S. P.; Townsend, L. B.; McCloskey, J. A. *J. Org. Chem.* **1982**, *47*, 3923.
(14) Slowikowski, D.; Schram, K. H. *Nucleosides Nucleotides* **1985**, *4*, 309.
(15) McCloskey, J. A. In *Mass Spectrometry in the Health and Life Sciences*; Burlingame, A. L., Castagnoli, N., Jr., Eds.; Elsevier: Amsterdam, 1985; p 521.
(16) Knapp, D. R. *Methods Enzymol.* **1990**, *193*, 314.
(17) McCloskey, J. A. In *Mass Spectrometry in Biomedical Research*; Gaskell, S. J., Ed.; Wiley: New York, 1986; p 75.
(18) McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 771.
(19) Schram, K. H. *Methods Enzymol.* **1990**, *193*, 791.
(20) von Minden, D. L.; McCloskey, J. A. *J. Am. Chem. Soc.* **1973**, *95*, 7480.
(21) For example: (a) Isono, K.; Crain, P. F.; Odiorne, T. J.; McCloskey, J. A.; Suhadolnik, R. J. *J. Am. Chem. Soc.* **1973**, *95*, 5788. (b) Ludlum, D. B.; Kramer, B. S.; Wang, J.; Fenselau, C. *Biochemistry* **1975**, *14*, 5480. (c) Isono, K.; Crain, P. F.; McCloskey, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 943. (d) Pang, H.; Ihara, M.; Kuchino, Y.; Nishimura, S.; Gupta, R.; Woese, C. R.; McCloskey, J. A. *J. Biol. Chem.* **1982**, *257*, 3589. (e) Chheda, G. B.; Dutta, S. P.; Mittelman, A.; Montgomery, J. A.; Sethi, S. K.; McCloskey, J. A.; Patrzyc, H. B. *Cancer Res.* **1985**, *45*, 5958. (f) Muramatsu, T.; Yokoyama, S.; Horie, N.; Matsuda, A.; Ueda, T.; Yamaizumi, Z.; Kuchino, Y.; Nishimura, S.; Miyazawa, T. *J. Biol. Chem.* **1988**, *263*, 9261.
(22) Liehr, J. G.; von Minden, D. L.; Hattox, S. E.; McCloskey, J. A. *Biomed. Mass Spectrom.* **1974**, *1*, 281.
(23) Liehr, J. G.; Weise, C. L.; Crain, P. F.; Milne, G. H.; Wise, D. S.; Townsend, L. B.; McCloskey, J. A. *J. Heterocycl. Chem.* **1979**, *16*, 1263.
(24) Sethi, S. K.; Gelijkens, C. F.; McCloskey, J. A.; Shapiro, R.; Brandes, B. A. *Biomed. Mass Spectrom.* **1983**, *10*, 665.
(25) Crain, P. F.; Yamamoto, H.; McCloskey, J. A.; Yamaizumi, Z.; Nishimura, S.; Limburg, K.; Raba, M.; Gross, H. J. *Adv. Mass Spectrom.* **1980**, *8*, 1135.
(26) McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 329.
(27) Biemann, K.; McCloskey, J. A. *J. Am. Chem. Soc.* **1962**, *84*, 2005.
(28) Sethi, S. K.; Smith, D. L.; McCloskey, J. A. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 126.

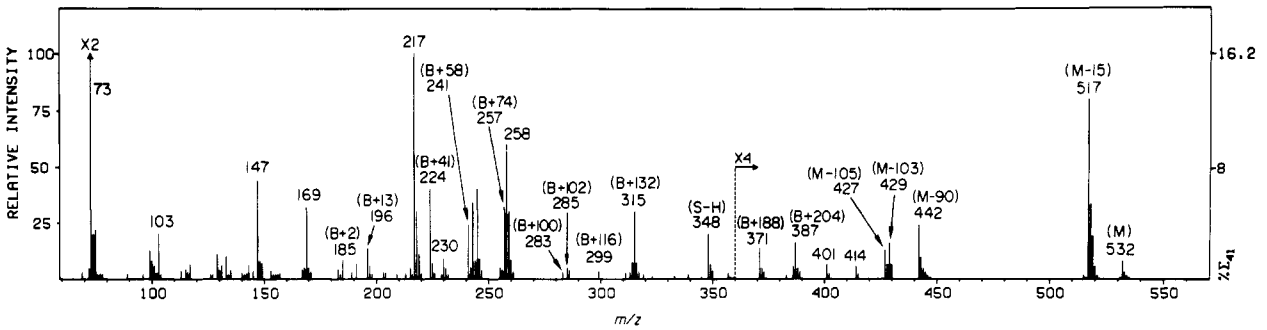

Figure 2. Electron-ionization mass spectrum of the trimethylsilyl derivative of uridine: M, molecular ion; B, heterocyclic base (uracil) fragment; S, sugar (ribosyl) fragment. Reprinted with permission from ref 13. Copyright 1982 American Chemical Society.

mobile phases.[29] Application of deuterium exchange methods for nucleoside characterization using the FAB and LC/MS methods has become routine (e.g., refs 30–32) and is advantageous in providing a constant excess of labile D to minimize back-exchange reactions resulting from surface collisions. The incorporation of up to about eight deuterium atoms can be accurately determined by visual inspection of the mass spectrum, while incorporation levels over D_{20} can be established by comparison with computer-calculated isotopic abundance patterns.[33] Deuterium exchange of H-8 of the imidazole ring of purines by heating in D_2O, followed by back-exchange of active hydrogen using H_2O at room temperature, constitutes a simple test for the presence of an unsubstituted (at C-8) purine ring.[34-36]

Chemical conversions can also be used as functional-group- or structure-specific tests for nucleosides, and they permit a number of reactions to be conducted using a total of several micrograms of material when measured by mass spectrometry. If the component of interest is present in a mixture, the reaction products can be analyzed by LC/MS, a method that is also useful for rapid surveys of conventional-scale nucleoside synthesis reactions.[37] Some examples of microscale reactions used to selectively probe for certain structural features in nucleosides are shown in Figure 1: ring oxidation of purines during trimethylsilylation as a test for N-7 methylation[21b,38,39] (eq 1); "overmethylation" of pyrimidines as a test for the 5,6-dihydropyrimidine moiety[40] (eq 2); and *O*-isopropylidene formation for a test of the presence of *cis*-diols[34,41] (eq 3).

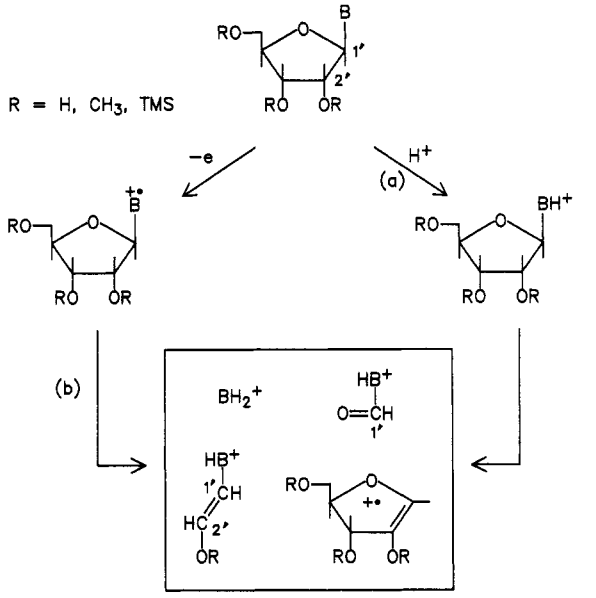

Figure 3. Principal dissociation reactions of ionized nucleosides, showing the similarity of products formed from different molecular ion precursors.

Gas-Phase Ion Chemistry of Nucleosides

The mass spectra of nucleosides have been studied in some detail[6] and provide an extensive base of information for applications to structure elucidation. Fortunately, the emergence of new techniques in recent years has provided an extensive menu of experimental parameters from which to choose: detection of positive or negative ions; production of odd-electron ($M^{\bullet+}$) or protonated (MH^+) molecular ions; and spontaneous- vs collision-induced dissociation (CID) of the molecular ions formed. The selection of ionization mode (and hence the ion chemistry) that will be most advantageous depends to some extent on the nature of information sought. However, EI of TMS derivatives will, in general, offer the greatest structural detail from a single experiment. For example, the EI mass spectrum of trimethylsilylated uridine, Figure 2, exhibits a complex and informative fragmentation pattern for which nearly every ion has been assigned, as a result of extensive D and ^{13}C labeling, measurements of exact mass, and study of pathways and mechanisms of dissociation.[13]

The principal reaction paths of nucleoside molecular ions ($M^{\bullet+}$) were established in early work on unblocked

(29) Edmonds, C. G.; Pomerantz, S. C.; Hsu, F. F.; McCloskey, J. A. *Anal. Chem.* **1988**, *60*, 2314.
(30) Isono, K.; Uramoto, M.; Kusakabe, H.; Miyata, N.; Koyama, T.; Ubukata, M.; Sethi, S. K.; McCloskey, J. A. *J. Antibiot.* **1984**, *37*, 670.
(31) Edmonds, C. G.; Crain, P. F.; Hashizume, T.; Gupta, R.; Stetter, K. O.; McCloskey, J. A. *J. Chem. Soc., Chem. Commun.* **1987**, 909.
(32) Ubukata, M.; Isono, K.; Kimura, K.; Nelson, C. C.; McCloskey, J. A. *J. Am. Chem. Soc.* **1988**, *110*, 4416.
(33) Verma, S.; Pomerantz, S. C.; Sethi, S. K.; McCloskey, J. A. *Anal. Chem.* **1986**, *58*, 2898.
(34) Kasai, H.; Ohashi, Z.; Harada, F.; Nishimura, S.; Oppenheimer, N. J.; Crain, P. F.; Liehr, J. G.; von Minden, D. L.; McCloskey, J. A. *Biochemistry* **1975**, *14*, 4198.
(35) Shapiro, R.; Dubelman, S.; Feinberg, A. M.; Crain, P. F.; McCloskey, J. A. *J. Am. Chem. Soc.* **1977**, *99*, 302.
(36) Yamaizumi, Z.; Nishimura, S.; Limburg, K.; Raba, M.; Gross, H. J.; Crain, P. F.; McCloskey, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 2224.
(37) Hashizume, T.; Nelson, C. C.; Pomerantz, S. C.; McCloskey, J. A. *Nucleosides Nucleotides* **1990**, *9*, 355.
(38) von Minden, D. L.; Stillwell, R. N.; König, W. A.; Lyman, K. J.; McCloskey, J. A. *Anal. Biochem.* **1972**, *50*, 110.
(39) Ro-Choi, T. S.; Choi, Y. C.; Henning, D.; McCloskey, J. A.; Busch, H. *J. Biol. Chem.* **1975**, *250*, 3921.
(40) Panzica, R. P.; Townsend, L. B.; von Minden, D. L.; Wilson, M. S.; McCloskey, J. A. *Biochim. Biophys. Acta* **1973**, *331*, 147.
(41) Phillipson, D. W.; Edmonds, C. G.; Crain, P. F.; Smith, D. L.; Davis, D. R.; McCloskey, J. A.; *J. Biol. Chem.* **1987**, *262*, 3462.
(42) This footnote was deleted in revision.
(43) This footnote was deleted in revision.

nucleosides.[27,44] The basic chemistry of protonated nucleoside ions (MH^+) was first established by using chemical ionization[45,46] and is in general followed in all desorption ionization methods. Mass spectra of deprotonated nucleoside molecular ions $(M - H)^-$, first studied by Smith et al.,[47] reflect structural features associated with negative charge delocalization and stabilization, but offer no obvious advantages over positive ions, although the differentiation of some methylated isomers of guanosine by CID of negative ions is promising.[48] In general, dissociation reactions of collisionally activated ions[49] are qualitatively similar to those from spontaneous dissociation, with the advantages of CID being those inherent to MS/MS in general.[50]

Several characteristics are common to the chemistry of nucleosides in the gas phase, regardless of charge state and method of ion preparation: (1) The charge generally resides in the heterocyclic base, which is the principal controlling element of most dissociation reactions of $M^{\bullet+}$ ions by virtue of its low ionization potential compared with the sugar, and of MH^+ ions as a result of its high proton affinity (~208–227 kcal/mol).[46,51] (2) The stability of the ionized base, whether odd- or even-electron, yields fragment ions in which the base moiety remains intact, bound through C-1′ to portions of the sugar, as illustrated in Figure 3. Glycosidic bond cleavage commonly occurs, leading to the free base or protonated base (positive ions), or to the deprotonated base (negative ions) which is stabilized through extensive charge delocalization.[47] Ions produced solely from the sugar are generally of lower abundance and derive from a minor population of molecular ions in which the charge resides initially in the sugar. The abundances of sugar ions may increase if the sugar contains basic or charge-stabilizing sites, as in some nucleoside antibiotics, or in blocked nucleosides (e.g., TMS derivatives). (3) Intramolecular hydrogen transfer reactions are common, occurring mainly from the sugar to the base, which initiates bond cleavage reactions in the sugar skeleton. (4) Interactions between the base and H-2′ or OH-2′ are important and reflect the steric accessibility of C-2′ substituents to the base. As one consequence of these interactions, α- and β-anomers can be distinguished through fragment ion abundances.[13]

Interestingly, the principal product ions of $M^{\bullet+}$ (from EI) are structurally, and to some extent mechanistically, similar to those of MH^+ (from CI, FAB, etc.), as diagramed in Figure 3. Ionization by CI, FAB, or similar means occurs by intermolecular proton transfer (path a), followed by spontaneous or collision-induced dissociation to product ions, four of which are shown in Figure 3. By contrast, the odd-electron $M^{\bullet+}$ ion, which is produced in the gas phase and is isolated from external sources of hydrogen, can form a protonated base by intramolecular abstraction of $H^\bullet$ from sugar hydroxyl groups (path b when R = H). When the sugar is blocked (as in Figure 2), hydrogen abstraction reactions occur solely from the sugar skeleton, primarily from C-2′ (due to the steric interactions discussed above).

As a consequence of the stability of the ionized base, there are fewer product ions with which to study structural variations in the base skeleton or to assign sites of side-chain substitution, compared with the sugar. If the corresponding base can be liberated without degradation by acid hydrolysis (a facile reaction with 2′-deoxynucleosides; more difficult with ribonucleosides), the base can be examined separately and provides a clearer means of assigning sites of substitution or other modifications (for example, refs 52 and 53). Side-chain substitution in nucleosides may yield ions derived from the substituent, which may compete favorably with reaction paths associated with the nucleoside skeleton. A catalog of common side chain fragment ions from EI of RNA and DNA constituents has been published, and methods for their recognition as components of nucleosides have been discussed.[54]

Posttranscriptionally Modified Nucleosides from Transfer RNA

Transfer RNA (tRNA) contains a remarkable number of modified nucleosides, now known to number 74, of which 52 have been located at specific sites in 455 reported sequences.[55] The importance of mass spectrometry in the characterization of new nucleosides in tRNA dates from the late 1960s, when nucleosides were individually isolated prior to structural characterization,[8] through the present time, when the necessity of isolation is often obviated by use of LC/MS (discussed in the following section).

The sequence locations of modified residues are indicated in Figure 4, which shows the general secondary structure of tRNA consisting of loops and stems formed by Watson–Crick base pairs. Although the principal function of tRNA is the transportation of amino acids (covalently bound at position 76) for protein assembly, it is also implicated in other biological roles,[56] in part through fine-tuning of the tRNA tertiary structure. Because the total tRNA pool is a mixture of 30–60 tRNA species, each typically containing 4–14 modified residues, a single modified nucleoside (such as ms^2t^6A at position 37 in Figure 4) comprises about 1% (about one nucleoside in 80 nucleotides) of an individual tRNA, but only about 0.001–0.4% of the total tRNA nucleoside population. As a consequence, the amounts of individual nucleosides that can be isolated for structure work rarely exceed 5–10 μg, so that mass spectrometry, often in conjunction with various chemical conversions, usually constitutes the principal source of data from which the structure must be derived.

Examples of the approaches used are given by three cases whose structures are shown in Figure 5: 2-

(44) Shaw, S. J.; Desiderio, D. M.; Tsuboyama, K.; McCloskey, J. A. *J. Am. Chem. Soc.* **1970**, *92*, 2510.
(45) Wilson, M. S.; Dzidic, I.; McCloskey, J. A. *Biochim. Biophys. Acta* **1971**, *240*, 623.
(46) Wilson, M. S.; McCloskey, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 3436.
(47) Smith, D. L.; Schram, K. H.; McCloskey, J. A. *Biomed. Mass Spectrom.* **1983**, *10*, 269.
(48) Hettich, R. L. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 265.
(49) Crow, F. W.; Tomer, K. B.; Gross, M. L.; McCloskey, J. A.; Bergstrom, D. E. *Anal. Biochem.* **1984**, *139*, 243.
(50) Busch, K. L.; Glish, G. L.; McLuckey, S. A. *Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry*; VCH Publishers: New York, 1988; Chapter 5.
(51) Meot-Ner, M. *J. Am. Chem. Soc.* **1979**, *101*, 2396.
(52) White, E., V; Krueger, P. M.; McCloskey, J. A. *J. Org. Chem.* **1972**, *37*, 430.
(53) Sethi, S. K.; Gupta, S. P.; Jenkins, E. E.; Whitehead, C. W.; Townsend, L. B.; McCloskey, J. A. *J. Am. Chem. Soc.* **1982**, *104*, 3349.
(54) McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 825.
(55) Sprinzl, M.; Hartmann, T.; Weber, J.; Blank, J.; Zeidler, R. *Nucleic Acids Res.* **1989**, *Suppl. 17*, r1.
(56) Björk, G. R.; Ericson, J. U.; Gustafsson, C. E. D.; Hagervall, T. G.; Jönsson, Y. H.; Wikström, P. M. *Annu. Rev. Biochem.* **1987**, *56*, 263.

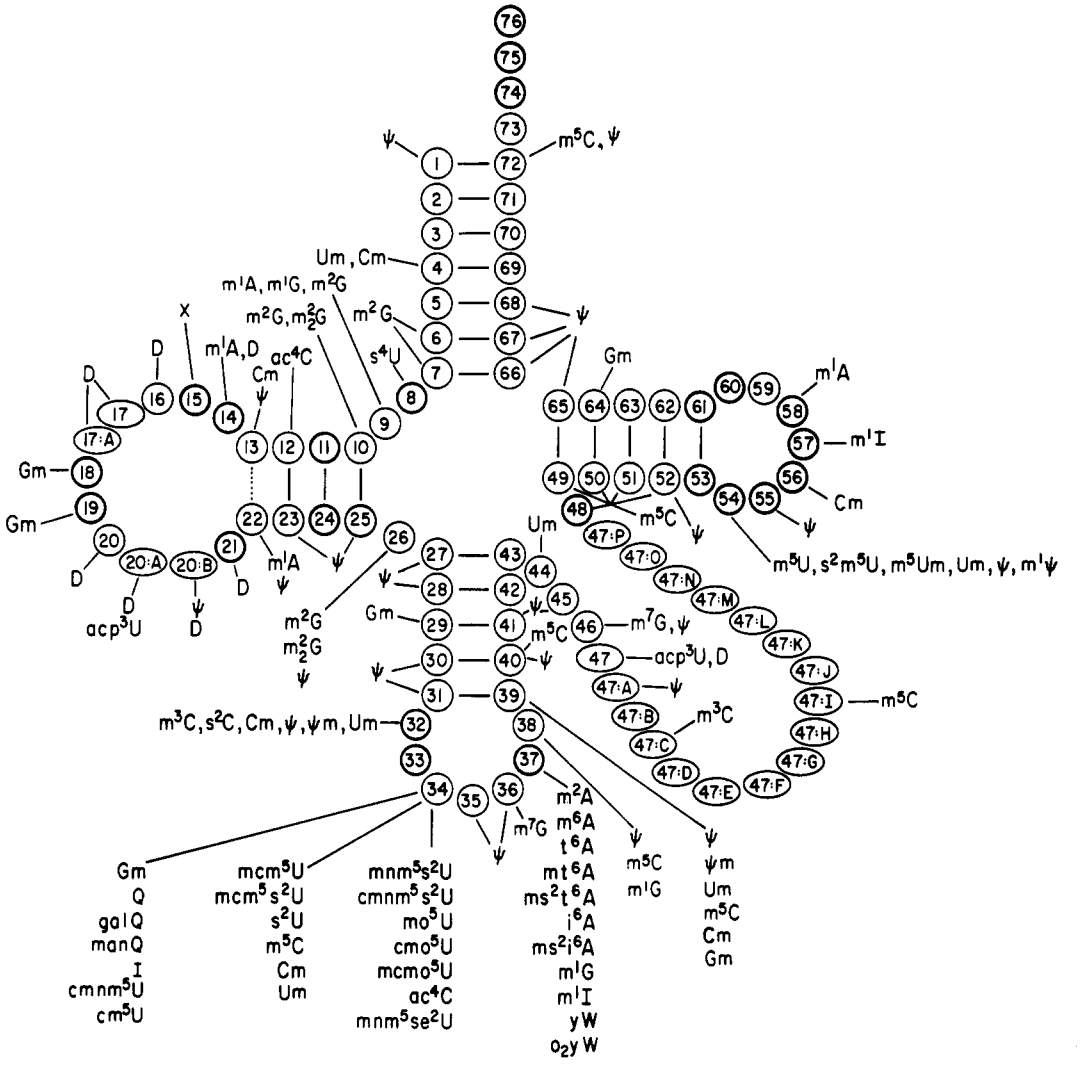

Figure 4. Nucleotide modification patterns in transfer RNA, showing the universal cloverleaf structure dictated by Watson–Crick base pairs. Circles represent positions that are always occupied; thick-edged circles represent invariant or semivariant residues. For modification nomenclature, see ref 55. Adapted from ref 15.

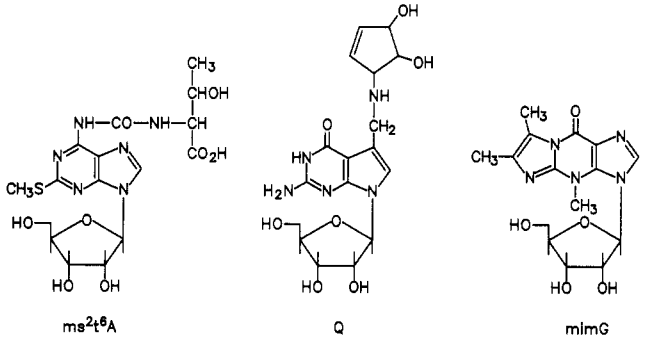

Figure 5. Examples of hypermodified nucleosides from tRNA. For nomenclature, see text.

(methylthio)-N^6-(carbamoylthreonyl)adenosine (ms^2t^6A),[36] queuosine (Q),[34] and 3-(β-D-ribofuranosyl)-4,9-dihydro-4,6,7-trimethyl-9-oxoimidazo-[1,2-*a*]purine (mimG).[57] These examples emphasize the utility of full high-resolution mass spectra, in which exact masses of all ions are measured, which is usually the single most useful method for determination of nucleoside structure. Although high-resolution measurements can be made directly on enzymatic digests of tRNA[58] (and as well on mononucleotides), exact mass measurements on isolated nucleosides using EI are generally the most practical and accurate. Derivation of the correct elemental composition from high-resolution data is a key step because the heteroatom content of the base and its fragment ions will usually define the ring system (purine or pyrimidine), which, along with other inferences from structural features common to nucleic acids,[9] aids in reducing the number of structural candidates. Selection of likely nucleoside candidates from computer-derived elemental compositions is aided by the application of a set of simple structural rules, which include allowable rings and double bond values and minimum heteroatom content, to every computer-generated composition candidate.[25]

In unusual cases the number of possible elemental compositions derived from exact mass values may still be too large to permit unambiguous selection of the correct one. Such an example was encountered in the structure determination of the nucleoside ms^2t^6A (see Figure 5) which had been isolated from rabbit liver tRNA.[36] In this case it was necessary to characterize

(57) McCloskey, J. A.; Crain, P. F.; Edmonds, C. G.; Gupta, R.; Hashizume, T.; Phillipson, D. W.; Stetter, K. O. *Nucleic Acids Res.* **1987**, *15*, 683.

(58) Pang, H.; Smith, D. L.; Crain, P. F.; Yamaizumi, K.; Nishimura, S.; McCloskey, J. A. *Eur. J. Biochem.* **1982**, *127*, 459.

the nucleoside (2-(methylthio)adenosine) separately from the side chain (threonine) in order to recognize the unexpected presence of sulfur and to establish the correct overall elemental composition.

In rare but important instances, exact mass measurements will reveal unusual structural features that would otherwise be misassigned or would require substantially more material for characterization. The Q nucleoside, whose structure was reported in 1975,[34] is unprecedented in being the only known base in any nucleic acid that is neither a purine nor a pyrimidine.[59] On the basis of biological evidence, Q was thought to be a derivative of guanosine (G), and indeed, the elemental composition determined from high-resolution mass data showed the presence of five nitrogen atoms (as required for G), but revealed two unprecedented structural features. First, a number of fragment ions showed N_4, and not N_5, as part of the central structural unit, implying that the base moiety was not a simple purine, and second, the presence of a $C_5H_7O_2$ side chain not previously known in nucleic acids was indicated. Determination of the total structure, since confirmed by X-ray crystallography[60] and total synthesis,[61] required a scaled-up isolation for NMR measurements. Other interesting structures associated with the novel Q family include the following: the free base queuine[62] (which was unexpectedly discovered in amniotic fluid[63]); mannose and galactose derivatives of Q from eukaryotic tRNA;[64] and the epoxide oQ (structure in eq 3, Figure 1) found in *Escherichia coli* tRNA.[41]

The structure determination of the fluorescent tricyclic base-containing nucleoside mimG provides an example of the importance of known structural precedents in arriving at a structural candidate suitable for direct testing. mimG was discovered in the tRNA of the thermophilic microorganism *Sulfolobus solfataricus* by screening of unfractionated tRNA enzymatic hydrolysates by LC/MS, from which the mass spectrum revealed a molecular weight of 349, no apparent side chain, and unmodified ribose.[57] Following isolation of approximately 10 μg of nucleoside, high-resolution mass measurements made on the TMS derivative indicated a $C_{10}H_{10}N_5O$ base moiety, with two unusual features: eight rings and double bonds and no active hydrogen atoms. The number of possible heterocyclic structures that fit these data is potentially large, but is greatly reduced if the molecule is assumed from its composition to include a purine skeleton. Due to its fluorescent properties, the compound was recognized as a possible member of the wye (Y) family of tricyclic tRNA nucleosides which occur at position 37 of $tRNA^{Phe}$, of which five other members are known.[65] This inference (tricyclic base derived from guanine) further reduced the structural possibilities to a single likely candidate. Confirmation of the structure was made using 0.5 μg of nucleoside, by mild acid hydrolysis to liberate the base, and comparison with a sample of the synthetic base,[66] by GC/MS of the respective TMS derivatives. If volatile derivatives can be prepared, direct comparisons using capillary column GC and EI mass spectrometry in this manner constitute the single most useful test of identity at the submicrogram level.

Screening of Nucleic Acids for New Forms of Natural Modification

The discovery and characterization of new nucleosides from nucleic acids or other sources reflects issues common to other fields such as natural product chemistry, in which the components of potential interest must initially be distinguished from related (but structurally known) compounds and unrelated constituents, prior to isolation and further characterization. In the case of tRNA constituents, many of the common modified nucleosides were identified in early work after large-scale isolations from bulk nucleic acid.[67] However, most of the more highly modified and structurally interesting nucleosides were first recognized during the course of tRNA sequencing,[8] a procedure that is presently much less frequently performed due to reliance on sequencing of the corresponding gene (which is unmodified). In any event the nucleoside composition of a single sequenced tRNA species presents a very limited picture of nucleosides present in a total population of perhaps 40–50 different tRNAs, such as in the example shown in Figure 6. A powerful new approach to this problem has been provided by the development of thermospray LC/MS,[69] in which ions are formed by evaporation of droplets from a heated spray of the HPLC effluent. The tRNA nucleosides, oQ (Figure 1), mimG (Figure 5), and those shown in the inset of Figure 6, were initially discovered by LC/MS of enzymatic hydrolysates of tRNA. The identification of known nucleosides by LC/MS is based on (1) HPLC retention times, which for RNA nucleosides have been extensively cataloged; (2) UV absorbance characteristics, measured by using a UV detector placed in series between the liquid chromatograph and the mass spectrometer; and (3) mass spectra, recorded every 2–3 s during the full duration of a chromatographic run.[70] Due to the selectivity of the mass spectrometer as an HPLC detector, minor nucleoside components can be recognized and partly characterized even if they are chromatographically unresolved.

The screening of tRNA enzymatic digests in this manner[71] permits rigorous assignment of all known nucleosides in a single experiment and provides limited structure information on unknown components, which are then effectively targeted for isolation or other experiments. In favorable cases, sufficient evidence can

(59) For reviews of the occurrence, biosynthesis, and function of queuosine, see: (a) Nishimura, S. *Prog. Nucleic Acid Res. Mol. Biol.* **1983**, *28*, 49. (b) Kersten, H. *BioFactors* **1988**, *1*, 27.
(60) Yokoyama, S.; Miyazawa, T.; Iitaka, Y.; Yamaizumi, Z.; Kasai, H.; Nishimura, S. *Nature* **1979**, *282*, 107.
(61) Ohgi, T.; Kondo, T.; Goto, T. *Tetrahedron Lett.* **1977**, 4051.
(62) Crain, P. F.; Sethi, S. K.; Katze, J. R.; McCloskey, J. A. *J. Biol. Chem.* **1980**, *255*, 8405.
(63) Katze, J. R.; Basile, B.; McCloskey, J. A. *Science* **1982**, *216*, 55.
(64) Kasai, H.; Nakanishi, K.; Macfarlane, R. D.; Torgerson, D. F.; Ohashi, Z.; McCloskey, J. A.; Gross, H. J.; Nishimura, S. *J. Am. Chem. Soc.* **1976**, *98*, 5044.
(65) Dirheimer, G. *Recent Results Cancer Res.* **1983**, *84*, 1.
(66) Itaya, T.; Mizutani, A. *Tetrahedron Lett.* **1985**, 347.
(67) Hall, R. In *The Modified Nucleosides in Nucleic Acids*; Columbia University Press: New York, 1971.
(68) Edmonds, C. G.; Crain, P. F.; Gupta, R.; Hashizume, T.; Hocart, C. H.; Kowalak, J. A.; Pomerantz, S. C.; Stetter, K. O.; McCloskey, J. A. *J. Bacteriol.*, submitted.
(69) Yergey, A. L.; Edmonds, C. G.; Lewis, I. A. S.; Vestal, M. L. *Liquid Chromatography/Mass Spectrometry, Techniques and Applications*; Plenum: New York, 1990; Chapters 4 and 6.
(70) Edmonds, C. G.; Vestal, M. L.; McCloskey, J. A. *Nucleic Acids Res.* **1985**, *13*, 8197.
(71) Pomerantz, S. C.; McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 796.

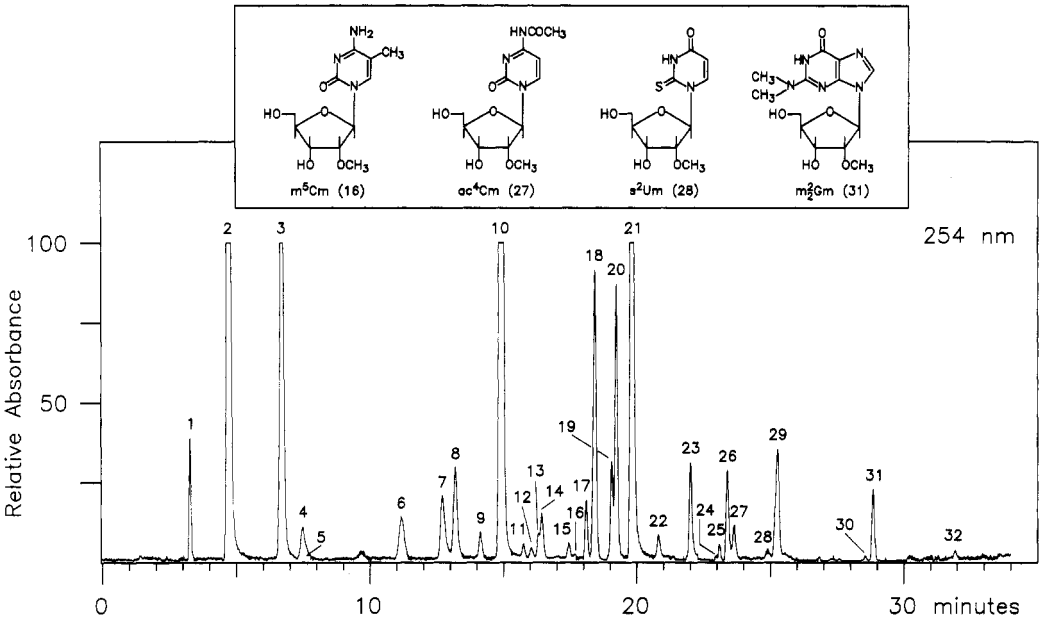

Figure 6. Reversed-phase chromatographic separation of nucleosides from LC/MS analysis of an enzymatic digest of 15 μg of unfractionated *T. neutrophilus* tRNA (adapted from ref 68); UV detection at 254 nm. Peak identities are as follows: 1, ψ; 2, C; 3, U; 4, $m^1\psi$; 5, dC; 6, m^5C; 7, m^1A; 8, Cm; 9, I; 10, G; 11, m^7G; 12, s^4U; 13, Um; 14, dG; 15, dT; 16, m^5Cm; 17, m^1I; 18, m^1G + Gm; 19, ac^4C; 20, m^2G; 21, A; 22, dA; 23, m_2^2G; 24, Am; 25, t^6A; 26, uncharacterized nucleoside; 27 , ac^4Cm; 28, s^2Um; 29, m^6A; 30, ms^2t^6A; 31, m_2^2Gm; 32, mimG. See ref 55 for nomenclature. Unnumbered peaks were shown from their mass spectra not to be nucleosides. Inset: new ribose-methylated nucleosides discovered by LC/MS in tRNA from organisms such as *T. neutrophilus* which grow at exceptionally high temperatures, up to 105 °C. Numbers refer to peaks in the chromatogram.

be gained solely from LC/MS data for a structural candidate to be proposed, synthesized, and tested without isolation of the nucleoside of interest. For example, N^4-acetyl-2′-O-methylcytidine (inset, Figure 6) was tentatively characterized (and later verified by synthesis) with a single mass spectrum from a hydrolysate of unfractionated *Thermoproteus neutrophilus* tRNA,[31] from two key features: loss of 42 u from both the MH^+ and BH_2^+ ions, assigned as the characteristic expulsion of ketene from *N*- or *O*-acetates; and the mass difference between MH^+ and BH_2^+ ions (146 u), requiring methylribosyl rather than the usual ribosyl loss (132 u) found in ribonucleoside mass spectra.

In the event that common side-chain substituents are encountered, they can usually be recognized by comparison with spectra from earlier studies on nucleoside models. For example, a 16 mass unit shift of the side-chain ion *m/z* 132 from the epoxide derivative oQ (structure in Figure 1), compared with the *m/z* 116 ion from nucleoside Q (structure, Figure 5), provided the initial evidence that oQ was related to Q by a difference of one oxygen atom in the side chain. This inference was confirmed by isolation of oQ using HPLC, followed by trimethylsilylation and measurement of exact molecular mass, 929.4348, which is 15.9984 u greater than that of Q (oxygen = 15.9949).[41]

The structures shown in Figure 6 are new nucleosides from tRNA, which were not known compounds, discovered by screening of enzymatic digests using LC/MS.[31,57] They are derived from a remarkable group of microorganisms that grow optimally at temperatures up to 105 °C.[72] These nucleosides are unusual in exhibiting modification both in the base and at O-2′ in the sugar. This structural feature is presumably related to the ability of the RNA in these organisms to retain extraordinary structural integrity at high temperatures, a topic under current investigation in our laboratory.

Emerging Trends

Recent advances in mass spectrometry instrumentation and in techniques for production and study of gaseous ions will continue to have considerable influence on methods available for structural studies of polar molecules, of which nucleosides are but one example. In this context, three areas are particularly noteworthy. The first of these is continuing refinements in the overall field of chromatography–mass spectrometry, now extended to include such methods as capillary electrophoresis–electrospray mass spectrometry[73] and microbore HPLC–FAB mass spectrometry.[74] Overall, the principal experimental obstacles to direct combinations of separation methods and mass spectrometry are twofold: problems associated with the pressure differential between the separation/ionization and mass analysis stages; and problems associated with involatile salts, present either in the sample or the separation system, which can cause a marked decrease in ionization efficiency.

A second area of importance is the *applications* of MS/MS to nucleoside structural problems, which have been notably few compared with the number of model studies that have demonstrated the utility of the method.[11] The suitability of MS/MS for the analysis of mixtures decreases as the number of components increases, so that chromatographic preseparation (as in LC/MS) becomes more practical, especially when isomers are present. A potential strength of MS/MS lies in applications to particularly complex nucleosides, such as antibiotics, where unusual structural elements may

(72) Stetter, K. O. In *Thermophiles: General, Molecular and Applied Microbiology*; Brock, T. D., Ed.; Wiley: New York, 1986; Chapter 3.

(73) Edmonds, C. G.; Loo, J. A.; Barinaga, C. J.; Udseth, H. R.; Smith, R. D. *J. Chromatogr.* **1989**, *474*, 21.

(74) Caprioli, R. M. *Methods Enzymol.* **1990**, *193*, 214.

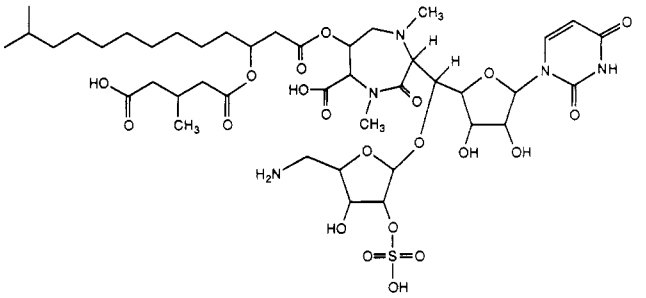

Figure 7. Liposidomycin B, a nucleoside antibiotic isolated from *Streptomyces* sp.

be encountered, and larger sample quantities can usually be isolated than in the case of nucleosides from nucleic acids. In such studies, CID has two main functions: to generate fragment ions which may bear structural information but which are not formed by spontaneous dissociation, and to establish the origins or decomposition pathways of fragment ions. For example, CID spectra of the complex nucleoside liposidomycin B (Figure 7) were used to establish the order in which structural subunits were lost by dissociation, thereby placing constraints on the interconnectivity of subunits.[32] Such information is complementary to that obtained from 2D NMR experiments, but requires less material. If a detailed CID map is determined for one member of a family of compounds, subsequent measurements made on related molecules can be used to rapidly assign sites of structural differences.

The recent discovery of methods for vaporization and ionization of very polar molecules has led to unforeseen results in which proteins in the range to 236 kilodaltons (kDa) and intact RNAs up to 25 kDa have been converted to solventless, gaseous ions in high yield and detected by mass spectrometry.[75,76] The exceptional polarity of polynucleotides, which is conferred by the polyphosphate backbone, has long constituted a significant obstacle to the applications of mass spectrometry to nucleic acids and to many biological problems that require work at the polymer, rather than nucleoside or monomer, level. It appears certain that this obstacle as such is rapidly diminishing, and a new generation of applications of mass spectrometry to nucleic acids will follow.

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(75) Hillenkamp, F.; Karas, M.; Ingendoh, A.; Stahl, B. In *Biological Mass Spectrometry*; Burlingame, A. L., McCloskey, J. A., Eds.; Elsevier: Amsterdam, 1990; p 49.
(76) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882.

Spin Control in Organic Molecules

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Magnetism has fascinated and served humanity for almost 3000 years.[1] Since the discovery of the lodestone ($FeO-Fe_2O_3$), many different magnetic materials have been developed, almost all based on transition metals and/or rare-earth elements. Technological application of magnetism also has a long history, from the compass to today's sophisticated magnetic memory systems. In contrast, the theory of magnetism has progressed more slowly, despite the efforts of great minds throughout history. The reason early theoretical models were not very valuable is now clear. Any viable theory of magnetism must be based on two inherently quantum mechanical concepts: electron spin and the Pauli exclusion principle. As such, only the 20th century has produced a competent model for magnetism, and that model continues to evolve. Studies over the last 20 years have revealed a bewildering array of new magnetic phenomena that continue to challenge our understanding of solid-state physics.[2]

In recent years, our group and others have embarked on a long-range program to prepare magnetic materials based on organic molecules and/or polymers. One can imagine that such materials would have fundamentally new properties that would provide valuable insights into the nature of magnetism. There may also be practical value in such materials. From our perspective, though, the use of molecular structures[3] in the design of new magnetic materials represents an intellectual challenge that defines a frontier of organic chemistry and needs no further justification.

Ultimately, the magnetic properties of any material are determined by the ways in which unpaired electrons in the material interact with each other. For example, a ferromagnet results if, over large regions of the material (domains), unpaired electrons are high-spin coupled (spins parallel) to each other. As with all forms of magnetism, ferromagnetism is strictly a solid-state phenomenon. There is no such thing as a ferromagnetic molecule. It is possible, however, for a condensed state of certain molecules to be ferromagnetic. The issue is spin control: the qualitative and quantitative aspects of spin-spin interactions among electrons. The present

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(1) For valuable introductions to magnetism, see: Mattis, D. C. *The Theory of Magnetism I*; Springer-Verlag: Berlin, 1981 (see especially Chapter 1 for a fascinating account of the history of magnetism). Carlin, R. L. *Magnetochemistry*; Springer-Verlag: Berlin, 1986.
(2) Hurd, C. M. *Contemp. Phys.* **1982**, *23*, 469-493.
(3) Most magnetic materials are not molecular solids. Rather, they involve extended structures in the solid state, such as seen in metallic, ionic, or network solids.

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