Recent Developments in Mass Spectrometry for the Characterization of Nucleosides, Nucleotides, Oligonucleotides, and Nucleic Acids

Joseph H. Banoub,*[,†,‡] Russell P. Newton,[§] Eddy Esmans,[||] David F. Ewing,[⊥] and Grahame Mackenzie[⊥]

Fisheries and Oceans Canada, Science Branch, Special Projects, P.O. Box 5667, St. John's NL A1C 5X1, Canada, Department of Biochemistry, Memorial University of Newfoundland, St. John's NL A1B 3X1, Canada, Biochemistry Group & Biomolecular Analysis Mass Spectrometry Facility, University of Wales Swansea, Wallace Building, Singleton Park, Swansea SA2 8PP, United Kingdom, Department of Chemistry, University of Antwerp, Nucleoside Research and Mass Spectrometry Unit, Groenenborgerlaan, 171, B-2020 Antwerp, Belgium, and Department of Chemistry, University of Hull, Hull HU6 7RX, United Kingdom

Received November 10, 2004

Contents

* To whom correspondence should be addressed. Phone: 709-772-4928. Fax: 709-772-5315. E-mail: banoubjo@dfo-mpo.gc.ca.
† Fisheries and Oceans Canada.
‡ Memorial University of Newfoundland.
§ University of Wales Swansea.
|| University of Antwerp.
⊥ University of Hull.

10.1021/cr030040w CCC: $53.50
Published on Web 04/23/2005

Joseph Banoub was born in 1947 and obtained his B.Sc. (Hons.) degree in 1969 from the University of Alexandria, Egypt. In 1976 he obtained his Ph.D. degree in Organic Chemistry from the University de Montreal, under the supervision of Stephen Hanessian. He spent 3 years as a Research Associate with MDS Health Group, Ottawa. In 1979 he moved to St. John's, Newfoundland, to take a position as a Research Scientist with the Federal Government of Canada, Department of Fisheries and Oceans. He is currently Senior Scientist in the Department of Fisheries and Oceans. He has held the appointment of Adjunct Professor, Department of Biochemistry, Memorial University of Newfoundland, since 1984. His research interests include tandem mass spectrometry of biologically active molecules.

Russ Newton is Professor of Biochemistry at the University of Swansea. A native of Birkenhead, Wirral, England, he obtained his B.Sc. degree in Biochemistry at the University of Liverpool in 1968 and also his Ph.D. degree in 1971 for studies of Vitamin E biochemistry in plants. He joined the University at Swansea as a postdoctoral researcher in 1971 and progressed through the ranks to be awarded a Personal Chair in 2000. In 1996 he was awarded a D.Sc. by the University of Liverpool for "an outstanding contribution to knowledge of cyclic nucleotide biochemistry"; he also has fellowships of the Royal Society of Chemistry and the Institute of Analysts and Programmers. In 2000 he was a Visiting Professor at the University of Antwerp, where he retains active and productive collaborations with Professors Eddy Esmans (Mass Spectrometry) and Harry van Onckelen (Plant Biochemistry). In addition to being Head of the Biochemistry Group at Swansea University he is Lead Director of the Biomolecular Analysis Mass Spectrometry Facility: major research interests are the biochemistry of second messengers in both plants and animals and the development and application of modern mass spectrometric methods in biomolecular analysis.

1. Introduction

Nucleic acids and their components play an important role in a variety of fundamental biological processes. Over the last century it has been shown that genetic information is used to make functional macromolecules: deoxyribonucleic acid (DNA) is the carrier of genetic information in all cells and many viruses[1] with the initial products of all genes being ribonucleic acids (RNAs). RNA is similar to DNA in that both are heteropolymers of heterocyclic bases; linked via a ribose moiety to a phosphodiester link, they differ in that D-ribose is the sugar glycone in RNA instead of 2-deoxy-D-ribose in DNA, the nucleobase uracil in RNA replaces thymine in DNA, while DNA is composed of twin, base-paired strands, and RNA is single stranded.

The elucidation of the molecular structure of DNA in 1953 by Watson and Crick[2] led to the so-called central dogma of molecular biology, that DNA makes RNA which makes proteins, better described as the flow of genetic information from gene to RNA to proteins. DNA is transcribed to form messenger RNA (mRNA) of complementary sequence and also directs its own replication. The sequence of nucleobases in the RNA is then translated to the corresponding sequence of amino acids to form a protein.[2] In addition to mRNA, two other RNA species, transfer (tRNA) and ribosomal (rRNA), are required for this translation process. Nucleic acid research has consequently had a most profound impact on molecular biology and initiated an avalanche of new disciplines.

Eddy Esmans is Professor of Organic Chemistry at the University of Antwerp. He was born in Berchem, a suburb of Antwerp, in 1947 and obtained his B.Sc. degree at the University of Antwerp in 1968 and his M.Sc. degree at the University of Ghent in 1970. He received his Ph.D. degree from the University of Antwerp on the synthesis of nucleoside analogues in 1976 and his Special doctor degree for studies on DLI LC/MS of nucleosides in 1986. He joined the University of Antwerp in 1976 and was appointed as an assistant. He was promoted to Research Leader in 1981 and Associate Professor in 1992. In 1996 he was appointed Full Professor at the Department of Chemistry. At this time he is Director of the Centre for Mass Spectrometry and Proteomics of the University of Antwerp together with Professor H. Van Onckelen. He is also Dean of the Faculty of Science. He has international collaborations with the groups of Professor Newton and Professor Törnqvist of the University of Stockholm, and his major interest lies in the field of mass spectrometry of biomolecules, miniaturized LC/MS, and method development and the detection of DNA adducts from in vivo samples

David F. Ewing received his B.Sc. and Ph.D. degrees from St. Andrews University, Scotland, and then spent 1 year in the Ottawa laboratories of the National Research Council of Canada. He held fellowships at the University of Hull, U.K., and then joined the staff there in 1967. His research interests have mainly involved synthesis of heterocyclic compounds and application of spectroscopic methods to the elucidation of structure. He has published ca. 130 papers and reviews in these areas. He recently formally retired but continues research collaboration with colleagues.

Grahame Mackenzie graduated with his B.Tech. and Ph.D. degrees at the University of Bradford. His postgraduate and postdoctoral research fellowship work was with Gordon Shaw at the University of Bradford and then with George Brown at the Sloan Kettering Cancer Research Institute, New York. He took an appointment as Senior Lecturer at the University of Lincoln and then moved to the University of Hull, first as Senior Lecturer and then as Reader in Bioorganic Chemistry. He has been a Visiting Professor at the University of Hokkaido, Japan, and CNRS Post Rouge, Director de Recherche at the Université de Lyon, France. Since 1992 he has been a Visiting Professor at the Université de Picardie, Jules Verne, France. He has published ca. 130 papers in the fields of carbohydrate and nitrogen heterocyclic chemistry, particularly in relation to the synthesis of nucleosides and glycolipids. More recently his work has focused on the chemistry of plant spore materials.

The field of genomics has evolved over the last few years, culminating in the sequencing and characterization of the entire human genome.[3] Current challenges include the unraveling of the relationship between the genome and the proteome: while the genome can be used to predict the potential proteome, all the proteins which a cell is capable of producing, the proteome is the protein complement actually expressed at a specific time, including all modifications carried out after the initial translation from mRNAs to protein. A key component of proteomics thus involves characterizing the regulation involved in progressing from gene to functional protein,[4] an area where mass spectrometry will have a major role.

The analysis of nucleic acids and derived products isolated from complex biological matrixes is synonymous with analysis in very low concentration. Thus, tremendous efforts have been made to develop and implement methodologies and mass spectrometric methods for the study of the monomeric constituents of nucleic acids (nucleobases, nucleosides, nucleotides, oligonucleotides, and derivatives).[4−7] Detailed fragmentation pathways were elucidated for the major ions present in both electron impact (EI) and chemical ionization (CI) mass spectra of simple purine and pyrimidine nucleosides by Biemann and McCloskey.[8−10] Derivatization, which is of value in many cases, requires an additional sample manipulation step and may produce unwanted and unexpected side products.[11−14]

Volatility restrictions for nucleic acid analysis have been circumvented by the use of desorption techniques which have allowed the production of quasimolecular ions of thermally fragile biomolecules. The hugely increased mass range made accessible by new ionization techniques has marked much of the progress in the field of mass spectrometry over the last two decades. The key ionization techniques include field desorption (FD),[252] Cf-plasma desorption (PD), desorption chemical ionization (DCI), secondary ion mass spectrometry (SIMS), and fast atom bombardment (FAB) with significant contributions being made by each of those methods.[15−25] Two further new soft ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), have made such an important contribution that they have resulted in the award of the Nobel Prize in Chemistry (2002) to John Fenn[26] (for ESI) and Koichi Tanaka[27] (for MALDI).

ESI is a well-established, robust technique when interfaced with liquid chromatography (LC/MS) or with similar techniques such as capillary electrophoresis (CE-MS).[28,29] It allows rapid, accurate, and sensitive analysis of a wide range of analytes from low-mass polar compounds (<200 Da) to biopolymers (>200 kDa).[30,31] A useful feature of ESI is its ability to generate multiply charged quasimolecular ions in the gas phase without fragmentation. These ions can have high mass, but the *m/z* values are sufficiently low to allow their analysis in quadrupole and quadrupole−ion-trap instruments, normally limited to mass ranges below 6000 Da for singly charged ions. It is an excellent means of characterization of DNA−oligonucleotides varying in size from around 70 residues[31] to DNA with masses greater than 10^6 Da.[32] Electrospray ionization appears to be almost unlimited in the size of molecules that can be ionized, and recent reports describe macromolecular ions with masses varying from 400 000 Da for polystyrene molecules to other polymer ions ranging up to 5 000 000 Da with nearly 5000 charges.[33] These megadalton macromolecules are now routinely analyzed in commercial mass spectrometers with a wide range of different analyzers that can be interfaced to an ESI source.

Recently, the development of novel mass analyzers has been especially important for biomolecule analysis. The performance characteristics of Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), triple-quadrupole mass spectrometry (TQ-MS), and quadrupole ion-trap mass spectrometry (QIT-MS) have been thoroughly reviewed by Yates[34] and McLuckey and Wells.[35] Over the past decade the MALDI technique has been coupled to time-of-flight mass spectrometry (MALDI-TOF-MS) as a potential tool to replace conventional gel electrophoresis for rapid DNA and RNA oligonucleotide sequencing and sizing studies.[36−38] For MALDI work samples are usually prepared by cocrystallization of the analyte with a large molar excess of a photoabsorbing organic compound. Laser desorption and ionization of the embedded analyte provide a relatively gentle method of generating large intact molecular ions in the gas phase.[39−42] The ions produced in this single desorption event are then separated and detected effectively by simple time-of-flight mass spectrometry. Therefore, MALDI-TOF-MS has the advantage of speed and accuracy for the mass determination of large biomolecules such as proteins, DNA, and RNA oligomers.[42,43] Structural information deduced from ion fragmentation is also important, and two different types of fragmentation events have been observed in MALDI-TOF mass spectrometers. The first is metastable decomposition occurring in the field-free region of the TOF flight tube. This process is called post-source decay (PSD).[41,42] The second type, the in-source collision-induced dissociation (CID), can be performed on MALDI-TOF-MS by employing higher potential differences in the source to induce energetic collisions. However, induction of fragmentation of ions prior to entering the flight tube decreases the potential for mixture analysis because individual components are dissociated prior to an ion selection stage.[34]

In addition to single-stage mass spectrometry, tandem mass spectrometry or mass spectrometry/mass spectrometry (MS/MS) has become a particularly important analytical application in the structural characterization of nucleobases, 2′-deoxynucleosides, and 2′-deoxynucleotides.[44a,b] The first example of an MS/MS of an oligonucleotide tetramer was reported by Sindona's group in 1983,[45] which also suggested the "zwitterionic structure" of gaseous oligonucleotides. Subsequently, DNA and RNA oligonucleotides sequences were easier to generate using tandem mass spectrometry,[35,46] where the oligonucleotide ions were selectively fragmented in a collision cell within the mass spectrometer. These fragmentations are primarily induced in collisions with neutral gas molecules. A multitude of reports have been published concerning the use of tandem mass spectrometry to unravel complete DNA sequencing information. These tandem mass spectrometric methods used several different kinetic MS/MS instruments which involved metastable kinetic ion decomposition (MIKE)[47] as well as low-energy[48] and high-energy collision-induced dissociation (CID) analyses of the deprotonated DNA anions, including sector instruments, and Fourier transform ion cyclotron mass spectrometry.[48−53]

Recently, there has been a plethora of new technological developments in high-resolution hybrid tandem mass spectrometry arising from the availability of new state-of-the-art instruments such as MALDI-TOF-TOF, MALDI-FTICR-MS^n, MALDI-quadrupole orthogonal time-of-flight (Q-TOF), MALDI-quadrupole ion-trap orthogonal time-of-flight (QIT-TOF), and quadrupole ion-trap-Fourier transform ion cyclotron (QIT-FTICR-MS).[44−46,54] These instruments combine the dual advantages of high sensitivity and the comprehensive fragmentation information provided by either high- or low-energy collision-induced dissociation tandem mass spectrometry.[23,53]

Intense interest in the mass spectrometric analysis of nucleosides, nucleotides, DNA, and RNA, especially in the past decade, has produced a wealth of published work, and it is not possible to provide comprehensive coverage of the relevant literature in this review. Our major objectives are to put into perspective the progress in current biotechnological research in the area, highlight the most popular ionization methods, especially when used in conjunction with tandem mass spectrometry, and illustrate the diversity of strategies employed in the characterization and sequencing of DNA and RNA oligomers, including nucleosides, nucleotides, and adducts of biomedical importance. Coverage includes some work from 2004.

2. Characterization of Nucleosides and Nucleotides by Mass Spectrometry

Nucleotides are the biosynthetic precursors and catabolites of oligomeric DNA and RNA and represent the primary level for the storage and transmission of genetic information. They not only are crucial to the organization and execution of protein synthesis[55] but also function as secondary messengers,

metabolic regulators, and components of vitamins and act as high-energy intermediates to drive thermodynamically unfavorable enzyme-catalyzed reactions. Two classes of heterocyclic nitrogenous bases are found in nucleic acids; the two purine derivatives, adenine and guanine, and the three pyrimidine derivatives, uracil, thymine, and cytosine, are the standard bases of RNA and DNA. Much effort has been devoted to separation, isolation, and purification of nucleic acids from biological samples as well as separation and identification of constituents of hydrolyzed RNA and DNA at the nucleotide, nucleoside, and nucleobase level.[56] Mass spectrometry has played a key role in this area, and elucidation of the fragmentations of simple nucleosides and nucleotides is a key step in developing an understanding of the fragmentations of the more complex oligonucleotides and nucleic acids. Seminal reviews by McCloskey and collaborators[57−59] have established the fundamental importance of MS techniques and applications to nucleic acids and related biotechnological fields.

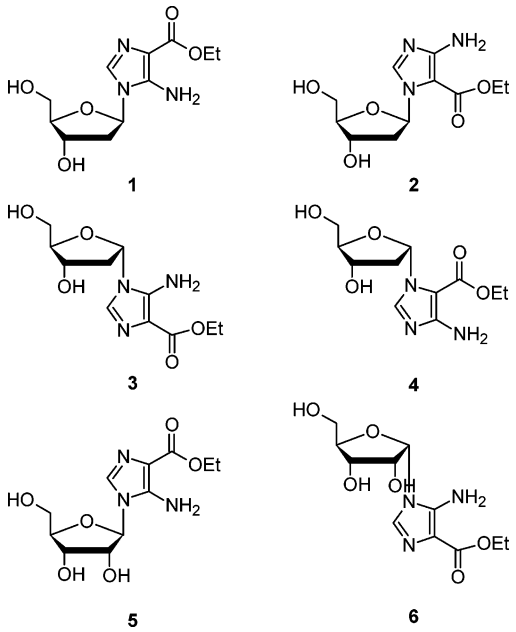
Nucleic acids and their constituents have long presented a considerable challenge for analysis by mass spectrometric techniques, owing to their polarity and thermal lability. Although mixtures of a variety of nucleobases and nucleosides can be analyzed by combined gas chromatography−mass spectrometry (GC-MS), it is usually necessary to convert to more volatile derivatives, by trimethylsilylation, for example. The GC-MS analysis of more complex nucleosides containing more polar modifications in the glycone or the nucleobase (aglycone) has not usually been successful.[60] However, the last two decades have seen the development of new ionization methods in mass spectrometry which allow the analysis of nucleosides, nucleotides, and related large oligonucleotides (DNA and RNA) without derivatization. In this section the past decade is reviewed with special emphasis on some unique nucleosides with important medicinal applications.

2.1. Fast Atom Bombardment MS and Tandem Mass Spectrometry

2.1.1. Ribofuranosyl and 2′-Deoxyribofuranosyl Imidazole Nucleosides and Related Species

It is well established that 5-aminoimidazole nucleosides are intermediates in the de novo biosynthesis of purine nucleosides and thus are of fundamental importance in all living systems.[61] Mackenzie and co-workers[62,63] synthesized a series of regioisomeric 2′-deoxynucleosides, ethyl 5-amino-1-(2-deoxy-β- and -α-D-erythropentofuranosyl)imidazole-4-carboxylate (β-N1 (**1**) and α-N1 (**2**), respectively) and ethyl 4-amino-1-(2-deoxy-β- and α-D-erythropentofuranosyl)imidazole-5-carboxylate (β-N3 (**3**) and α-N3 (**4**)), which are analogues of the pathway intermediate 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylic acid 5-*O*-phosphate (CAIR). The novel nucleosides, ethyl 5-amino-1-β- and -α-D-ribofuranosylimidazole-4-carboxylate (**5** and **6**, respectively) were also prepared as potential precursors of CAIR (Chart 1). For all species the fragmentation appears to involve the protonated molecules $[M + H]^+$ and fragmentation is initiated by the intramolecular

Chart 1



transfer of a hydrogen atom from the sugar glycone to the nucleobase which was originally postulated by Sindona's group.[64] This 1,2-elimination process, even if indirectly proven, has been applied in most publications dealing with nucleosides and oligonucleotide fragmentation.[65] This affords the $[BH_2]^+$ fragment ion (the base peak in the majority of cases) by cleavage of the glycone−nucleobase bond with charge retention on the glycone $[S]^+$ moiety as shown for the $[M + H]^+$ ion from **5** (Chart 2). Using positive-ion FAB tandem MS, high-energy MIKE and CID-MIKE, and

Chart 2

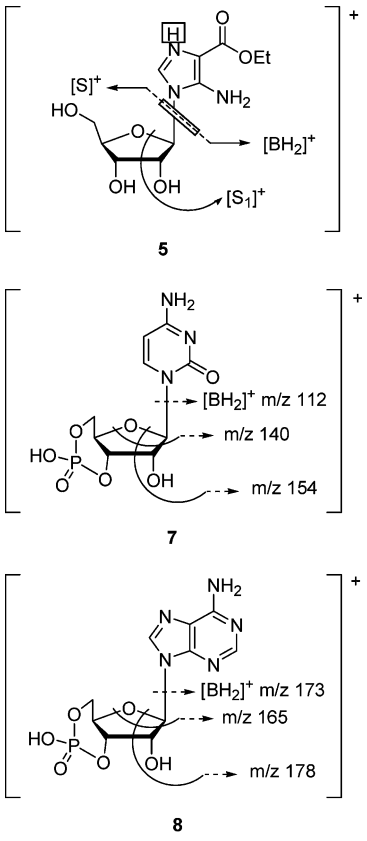


Table 1. Peak Intensities (%) in MIKE and CID-MIKE Product Ion Spectra of the Protonated Molecules $[M + H]^+$ at *m/z* 272 for Imidazole Nucleosides 1−4

		MIKE				CID-MIKE			
product ions	*m/z*	**1**(β-N_1)	**2**(β-N_3)	**3**(α-N_1)	**4**(α-N_3)	**1**(β-N_1)	**2**(β-N_3)	**3**(α-N_1)	**4**(α-N_3)
$[MH - NH_2]^+$	256			7	12			4	
$[MH - NH_2 - H_2O]^+$	238			13	4			10	
$[MH - EtOH]^+$	226	1	4		1	3	8	6	4
$[MH - 90]^+$	182					6	4	9	7
$[BH_2]^+$	156	100	100	100	100	100	100	100	100
$[MH - 90 - EtOH]^+$	136					2		6	
$[S]^+$	117					6		9	4
$[BH_2 - EtOH]^+$	110					9		11	13

low-energy CID-MS/MS (MS/MS hybrid) spectra of the protonated molecular ions $[M + H]^+$ were recorded for the whole series. The main product ions obtained from the MIKE and CID-MIKE analysis of the protonated molecules $[M + H]^+$ are indicated in Table 1. It is evident that each of the nucleoside analogues **1**−**4** can be distinguished by these spectra.[64]

FAB-MIKE and FAB-CID-MIKE spectra have also been applied[66,67] to the characterization of several ribosylamine derivatives, precursor species in the formation of bredinin, a nucleoside with immunosuppressive activity.[68]

2.1.2. Cyclic and Modified Nucleotides

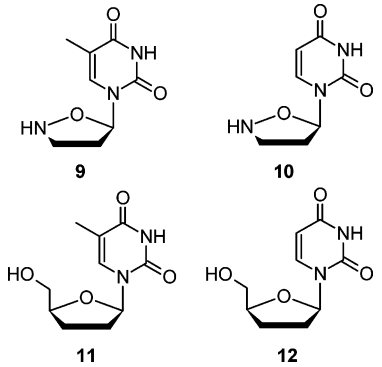
Newton and co-workers[69] investigated the presence of cyclic nucleotides in the culture medium of *Corynebacterium murisepticum* by FAB-MS/MS. Earlier reports had suggested that the culture media contained cytidine-3′,5′-cyclophosphate (**7**) and inosine-3′,5′-cyclophosphate (**8**). Using a BE sector instrument with collisionally induced dissociation/mass-analyzed ion kinetic energy spectrum scanning, the presence of **7** and **8** was confirmed and the corresponding uridine, adenosine, and guanosine cyclonucleosides were also identified unambiguously. These nucleotides were found both within the bacterial cells and in the growth medium. The fragmentation of the $[M + H]^+$ ion obtained by FAB-MS/MS was similar for each of this series of cyclonucleosides and is illustrated for **7** and **8** in Chart 2.[69]

Takeda et al.[70] made a comparison of ribonucleosides in urine and serum from uremic and healthy subjects. Using LC-frit-FAB-MS 20 nucleosides were detected in serum and 23 in urine. Most of the detected species were identified as breakdown products of tRNA on the basis of the $[M + H]^+$ and $[BH_2]^+$ ions. The levels of some nucleosides were higher in uremic subjects, but the main difference between the two groups was the presence of metabolites of the drug allopurinol used for uremia therapy.

2.1.3. Isoxazolidinyl Nucleobases

Recently, Sindona and co-workers[71] investigated the synthesis of the isoxazolidinyl nucleosides by the 1,3-dipolar cycloaddition of nitrones to vinylnucleosides. They obtained 1-(isoxazolidin-5-yl)thymine (AdT) (**9**) and the analogous uracil (AdU) (**10**), which are analogues of 3′-deoxythymidine (ddT) (**11**) and 2′,3′-dideoxyuridine (ddU) (**12**) (Chart 3). AdT and AdU are representative of a new class of antiviral

Chart 3



agent and were characterized by FAB-MS/MS using a V6-ZAB-2F multisector instrument. The tandem mass spectra of **9** and **10** were similar to those of the dideoxyribose nucleosides, displaying fragment ions corresponding to $[BH_2]^+$ and $[M - BH]^+$ species. They also show a $[B + 27]^+$ species which appears to be formed from a retrocycloaddition process, which was

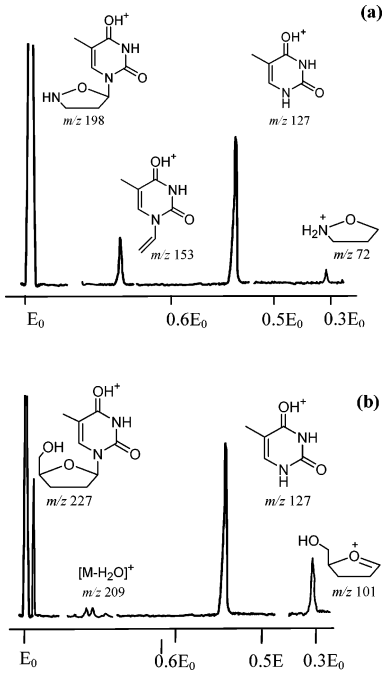


Figure 1. FAB tandem mass spectra (MIKE) of (a) the $[M + H]^+$ species from AdT (**9**) and (b) the $[M + H]^+$ species from ddT (**11**). Glycerol was used as the matrix, and spectra were recorded in the absence of collision gas in the second field-free region of a B−E sector instrument. (Adapted with permission from ref 71. Copyright 2001 John Wiley and Sons, Ltd.)

obviously less evident in the dideoxyribose nucleosides (Figure 1). This behavior appeared to be consistent with protonation of the analytes at the pyrimidine rings. Model isoxazolidines, in which the nucleobase was replaced by a phenyl or a naphthyl moiety, displayed the expected behavior of species with a localized charge on the N−O moiety of the isoxazolidine ring.[71]

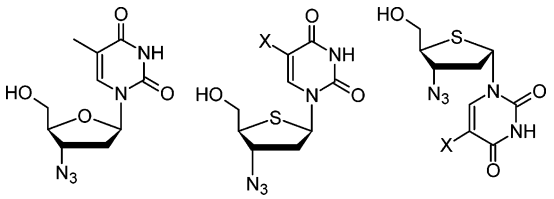
2.2. Electrospray Ionization MS and Tandem Mass Spectrometry

2.2.1. Antiviral Nucleosides and Nucleotides

The human immunodeficiency virus (HIV) has been identified as the etiological agent of acquired immunodeficiency syndrome (AIDS).[72] Nucleoside analogues such as AZT (**13**) and other 2′,3′-dideoxynucleosides are potent inhibitors of this retrovirus in vitro and in vivo.[73−75] Consequently, there is an ongoing search for novel nucleosides which are effective against the AIDS virus;[75] systematic structure−reactivity relationship studies of AZT analogues have shown a pronounced enhancement of the anti-HIV activity when either the 5-position of the pyrimidine ring is substituted by halogens such as Br or I or the O-4′ atom has been replaced by either a methylene group or a sulfur atom.[76,77]

Banoub and co-workers reported[78] the use of electrospray MS for structural characterization of AZT and a novel series of 3′-azido-2′,3′,4′-trideoxy-5-halogeno-4′-thio-β-D-uridine nucleosides and their respective α-anomers (**14**−**19**) (Chart 4). Low-energy

Chart 4



CID-MS/MS analysis of the protonated molecules $[M + H]^+$ confirmed the predicted fragmentation route for AZT and the series of uridines and also provided characteristic fingerprint patterns which permitted differentiation of anomers. The mass spectra of the anomeric pair **14** and **17**, obtained from the protonated molecule $[M + H]^+$ at *m/z* 348, are summarized in Table 2, illustrating the clear differentiation between the anomers. The variation in relative abundance of the same product ions reflects differences in chemical free energy due to stereochemical effects.[78]

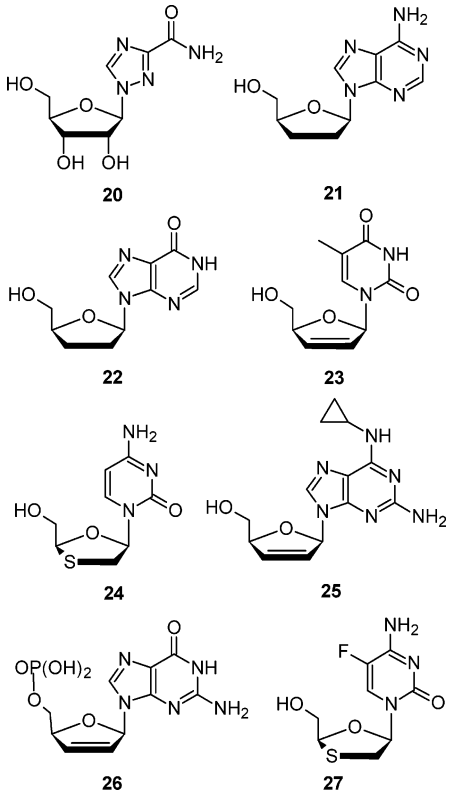
Ribavirin (**20**) (Chart 5) is a synthetic nucleoside with a broad spectrum of antiviral activity. Recently, ribavirin, in combination with pegylated interferons α-2b and α-2a, has been used successfully for the treatment of viral Hepatitis C.[79] Shou and collaborators[80] developed an ESI-CID-MS/MS method for the analysis of ribavirin in human plasma and serum

Table 2. Positive Electrospray Mass Spectra of the β- and α-Anomers of 3′-Azido-5-bromo-2′,3′,4′-trideoxy-4′-thiouridine (14 and 17)

type of ion[a]	*m/z*	β-anomer	α-anomer
$[2M_1 + H]^+$	699	31.7	33.9
$[M_1 + M_2 + H]^+$	697	50.7	56.2
$[2M_2 + H]^+$	695	26.9	31.2
$[699 - 81]^+$, $[697 - 79]^+$	618	10.8	15.0
$[697 - 81]^+$, $[695 - 79]^+$	616	9.1	10.1
$[M_1 + NH_4]^+$	367	12.5	7.1
$[M_2 + NH_4]^+$	365	7.5	6.3
$[M_1 + H]^+$	350	100	100
$[M_2 + H]^+$	348	96.8	93.0
$[M + H - Br]^+$	269	28.2	27.0

[a] $M_1 = C_9H_{10}N_5O_3S^{81}Br$. $M_2 = C_9H_{10}N_5O_3S^{79}Br$.

Chart 5



using multiple reaction monitoring (MRM) of the MS/MS transition $[M + H]^+$ at *m/z* 245 → $[BH_2]^+$ at *m/z* 113.

Capillary (zone) electrophoresis coupled with ESI-MS/MS has been shown by a French group[81−83] to be an excellent technique for the separation and quantification of various anti-HIV drugs and their metabolites in the presence of the common nucleosides. Protocols were developed within this methodology to estimate 2′,3′-dideoxyadenosine (ddA, **21**) a metabolite of didanosine (ddI, **22**),[81] and produce the simultaneous detection of two or more anti-HIV nucleosides, such as stavudine (d4T, **23**) and zidovudine (**13**) or lamivudine (**24**) and ddA, all potent HIV reverse transcriptase inhibitors (RTIs) often used in combination therapy.[82,83] The CID tandem mass spectrometry used in this method relied on the MRM analysis of the $[BH_2]^+$ ion; typical transitions include for ddI ($C_{10}H_{12}O_3N_4$, 236), $[M + H]^+$ at *m/z* 237 → $[BH_2]^+$ at *m/z* 137; for d4T ($C_{10}H_{20}O_4N_2$, 224), $[M + H]^+$ at *m/z*

Table 3. Electrospray Mass Spectra[a] of the Four Stereoisomers of 1-(3-Benzoyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)uracil (30−33)

characteristic ions	*m/z*	**30** (1*S*,3*R*)	**31** (1*R*,3*S*)	**32** (1*R*,3*R*)	**33** (1*S*,3*S*)
$[2M + Na]^+$	751	8.8	9.4	9.4	9.1
$[2M + H]^+$	729	9.5	10.1	26.4	22.3
$[2M + H - BH - PhCO_2H]^+$	495	9.4	2.9	2.9	(-)
$[M + Na]^+$	387	12.3	20.1	14.1	19
$[M + H]^+$	365	52.3	15.3	15.2	11.2
$[S]^+$	253	100	54	56.4	95.2
$[S - PhCO_2H]^+$	131	100	100	100	100

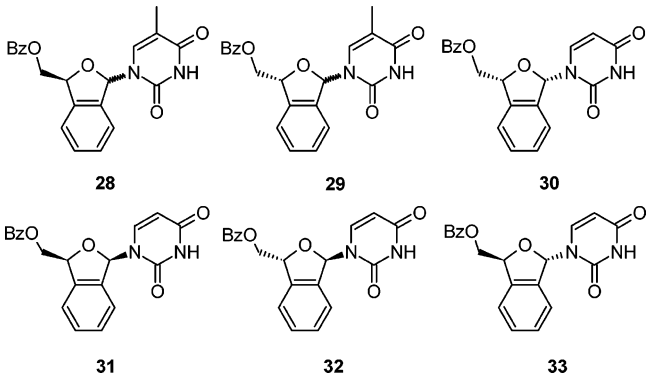
[a] Recorded with a cone voltage of 20 V.

225 → $[BH_2]^+$ at *m/z* 127; for ddA ($C_{10}H_{13}O_2N_5$, 235), $[M + H]^+$ at *m/z* 236 → $[BH_2]^+$ at *m/z* 136. The limit of detection is 20 ppb for d4T and in the range 2−5 ppb for the other analytes.[83] Benech and colleagues[84,85] used LC/MS/MS with MRM as an improved method for the simultaneous determination of the intracellular phosphorylated anabolites of d4T, 3TC, and ddI in human peripheral blood mononuclear cells of HIV-positive patients. This method was developed in order to satisfy the constant need for direct determination (i.e., without dephosphorylation) of these RTIs. The developed MRM method can be applied to routine analysis of clinical samples from HIV-positive patients receiving antiretroviral therapy containing d4T, ddI, and/or lamivudine 3TC.[82]

Ziagen (abacavir) (**25**) is a carbocyclic nucleoside analogue possessing in vitro activity and in vivo efficacy against HIV.[86−88] Fung and co-workers[89] reported an ion-pairing MS/MS method using positive-ion mode electrospray ionization to simultaneously quantify ziagen and three metabolites, carbovir monophosphate (**26**) and the corresponding di- and triphosphates. *N*,*N*-Dimethylhexylamine (DMHA) was used as the ion-pairing agent, allowing excellent separation of the four compounds by reverse-phase HPLC. This methodology has been successfully applied to the analysis of these compounds in human liver cells treated with ziagen.[89] The same four compounds were also analyzed simultaneously in cellular extracts of human peripheral blood mononuclear cells (PBMCs) by a capillary electrophoresis-ion-trap MS method with a detection limit of <2 μM for all analytes.[90] Ion-pair HPLC was also applied[91] using a mobile phase containing 10 mM ammonium phosphate with 2 mM tetrabutylammonium hydroxide and 15% acetonitrile to the analysis of intracellular triphosphates using ESI-CID-MS/MS, in particular, the metabolites of the antiviral agent ((2*R*,5*S*)-5-fluoro-1-[2-(hydroxymethyl-1,3-oxathiolan-5-yl]cytosine (**27**) in human PBMCs.

It is well established that nucleoside analogues possessing conformational rigidity, imposed by locking the conformation of the furanose (or carbocyclic) ring by the introduction of a second ring, are of great interest as potential RTIs. Efficient routes for the synthesis of d4T analogues of uracil, thymine, and cytosine based on a 1,3-dihydrobenzo[*c*]furan glycone core have been described by Mackenzie and coworkers.[92−95] These systems, aromatic analogues of the well-known antiviral 2′,3′-dideoxy-2′,3′-didehydronucleosides, contain two chiral centers (C-1′ and C-3′, Chart 6) corresponding to the α/β and D/L centers in a furanose sugar. The asymmetric synthesis protocol provided compounds which were stereochemically pure at the C-3′ site and a 1:1 mixture of both stereoisomers at the C-1′ site. Final resolution of each pair of diastereoisomers was easily achieved chromatographically to afford the two diastereoisomeric mixtures of 1-(3-benzoyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)thymine, i.e., **28** [(1′*R*,3′*S*) and (1′*S*,3′*S*)] and **29** [(1′*R*,3′*R*) and (1′*S*,3′*R*)], and the four optically pure stereoisomers of the analogous uracil (1′*S*,3′*R*) **30**, (1′*R*,3′*S*) **31**, (1′*S*,3′*S*) **32,** and (1′*R*,3′*R*) **33**.[95]

Chart 6

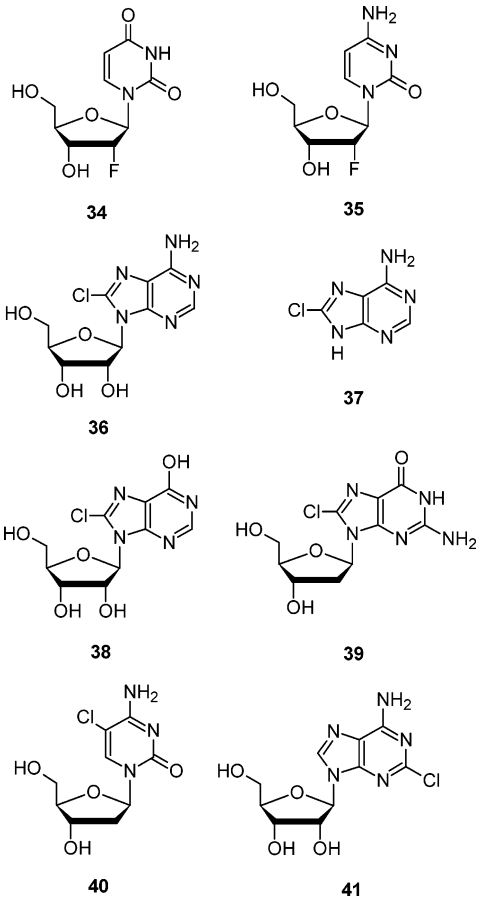


Low-energy collision tandem mass spectrometric analysis of the various protonated molecules $[M + H]^+$ and cluster ions such as $[M + Na]^+$, $[2M + H]^+$, and $[2M + Na]^+$ confirmed the characteristic fingerprint pattern obtained in the conventional electrospray spectra.[96] In the tandem mass spectra obtained for the precursor ion $[M + H]^+$ at *m/z* 365 isolated from nucleosides **28** and **29** the differences between the product-ion intensities within each enantiomeric pair were, as expected, not significant. However, the product-ion scan spectra obtained for the $[2M + Na]^+$, $[2M + H]^+$, and $[M + Na]^+$ ions at *m/z* 751, 729, and 387, respectively, obtained from the four pure enantiomers **30**−**33** showed discriminating differences in fingerprint ion intensities in each enantiomeric pair. This reflects differences in the tertiary structure of the precursor molecular ions arising in inequalities in chemical free energy and differences in the proton and sodium complexation of the nucleobase as influenced by the molecular stereochemistry. For example, Table 3 shows the differences in the product-ion scans obtained for the $[2M + Na]^+$ obtained for the four enantiomers **30**−**33**.[96]

2.2.2. Analysis of Halogenonucleosides

Nucleoside analogues 2′-deoxy-2′-fluorouridine (2′-FU; **34**) and 2′-deoxy-2′-fluorocytidine (2′-FC; **35**)

Chart 7



(Chart 7) have been used in the development of therapeutic aptamers. It is important to understand the biological fate of these nucleoside analogues in order to evaluate and increase their efficacy as aptamers in vivo by reducing their degradation half-life by endogenous nucleases. Richardson and co-workers[97] developed an ESI-MS/MS method to detect and quantify the nucleosides 2′-FU and 2′-FC in enzymatically hydrolyzed DNA, and using this methodology they have shown that both nucleosides are incorporated into the DNA of rats following a chronic treatment program.

The nucleoside 8-chloroadenosine (**36**) is currently being used for treatment of multiple myeloma and leukemias. Although accumulation of the phosphorylated drug product is known to occur within cell lines, its metabolic fate in plasma or circulating cells in animals is unclear. To study this Newman and collaborators[98] developed an ESI-MS method that permitted simultaneous determination of **36** and its metabolites, 8-chloroadenine (**37**) and 8-chloroinosine (**38**). The MS/MS spectra of the protonated molecules $[M + H]^+$ at *m/z* 303 and 302 were used to identify **36** and **38,** while MS/MS of the $[BH_2]^+$ ion at *m/z* 170 allowed the identification of **37**. Recently, Masuda et al.[99] investigated the reaction of 2′-deoxynucleosides with HOCl. Products such as 8-chloro-2′-deoxyguanosine (**39**) and 5-chloro-2′-deoxycytidine (**40**) were detected and quantified by ESI-MS/MS. Exposure of isolated calf thymus DNA or calf liver RNA to HOCl produces several chlorinated nucleosides including **39**, a reaction which is dramatically enhanced by the presence of nicotine. Since it is known that myeloperoxidase generates HOCl,[100] this work may indicate an important pathophysiological effect associated with tobacco habits. The stability of adenosine and two synthetic chlorine-containing analogues, 2-chloroadenosine (**41**) and 5′-chloro-5′-deoxyadenosine, has also been studied as a function of pH and temperature using HPLC/ESI-MS.[101]

2.2.3. Simultaneous Analysis of Mono-, Di-, and Triphosphates

Esmans et al.[102] developed a novel LC/MS method using ESI-MS (negative-ion mode) for the analysis of complex mixtures of nucleoside mono-, di-, and triphosphates. A short capillary column operated under ion-pair high-performance liquid chromatography conditions was hyphenated to a Q-TOF-MS/MS hybrid instrument. Separation of 12 nucleotides (the three phosphates of the four common DNA nucleosides) was achieved by a binary gradient elution using CH_3OH/H_2O and DMHA as ion-pairing agent. The influence of ion-pair concentration on the chromatographic and mass spectrometric performance was evaluated to establish optimal LC/MS conditions. Very recently ion-pair chromatography (with DMHA) coupled to positive-ion ESI-MS has been applied to the simultaneous analysis of AMP, ADP, and ATP in extracts from rat C6 glioma cells.[103]

2.2.4. Characterization of Urinary Nucleosides

Newton and his group[104−107] attempted to find an optimum combination of chromatography coupled with MS for the analysis of urinary nucleosides including modified nucleosides which may be markers for cancer and other diseases. They compared GC-MS, HPLC quadrupole ion-trap mass spectrometry (HPLC/QIT-MS), and capillary liquid chromatography/triple-quadrupole mass spectrometry for the qualitative and quantitative analyses of urinary nucleosides. It was concluded that all three methods were valuable techniques and that the systematic study of urinary nucleoside profiles in cancer patients is best served by a combination of analytical procedures.[104] The best ionization conditions for HPLC/MS were determined and fragmentation profiles further investigated in the positive mode since that gave a better quasi-molecular ion intensity.[105] Seventeen modified nucleosides have been identified by MS as possible tumor markers[107] including 5′-deoxycytidine, previously unknown in mammals.[106]

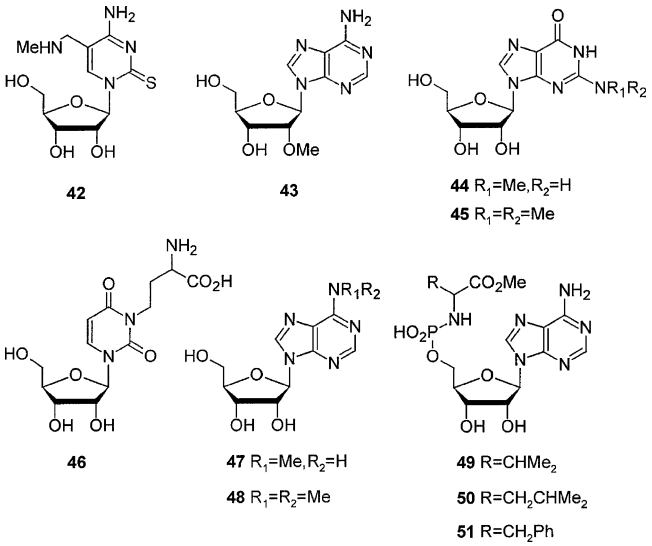
Very recently Cheung and co-workers[108] reported the analysis of nucleosides in urine by LC/ESI-MS using direct sample injection (no extraction). This methodology appears to have great potential for the detection of urinary nucleosides down to 0.2 nmol mL^{-1}.

2.3. Posttranscriptional Modifications of RNAs

2.3.1. Modifications of Archeal RNAs

A detailed study of several species of *Methanococci* (a lineage of methanogenic marine euryarchaea that grows over an unusual temperature range) has been reported by McCloskey and collaborators[109] to try to

Chart 8



establish whether posttranscriptional modifications of tRNAs reflect phylogenetic relationships. The tRNAs from *Methanococci vannielii*, *Methanococci maripaludis*, the thermophile *Methanococci thermolithotrophicus,* and hyperthermophiles *Methanococci jannaschii* and *Methanococci igneus* were digested and analyzed by LC/ESI-MS, revealing up to 24 modified nucleosides including the complex tricyclic nucleoside wyosine, characteristic of position 37 in $tRNA^{Phe}$ and two further members of this family of unknown structure. The hypermodified nucleoside 5-methylaminomethyl-2-thiouridine (**42**) (Chart 8), reported previously only in bacterial tRNA, was identified by LC/ESI-MS in four of the five organisms. The methylated nucleosides, 2′-*O*-methyladenosine (**43**), N^2,2′-*O*-dimethylguanosine (**44**), and N^2,N^2,2′-*O*-trimethylguanosine (**45**), were found only in hyperthermophile tRNA, consistent with their proposed roles in thermal stabilization of tRNA.[109] An important facet of these studies has been the greater accuracy in nucleoside assignments resulting from the combined use of HPLC relative retention times and ESI-MS.[110]

2.3.2. Modifications of 16S Ribosomal RNA from Haloferax volcanii

McCloskey and co-workers[111] reported an investigation of the modified sites in a small subunit (16S) rRNA (smrRNA) of *Haloferax volcanii*. Following total digest a census of all modified residues was obtained by LC/ESI-MS. Accurate molecular mass values of oligonucleotide products were obtained following rRNA hydrolysis by RNase T_1 and compared with values predicted from the corresponding gene sequence. Three modified nucleosides were distributed over four conserved sites in the decoding region of the molecule. These nucleosides were characterized as 3-(3-amino-3-carboxypropyl)uridine (acp^3U; **46**), N^6-methyladenosine-1501 (m^6U; **47**), and N^6,N^6-dimethyladenosine ($m_2{}^6U$; **48**), and the located sites are shown in Table 4. Nucleoside **46**, previously unknown in rRNA, occurred at a highly conserved site of modification in all three evolutionary domains

Table 4. Oligonucleotides[a] from smrRNA of *H. volcanii* Containing Modified Nucleosides Inferred from MS Data

molecular mass/Da	sequence[b]
2673.8	964-AC**acp**^{3}**U**CAACGp-971
2305.5	1498-UA**m**^{6}**A**CAAGp-1504
1995.3	1518-**m**$_2{}^6$**Am**$_2{}^6$**A**UCUGp-1523

[a] RNase T_1 hydrolysis. [b] *E. coli* numbering in conjunction with 16s rRNA gene sequence. Modified nucleotides are in bold.

for which no structural assignment in archaea has been reported. It was also shown that nucleoside **47**, not previously identified in archaeal rRNAs, frequently occurred at the analogous location in eukaryotic smrRNA. The authors[111] concluded that *H. volcanii* smrRNA appeared to reflect the phenotypically low modification level in the *Crenarchaeota* kingdom and is the only cytoplasmic smrRNA shown to lack pseudouridine.[112]

Although pseudouridine is a 'mass-silent' posttranscriptional modification of tRNA, it can be detected by cyanoethylation with acetonitrile with a consequential mass increment of 53 Da.[113] This methodology was used to identify a 4-thiouridine and a pseudouridine in $tRNA^{TyrII}$ from *E. coli.*

2.3.3. Characterization of Phosphoramidates, Modified Nucleosides, and Oligonucleotides

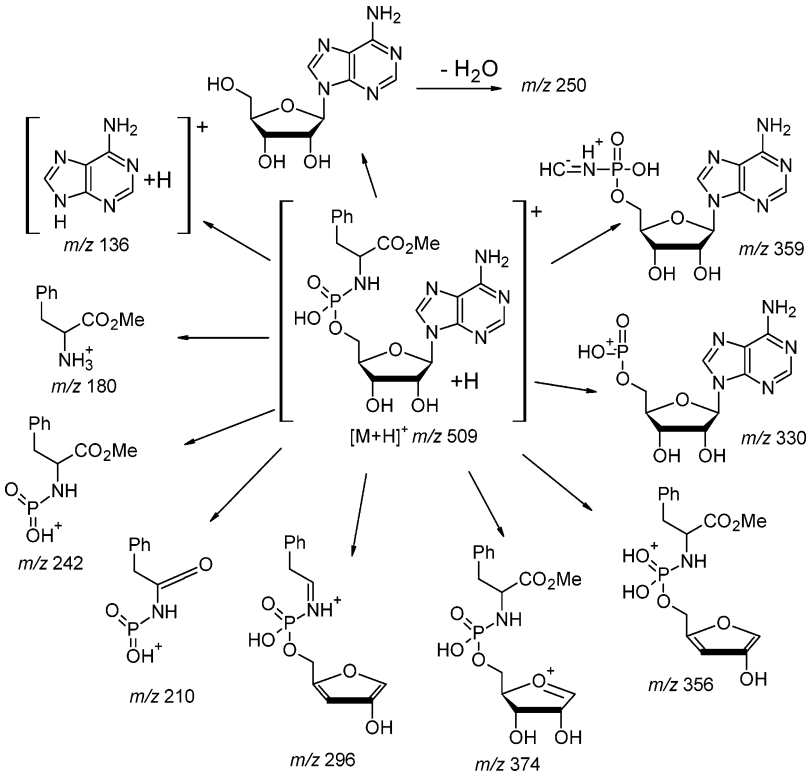
Prakash et al.[114,115] developed a versatile synthetic route for the synthesis of purine and pyrimidine nucleosides modified as the 2′-*O*-{2-[(*N*,*N*-dimethylamino)oxy]ethyl} derivatives. The synthetic oligonucleotides with this 2′-O modification were characterized by HPLC, capillary gel electrophoresis, and ESI-MS. They found that this modification enhanced the binding affinity of the oligonucleotides for the complementary RNA but not for DNA and produced excellent nuclease stability.

The structure of amino acid phosphoramidates of adenosine (**49**−**51**) has been established[116] by (positive and negative ion) ESI-MS and tandem mass spectrometry. Investigation of the fragmentation pathways indicated that ESI-MS (in both ion modes) gave product ions corresponding to losses of water, adenine, adenosine, and amino acids. Losses of the methyl esters and methyl alcohol were also observed. In addition, amino acid side chain cleavage and cleavage of the C−N bond between adenine and sugar were noted. Scheme 1 summarizes the fragmentation pattern for compound **51** and confirms that MS/MS is an excellent tool for structural determination of amino acid phosphoramidates.

2.3.4. Identification of Monophosphate Nucleotides from Champagne Wine

In the food industry mononucleotides, particularly 5′-nucleotides such as guanosine-5′-monophosphate (5′-GMP) and inosine-5′-monophosphate (5′-IMP), have been used as flavoring ingredients and can be produced by enzymatic RNA degradation during yeast autolysis.[117,118] RNA represents more than 95% of the total nucleic acid content of yeast cells[119] and

Scheme 1. Fragmentation Pattern For Amino Acid Phosphoramidate 70c



is known to degrade more than DNA during autolysis.[120] One aspect of the traditional production of Champagne is the long aging on lees during which yeast autolysis occurs,[121] releasing intracellular yeast constituents, such as the degradation products of nucleic acids, into the wine.[122] Formation of such nucleotides in Champagne wine will clearly affect its quality.[123,124] Aussenac and co-workers[125] established a method for the isolation, separation, and identification of monophosphate nucleotides and applied it to analyze Champagne wine aged on lees for 8 years. ESI-MS permitted the identification and analysis for the first time in Champagne wine of 5′-IMP, 5′-AMP, 5′-CMP, 5′-GMP, 5′-UMP, and the 3′- and/or 2′-isomers.

2.3.5. Selective Detection of Ribose-Methylated Nucleotides in RNA

The posttranscriptional methylation of ribose at position O-2′ is one of the most common and conserved types of RNA modification. The detailed functional roles of this type of modification are not as simple as originally thought, and because large numbers of this type of modified nucleotide occur in eukaryotic rRNAs (around 65 in human 28S rRNA, for example), there is much interest in their detection and location. Classical methods of mapping methylated ribose nucleotide suffer from experimental difficulties, which increase with the RNA molecular size, and with the complexity of mixtures resulting from nuclease digestion. Recently, Qiu and McCloskey[126] described a new and relatively rapid approach based on MS/MS using any of the four ion reaction pathways occurring in the mass spectrometer (Figure 2). The observed reaction pathways could be monitored and were highly specific for the presence of 2′-*O*-methylribose residues originating from further dissociation of ribose-methylated mononucleotide ions formed in the electrospray ionization region of the mass spectrometer. The instrument could be set for the detection of generic ribose methylation in oligonucleotides, selectively for each of the common methylated nucleosides Cm, Gm, Am, or Um, or for specific cases in which the base or sugar was further modified. An example of this MS/MS methodology is the detection of N^4,*O*-2′-dimethylcytidine in *E. coli* 16S rRNA (1542 nt) using 25 pmol of an RNase T1 digest.[126]

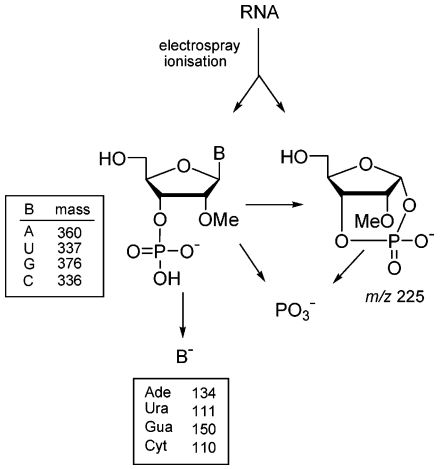


Figure 2. Ion dissociation pathways which can be used for the detection of 2′-O-methylation by tandem mass spectrometry. ESI of oligonucleotides produces diagnostic methylated nucleotide monophosphates and the cyclophosphate *m/z* 225, characteristic of generic methylation. (Adapted with permission from ref 216. Copyright 1999 Oxford University Press.)

2.3.6. Determination of Nucleosides in Rat Brain Microdialysates

Zhu et al.[127] reported an LC/MS/MS method for the determination of brain basal nucleosides (inosine, guanosine, and adenosine) in microdialysates from the striatum and cortex of freely moving rats. A microdialysis probe was surgically implanted into the striatum or cortex of individual rats; then Ringer's solution was used as the perfusion medium at a flow rate of 0.3 or 0.5 μL min^{-1}, directly analyzed by off-line LC/MS/MS experiments using multiple reaction monitoring. Analytes were detected by ESI-MS/MS in the positive-ion mode using MRM for the following MS/MS transitions: for inosine (*m/z* 269 → 137), guanosine (*m/z* 284 → 152), and adenosine (*m/z* 268 → 136). The detection limits for inosine, guanosine, and adenosine were 80, 80, and 40 pg on column, respectively.

3. Electrospray Ionization MS for Characterization of Oligonucleotides

In modern molecular biotechnology the analysis of oligonucleotides has become a topic of primary importance since establishing the size, purity, and sequence of nucleic acids is a prerequisite to their use as molecular probes in a medicinal field. Significant progress in the area of accurate mass determination, sequencing, and study of noncovalent interactions has been made possible by the use of novel mass spectrometric techniques such as MALDI-TOF and ESI coupled to triple-quadrupole (TQ), quadrupole ion-trap (QIT), time-of-flight (TOF), and FTICR-MS/MS instruments in addition to Q-TOF, QIT-TOF, and QIT-FTICR-MS/MS hybrid instruments.

In the following sections the focus is primarily on recent literature dealing specifically with the practical aspects of oligonucleotide analyses by ESI-MS.

3.1. Purification and Practical Aspects of Oligonucleotide Analysis by ESI-MS

Successful application of ESI-MS for the analysis of nucleotides has been shown to require careful control of sample and instrument parameters as these factors affect the quality of the spectrum obtained. Ascertaining the specific sample conditions for each analyte should be approached on an individual basis because, in many published studies, the extent to which optimization is achieved is often less than clear.[128] Sample and ion-source tuning parameters are important considerations, especially since instruments with different ESI/ion-source designs give rather different spectra for the same sample. Excellent reviews on sample preparation, solvent composition, additives and other enhancements, pH effect on charge state, and separation methods for sample introduction have been published recently.[60,128]

The factors of oligonucleotide structures, which are crucial to understanding the analytical requirements, can be summarized as follows.[128a] (1) The polyanionic backbone of oligonucleotides is usually fully dissociated in solution, and the pK_a of the internucleotide phosphates are <1. (2) Acidic nucleosides containing thymine and/or as well as guanine have been shown to have pK_a values of 9.26.[128b] A comprehensive study on the enolizable protons of guanine has been reported by Liguori et al.[128c] (3) Nucleic acids are extensively solvated, and each nucleotide subunit is complexed with about 20 water molecules. (4) The nucleobases contain basic functional groups with pK_a values that range from 3.5 to 5. Exceptionally, N-3 in uracil structures has a $pK_a \approx 10$. The phosphate group of the sugar glycone and the nucleobase aglycone both contain metal-ion binding sites. (5) There is significant Coulombic repulsion from interaction between adjacent charged phosphates, although it has been suggested that the negative charges can be shielded through proton sharing with the nucleobases.[48]

Native and modified synthetic oligonucleotides have been traditionally purified by reverse-phase HPLC using volatile ion-pairing mobile phases. For example, purification of 10−90 nmol of oligonucleotides in a single injection was demonstrated by Gilar et al.[129] using a 4.6 × 75 mm HPLC column packed with porous 2.5 μm C18 sorbent. Recently, this group reported[130,131] a protocol for the analysis of desalted oligodeoxynucleotides using carefully optimized LC/MS-TOF which allows a throughput of nearly 1000 samples in 24 h. The average mass accuracy was 80 ppm for oligonucleotides up to 110-mer length. Stults and Masters[31] reported a method for rapid purification of synthetic oligodeoxynucleotides to remove sodium counterions prior to ESI-MS. The oligomers, following purification by gel electrophoresis, were precipitated from ammonium acetate to replace sodium ions with ammonium ions and then dissolved in water. Negative-ion ESI-MS were presented for oligomers with up to 48 residues in which the most intense peaks correspond to the $[M - nH]^{n-}$ ions. Recently, Cavanagh et al.[132] described the use of a simple in-line gel cartridge for desalting of solutions prior to electrospray MS which is valuable for investigation of protein−DNA interactions. An evaluation of sample preparation methods has been carried out by Muddiman and his group.[133] As they point out, MS analysis of PCR products requires a clean-up protocol which is both rapid and amenable to automation (potentially hundreds of samples per day). They compare various combinations of the use of Geneclean (a commercial product), ethanol, or 2-propanol precipitation and microdialysis, characterizing each method by examining the quality of the ESI-FT-ICR spectra of an 82 base-pair PCR product. Although ethanol precipitation followed by microdialysis is the best procedure, other less time-consuming alternatives can give results that are almost as good (Geneclean with ethanol precipitation or tandem ethanol precipitation).[133]

The accuracy of the determination of the mass of *n*-mer fragments is illustrated by the work of Deroussent and co-workers.[134] An ESI-MS method for the analysis of desalted oligonucleotides used for pharmacological studies was evaluated for sensitivity and the accuracy with two antisense ODN sequences. The transformed and raw negative-ion ESI-MS for a 25-mer phosphorothioate (Figure 3) showed the $[M + H]^+$ of the 24-mer sequence (M_r 7456.25 Da), indicat-

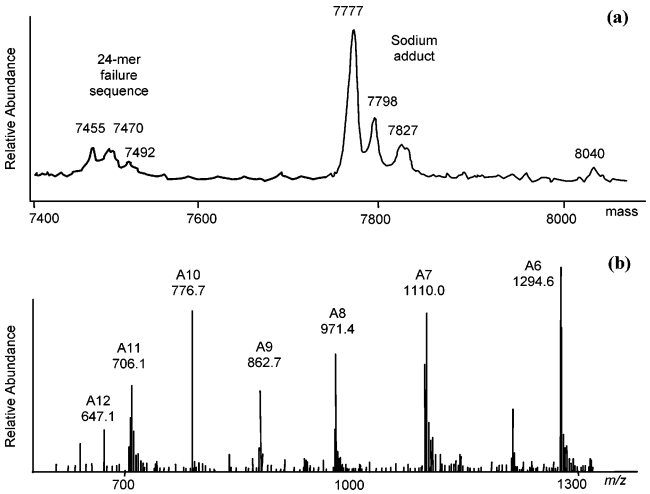


Figure 3. Transformed (a) and negative-ion spectra (b) of a 25-mer phosphorothioate oligonucleotide which targets the GAG region of the HIV-1 genome. The transformed spectrum shows the intact oligonucleotide whose mass was determined as 7777.58 ± 1.44 Da and the 24-mer species (M_r 7456.25) derived from loss of thymidine phosphorothioate from the 3′ end. (Adapted with permission from ref 134. Copyright 1995 John Wiley and Sons, Ltd.)

ing that thymidine phosphorothioate (M_r 320.2 Da) has been lost at the 3′ terminal end. Mass analysis of the 25-mer phosphorothioate [M_r 7776.58 ± 1.44 Da] was performed to within 0.001% accuracy (standard error of 0.05 Da) for a sample concentration of 12 pmol μL^{-1}. In contrast, the ESI-MS of a 77-mer (M_r 24 039 ± 1.21 Da) showed the $[M + Na]^+$ adduct as the predominant peak, indicating retention of only a single sodium ion.

Another method[135] for suppression of signals from alkali-adducted ions involves the addition of millimolar concentrations of a series of organic bases with solution pK_b values ranging from 11.5 to 5.5. Stronger bases such as triethylamine and piperidine were most effective in reducing the signals from bound sodium but also decreased the total ion current from the oligonucleotide. However, imidazole (pH ≈ 8.0) provided a modest suppression of sodium/potassium adduct ions with a 4-fold improvement in sensitivity. Co-addition of imidazole and triethylamine or piperidine produced high ion abundance and good suppression of cation-adducted species, especially for samples of phosphodiester or phosphorothioate oligomers which have not been desalted via preliminary precipitation or by HPLC. The addition of high concentrations of imidazole generated a bimodal distribution of charge states, which reflected the different gas-phase conformations for single-stranded oligomers.[135] The introduction of 0.1 M imidazole in acetonitrile after the HPLC column is also reported to afford increased sensitivity for the ESI-MS analysis of 20-mer to 50-mer oligonucleotides.[136]

A mixed-mode stationary phase has been developed by Van Breemen et al.,[137] particularly for LC/ESI-MS of oligonucleotides. The potential affinity ligand, 3-(thymid-1-yl)propanoic acid, was immobilized on an aminopropyl reverse-phase HPLC column to give a system with a combination of reverse-phase, ion-exchange, and affinity properties. Optimum column performance was achieved using gradients that utilized the potential affinity properties.

3.1.1. New MS Approaches for Oligonucleotide Sequencing Using Enzymatic Digestion

Janning and co-workers[138] describe a new approach for the enzymatic digestion of DNA yielding oligonucleotides ranging from dinucleoside monophosphate to octanucleoside heptaphosphates. Calf thymus DNA was digested by means of the benzon nuclease, an unspecific nuclease, and alkaline phosphatase to remove the terminal phosphate. The mixture of oligonucleotides was separated using capillary-zone electrophoresis with a buffer system with a strong electro-osmotic flow. The oligomers were separated into groups with nucleotides of the same chain length, typically 10 dimers (mass 516−596), 20 trimers (mass 805−925), 35 tetramers (mass 1094−1254), and 56 pentamers (mass 1383−1583). The CZE/ESI-MS were recorded in the negative-ion mode using a multisector-field mass spectrometer.

3.1.2. Improved Oligonucleotide Sequencing by Exonuclease Digestion

The combination of exonuclease digestion and mass spectrometry is an excellent sequencing method for oligonucleotides of greater than 25-mer. However, during an exonuclease digestion the rapid buildup of nucleotide concentration produces strong signals of nucleotide cluster ions in ESI-MS. This can result in poor signal/noise ratios in the reconstruction of the multicharged ions obtained into true molecular weights. Wu and Aboleneen[139] reported a procedure that eliminated the effect of the cluster ions. In this method alkaline phosphatase was added with snake venom phosphodiesterase to the oligonucleotide solution to convert the interfering nucleotides into noninterfering nucleosides. At the end of the digestion a CID-MS/MS of the dimeric oligonucleotide was obtained to determine the sequence of the last two bases at the 5′-terminus of the oligonucleotide. With this approach the signal/noise ratio of the reconstructed molecular weight spectrum was greatly improved (Figure 4) for relatively large oligonucleotides and only a single digestion was needed for sequencing.

Several exonuclease strategies have been proposed by Muddiman and co-workers[140] for the generation

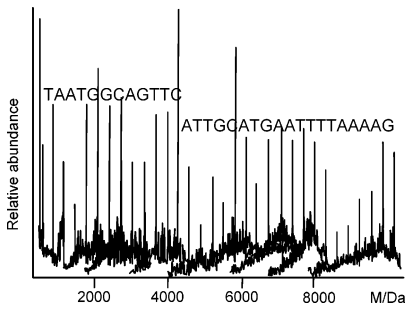


Figure 4. Overlaid reconstructed molecular mass spectrum of a 33-mer obtained from SVP and BAP digestion. The sequence of 31 bases is clearly established. (Adapted with permission from ref 139. Copyright 2001 Academic Press.)

of ladders of single-stranded DNA fragments leading to simple sequence identification including the presence of nucleotide polymorphs. These include the use of two enzymes (exonuclease III and lambda exonuclease) and restriction enzymes to screen specific amplicons for polymorphisms.

3.2. ESI Tandem Mass Spectrometric Analysis of Oligonucleotides

Elucidation of the sequence of oligonucleotides by tandem mass spectrometry of multiply charged DNA ions has been studied intensively over the past decade. McLuckey and collaborators[52,53,141] reported an elegant study of the fragmentation of multiply charged oligonucleotides by ESI in a QIT-MS^n instrument. They proposed a nomenclature for different oligonucleotide fragment types,[52,53] analogous to that developed for peptides, that has been widely accepted. For MS/MS of triply charged anions of the type $[d(A_4)]^{3-}$, preformed under gentle collision-induced activation conditions using low-amplitude supplementary rf signals, these workers established that the product ions resulted from fragmentations which were largely limited to adenine ion losses. They also established in the MS/MS of $[d(A_4)]^{3-}$ the concept of the formation of the $[A_4 - B_4(A)]^{2-}$ ion and its complement ion w_4^{1-} (using the McLuckey notation[53]). Under moderate CID conditions consecutive fragmentations occurred arising from further decompositions involving charge-separation reactions. Spectral interpretation was greatly facilitated by the fact that complementary ions arising from decompositions involving charge-separation reactions were usually observed in either MS/MS or MS^n.[52,141] The importance of the nucleobase type was illustrated by examination of a series of small oligonucleotides containing a single base type.[158] Dissociation behavior differs for each base type as well as depends on the parent ion charge.

The ESI MS[52] of the oligomer d(TGCATCGT) yielded the $[M - 7H]^{7-}$ ion with *m/z* 341.768, which was then resonantly excited for 20−40 ms to form the product-ion scan shown in Figure 5. The major fragmentation routes for the CID MS/MS of $[M - 7H]^{7-}$ were initiated by the loss of adenine anions (A^-), as already established for other adenine-containing DNA. Usually complementary ions can be identified in the MS/MS, which simplifies identification of the fragment ions. For example, the pair of product ions $\{A^-$ and $[M - 7H - A^-]^{6-}\}$ were complementary ions produced from the $[M - 7H]^{7-}$ activated precursor, while $\{w_4^{4-}$ and $[a_4 - B_4(A)]^{2-}\}$ and $\{w_4^{2-}$ and $[a_4 - B_4(A)]^{3-}\}$ were complementary ions activated by the $[M - 7H^+ - A^-]^{6-}$ product ion.[52]

McLuckey and his group[143] demonstrated that a duplex ion from a decamer dDNA can be stored in the quadrupole ion trap. Resonance excitation at the appropriate mass drastically reduced the duplex $[5^-]$ ion intensity within the large mass window obtained by CID MS/MS (Figure 6) and showed the presence of the product ions from both strands, 5′-d(ACATTCTGGC)-3′ and 5′-d(GCCAGAATGT)-3′. Both 2^- and 3^- ions were observed for each single-strand fragment from the excitation of the duplex 5^- ion.

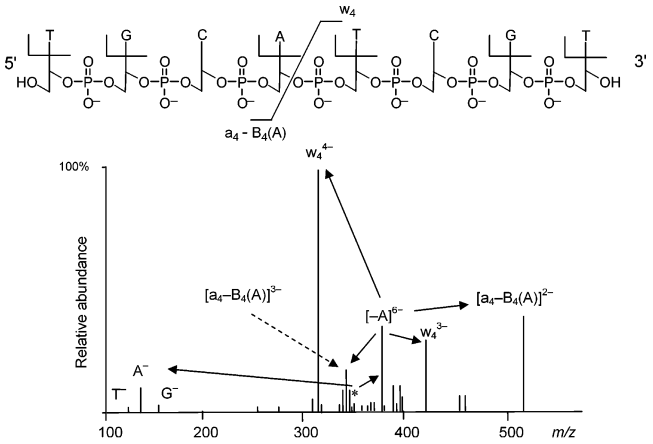


Figure 5. ESI-QIT-MS/MS analysis of 5′-d(TGCATCGT)-3′. The precursor ion $[M - 7H^+]^{7-}$ is indicated by an asterisk. The solid arrows indicate pairs of complementary ions derived from the precursor ion and two different dissociations of the ion derived by initial loss of adenine $[M - 7H^+ - A^-]^{6-}$. (Adapted with permission from ref 52. Copyright 1993 American Chemical Society.)

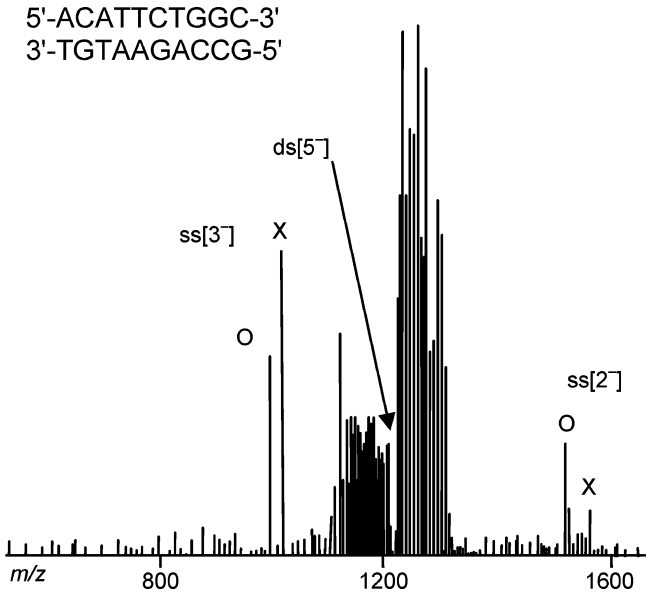


Figure 6. Mass spectrum of a decamer dDNA obtained by CID MS/MS. A range of ions encompassing the double-strand 5- ion was isolated in the ion trap and subjected to resonance excitation. The intensity of the ds[5-] is drastically reduced, and two sets of single-strand ions are produced corresponding to the two possible charge distributions. Peaks labeled X correspond to ions from 5′-(ACATTCTGGC)-3′, and those labeled O correspond to ions from 5′-d(GCCAGAATGT)-3′. (Adapted with permission from ref 143. Copyright 1994 American Chemical Society.)

This is convincing evidence that a decamer DNA ion can survive in the ion trap. Later, this group reported[144] the ESI-MS of 72-mer and 75-mer single-stranded oligonucleotides and a duplex DNA consisting of complementary strands with 72 base pairs. For the duplex DNA the ESI-MS was acquired from a 20 μM solution containing 10 mM ammonium acetate. The average charge on this duplex DNA was approximately −31. Although analytes electrosprayed with high salt concentrations in aqueous solutions showed reduced sensitivity, these data still demonstrated the utility of an ESI-QIT-MS instrument for

the analysis of large, double-stranded DNA and suggest that the ESI-QIT-MS is ideal for elucidating structural changes such as sites of alkylation in DNA. It should also be mentioned that oligonucleotides up to 76 residues had previously been electrosprayed and analyzed by Smith et al.[30] using a quadrupole mass spectrometer.

Recently, Tabet and colleagues[145] reported the study of low-energy collision MS/MS of multiply deprotonated products of mono- and single-strand oligonucleotides, prepared in a nanospray external source coupled to a QIT-MS^n instrument. For a selected anion the negative charge could be located by study of the fragmentation. Multideprotonated 5′-dephosphorylated nucleotides undergo isomerization into ion−dipole complexes prior to dissociation, leading to competitive [BH] and $[B]^-$ losses. In contrast, 5′-phosphorylated nucleotides yielded competitive HPO_3 and nucleobase eliminations. The proposed mechanisms were discussed in terms of the charge state of the precursor and the thermochemical gas-phase properties. This study established that the potential energy was minimal for distant charged phosphorylated groups. These findings suggested that a specific conformation is normally favored in order to minimize Coulombic repulsion during the desolvation process.[145]

On the basis of the small amounts of sample required and the unambiguous diagnostic fingerprints generated, FTICR-MS^n is emerging as the preferred platform for the investigation of the sequences of modified oligonucleotides. Detailed studies by McLafferty and colleagues[146−150] have provided a thorough understanding of the fragmentation of the multiply charged oligonucleotides using FTICR-MS. They used nozzle-skimmer (NS) dissociation and CID-MS/MS and MS^n experiments to investigate dissociation mechanisms of multiply charged larger oligosaccharides up to 108 nucleotides in length. To correctly sequence oligonucleotides containing disparate chemical modifications, an understanding of how the modifications influence the fragmentation pattern is essential. Sannes-Lowery and Hofstadler[151] explored how the glycone and backbone modifications affect fragmentation patterns observed from modified oligonucleotides which are fragmented by infrared multiple photon dissociation in the external reservoir of an ESI-FTICR-MS^n. They observed that the chemical modifications influence which fragment types dominate (i.e., $a_n - B$ versus C_n) and the ease with which the oligonucleotides are fragmented and sequenced.

Substantive studies of oligonucleotide sequencing by CID tandem mass spectrometry with triple-quadrupole or quadrupole−hexapole−quadrupole have also been performed.[149−151] Precursor ions with higher charges gave many low-mass, nonspecific fragment ions. In contrast, doubly and singly charged ions required higher collision energies owing to their greater stability, leading to an overall decrease in sensitivity. It has been generally accepted that the extent of fragmentation of the multiply charged precursor ion is dependent upon the amount of energy applied to it. This was predictably greater in triple-quadrupole instruments compared to ion-trapping analyzers. It is should be recognized that the QIT and FTICR analyzers have the capability for multiple stages of CID and mass analysis, which is very useful for the understanding of the genesis of the product ions.[128]

ESI-tandem mass spectrometric sequencing has been investigated by Gentil and Banoub[152] for a series of synthetic, self-complementary, isomeric DNA hexamers, namely, d(CAGCTG), d(CGATCG), and d(CGTACG) (M_r 1791) and d(CATATG), d(TGATCA), and d(TGTACA) (M_r 1790), using a quadrupole−hexapole−quadrupole instrument. As expected, the ESI-MS of these DNA hexamers exhibited identical series of multicharged deprotonated molecular ions. Low-energy collision CID-MS/MS analysis of the multicharged oligonucleotide anions $[M - 3H]^{3-}$ and $[M - 4H]^{4-}$ provided distinct characteristic fingerprint patterns which permitted discrimination among the individual isomeric DNA hexamers and allowed complete direct sequence determination. However, the inefficiency of these CID-MS/MS processes can be appreciated from the saturated precursor ion and the low abundance intensity of the product ions formed in the tandem mass spectra. Attempts to improve the efficiency of the CID resulted mostly in the formation of higher proportions of the lower *m/z* product ions of no diagnostic value. Although a wealth of information and complete bidirectional sequencing of the precursor $[M - 3H]^{3-}$ anion was possible, it was very difficult to rationalize the genealogy of the product ions since the MS/MS were complicated by ions arising from multiple fragmentations. Similar results were obtained in attempts to sequence series of 12-mer and 16-mer DNA oligonucleotides by CID-MS/MS with a newer quadrupole−hexapole−quadrupole MS/MS instrument, most probably due to higher pressure in the collision cell used to fragment the selected precursor ions.[152]

Generally it is considered that more complete sequence information can be obtained with a triple-quadrupole instrument than a QIT-MS/MS instrument.[128] Nevertheless, some workers prefer the use of QIT-MS^n compared to a TQ instrument since it enhances the predominant formation of the characteristic ions of the (w) and (a) series.[60] A useful comparison of instruments has been made by Ni and Chan,[153] who obtained spectra of the DNA oligonucleotide d(GATCTCGATC) by ESI-MS with quadrupole−orthogonal time-of-flight (Q-TOF)-MS, TQ-MS, and QIT-MS instruments. The ESI-MS are shown in parts a, b, and c of Figure 7, respectively. The five ions labeled A2, A3, A4, A5, and A6 represent the species $[M - nH]^{n-}$, $n = 2-6$. The calculated monoisotopic M_r of d(GATCTCGATC) was 3001.5417 Da. The M_r values obtained from the Q-TOF, TQ, and QIT instruments were 3001.6107, 3002.4, and 3002.5 Da, respectively, representing experimental errors of 0.002%, 0.03%, and 0.03% (Figure 7).[153] As expected, the isotopic peaks for the multicharged ions were resolved by the Q-TOF instrument only. A CID experiment on the $[M - 5H]^{5-}$ oligonucleotide anion (*m/z* 599.5) found that the Q-TOF spectra showed

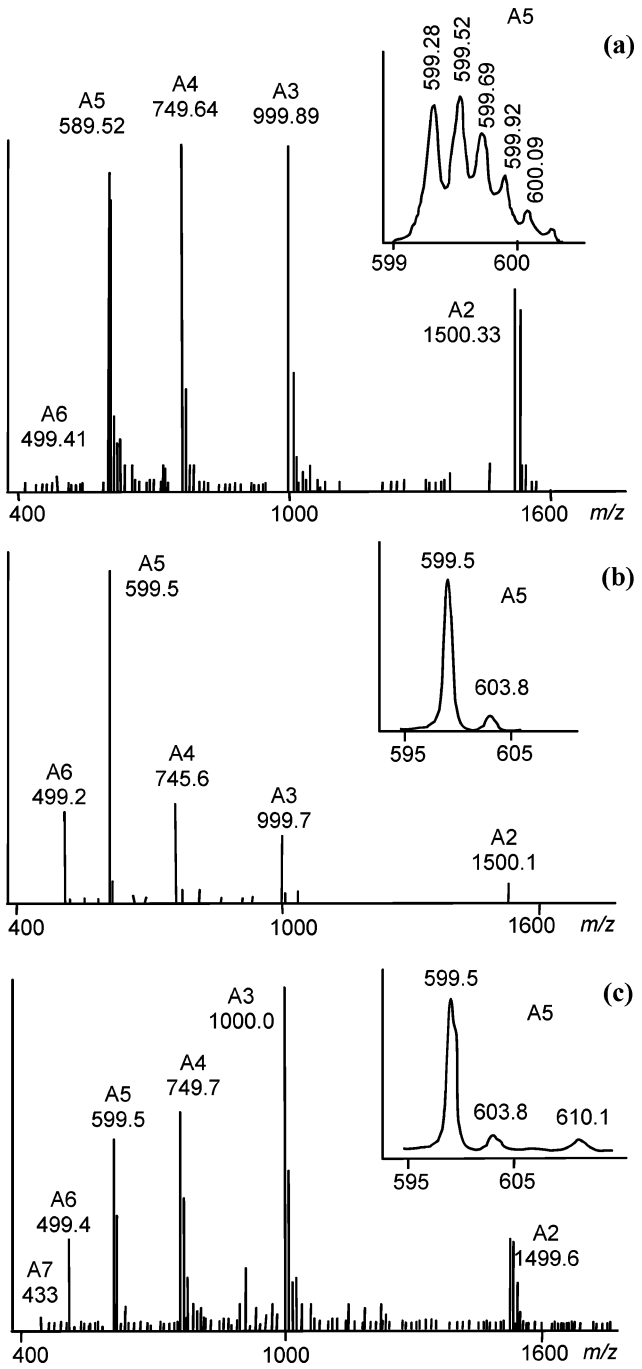


Figure 7. Comparative sequence analysis of an oligonucleotide with three different MS instruments. Resolution obtainable by quadrupole TOF-MS (a). Resolution of triple-quadrupole (b) and quadrupole ion-trap (c) instruments is at least 100 times worse. Notably, the latter instruments give different patterns of ion intensity. (Adapted with permission from ref 153. Copyright 2001 John Wiley and Sons Ltd.)

more low-intensity, sequence-specific fragment ions when compared to the other MS/MS instruments using similar collision energy, i.e., more sequence-related (w) series or (a − B) series ions were observed in the Q-TOF-MS/MS spectrum.[153]

The sequence of synthetic methylphosphonate oligodeoxynucleotides has been verified by Baker and co-workers[154] using ESI-MS/MS with sample introduction via flow injection. They examined product-ion scans from multiply protonated 4+ and 5+ precursors and detected ions from several series of fragments of different charge states which indicated the base sequence of the intact molecule. Oligomers containing as many as 18 bases have been successfully characterized.

3.3. Development of Ion/Ion and Ion/Molecule Reactions for ESI-QIT-MS^n

While most tandem mass spectrometry experiments continue to rely on endothermic reactions to obtain the desired information regarding the precursor ion, the use of exoergic reactions continues to expand. These types of reactions are attractive as no external means of excitation are needed. As a rule, ion acceleration is not only unnecessary but undesirable.[35] The most common use of exoergic reactions is in QIT-MS^n instrumentation operating at unit mass resolution. When these instruments were initially introduced there was a degree of uncertainty in determining charge states greater than two by isotope separation. Analysis of mixtures of relatively high-mass biomolecules by ESI was compromised by the tendency for multiple charging, yielding mass spectra too complex to interpret.[155] For this reason several ways have been tried to reduce the charge state of ions derived by ESI. Condensed-phase approaches to charge state reduction are the simplest, but their effectiveness is limited, and a compromise between charge states and yields is often required. Gas-phase ion/molecule reactions[156] are relatively simple to implement; however, clustering reactions compete with proton transfer, and thus it is usually difficult to drive biopolymer ions to either the 1+ or the 1− charge state whether the reactions take place external to or within the vacuum system of the mass spectrometer.

In contrast, ion/ion gas-phase reactions are a more robust means for manipulating charge states of high-mass ions and can be effected either external to or within the mass spectrometer.[157] Effecting reactions external to the mass analyzer is relatively simple, and a quadrupole ion trap allows the ion/ion reactions to be used in conjunction with MS^n strategies.[158] A good example[159] is the reaction between negatively charged anions of the single-strand deoxynucleotide 5′-d(AAAA)-3′ and protonated pyridine cations, formed in the QIT vacuum chamber. The single-strand DNA anions were injected into the QIT and stored with the pyridine cations for up to 1 s, during which time the ion−ion proton-transfer reaction occurred, converting the $d(A_4)^{3-}$ ion to the doubly charged species $d(A_4 + H)^{2-}$. The opposite polarity combination is illustrated by the use of anions derived from perfluorocarbons to study mixtures of positive oligonucleotide ions in direct analogy with positive protein ions.[160] The electric field of a positive charge-transfer agent can be used for storage of high-mass negative ions formed during the ion/ion reaction period.[160] Both oxygen cation and protonated isobutylene ($C_4H_9^+$) have been investigated as charge-transfer agents and found to have different stabilizing abilities.

McLuckey and co-workers[161] showed that an ion trap could readily be used to determine the mass and charge state of highly charged (positive or negative)

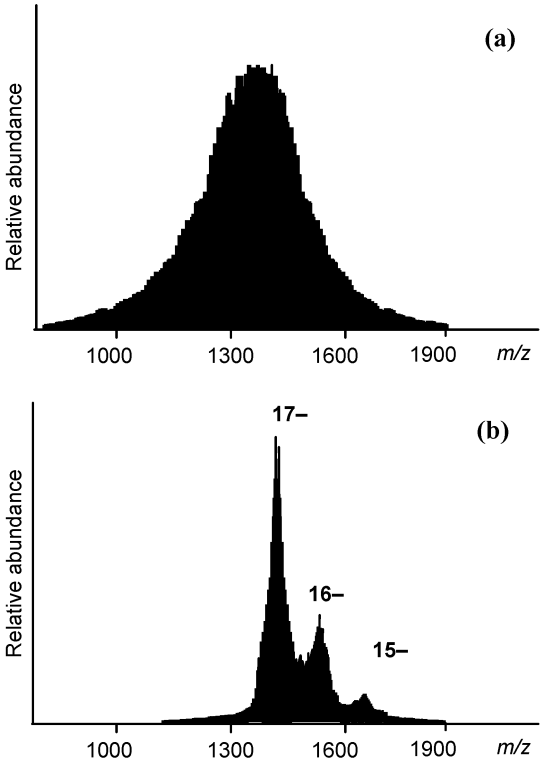


Figure 8. (a) ESI-MS of anions from *E. coli* t-RNA, strain W. (b) Product spectrum resulting from isolating a narrow mass range and then treated with TFA for 200 ms. The two higher distributions correspond to mono- and diprotonation. (Adapted with permission from ref 161. Copyright 1995 American Chemical Society.)

macromolecules by using ion−molecule reactions. Real macromolecule samples are seldom pure but are rather a heterogeneous mixture produced by incomplete biosynthetic pathways and often contain counterions, resulting in an overlap of adjacent mass-to-charge distributions. By isolating a narrow window from the broad overlapping distributions and then allowing a gas-phase reagent to react with these ions, the charge states could be readily determined. This is illustrated in Figure 8a, where tRNA from *E. coli* was electrosprayed into a QIT-MS/MS instrument, producing a very broad *m/z* profile. A narrow mass range of the anions was selected followed by reaction with trifluoroacetic acid for 200 ms. The consequent protonation of the isolated distribution produced two new higher *m/z* distributions as shown in Figure 8b. Assuming that the newly formed product distributions are produced by unit mass and unit charge changes (protonation), the mass and charge of the isolated precursor could be easily determined.

Extending from ion/ion reactions, recent MS/MS studies involving the reaction of electrons with slow, multiply charged ions derived from electrospray has shown that ion−electron reactions hold considerable analytical potential in MS/MS.[35]

3.4. Determination of Nearest Neighbors by ESI-Tandem Mass Spectrometry

The determination of nearest neighbors was a widely used method in early studies of DNA sequence and biosynthesis and for measurement of average dinucleotide frequencies.[162] This technique was based on polymerase-mediated incorporation of specific 5′-^{32}P-labeled nucleotides into the growing polynucleotide chain complementary to the DNA of interest in four parallel experiments. Nuclease digestion to 3′-mononucleotides directed the label into the adjacent residue which was then identified using 2D-TLC. This methodology is experimentally demanding, and the advent of an alternative ESI-MS method was welcome. The method of Rozenski and McCloskey[163] was based on the analysis of fragment ions of the nucleic acid formed in the ionization region by nozzle-skimmer fragmentation. The fragmentation of the oligonucleotide included the formation of cyclic phosphate dimers ($N^1pN^2 > p$), and subsequent analysis of their product ions ($N^1 > p$ or $N^2 > p$) by tandem MS indicated the nearest neighbors of any selected nucleotide whether it was modified. This method was applicable to RNA and DNA and directly to components of oligonucleotide mixtures through the use of LC/MS/MS.[163] In direct combination with an HPLC separation, components of complex mixtures could be successfully analyzed.[164]

3.5. Automatic Oligonucleotide Sequencing—SOS and Compass Programs

It is well known that the information content of product-ion spectra depends critically on the type of MS/MS instrument and on the experimental conditions used. Furthermore, spectrum complexity dramatically increases with the size of the fragmented oligonucleotide, resulting in considerable difficulties in spectrum interpretation.[165,166] Little et al.[149] outlined a sequencing strategy for manual interpretation of MS/MS spectra, but the deduction of sequence information was time consuming and highly technical and could only be performed in laboratories with extensive experience in MS/MS.[165] Hence, automation of procedures for interpretation of product-ion spectra is a prerequisite for the applicability of MS/MS in the routine nucleic acids sequence analysis.

Automating the analysis of the MS spectra oligonucleotides was first broached by McCloskey and coworkers.[167] This group developed a computer algorithm, based on the MS/MS fragmentation scheme described by McLuckey et al.[52,53,141] which allowed sequencing of oligonucleotides using the spectra from an ESI-CID triple-quadrupole tandem mass spectrometer. The method was intended for rapid sequencing of DNA or RNA oligonucleotides of completely unknown structure at approximately the 15-mer level and below. Identification of the sequence-relevant ions, produced from extensive fragmentation in the quadrupole collision cell, was based primarily on the recognition of 3′- and 5′-terminal residues as initial steps in mass ladder propagation. This method also used the alignment of overlapping nucleotide chains, constructed independently from each terminus, and depended additionally on the use of experimentally measured molecular mass for rejection of incorrect sequence candidates. These algorithms for sequence derivation were embodied in a computer program that requires <2 s for execution.[167] The efficacy of the procedure was demonstrated for se-

quence location of simple modifications in the base and sugar. The potential for direct sequencing of components of mixtures was shown using an unresolved fraction of unknown oligonucleotides from ribosomal RNA.[167]

The original computer algorithm was refined by Rozenski and McCloskey and transformed into the Simple Oligonucleotide Sequencing (SOS) program,[168] a simple user-interactive program for ab initio oligonucleotide sequencing by mass spectrometry. For ESI-CID mass spectra of oligonucleotides, this standalone computer program provided an effective means of sequence determination at the 20-mer level and below, allowing user-controlled rapid oligonucleotide sequencing from mass spectra on a residue by residue basis. Modifications could be defined in any combination for the base, sugar, or backbone. Sequence ladders were independently constructed in both 5′ → 3′ and 3′ → 5′ directions and graphically compared for homology and overlap. A particular advantage of this method was the ability to easily erase and rebuild alternate subsequences. The program was shown to be useful for the ab initio sequencing of modified or unmodified oligonucleotides, for rapid verification of sequence, and in studies of fragmentation processes of model oligonucleotide derivatives.[168]

Huber and co-workers[165,169] recently developed a program for the computer-aided interpretation of product-ion spectra obtained for collision-induced dissociation of multiply charged oligodeoxynucleotide ions generated by ESI. This algorithm for the comparative sequencing (COMPASS) of oligonucleotides was shown to be suitable for the sequence verification of nucleic acids ranging in length up to 80 nucleotides. The COMPASS program is based on matching the mass spectrum generated by CID-MS/MS to *m/z* values produced from a systematically varied reference sequence employing established fragmentation pathways. The potential and limits of the COMPASS program regarding the lengths, the charge state of the oligodeoxynucleotide, the selected collision energy, and the analyzed amount of sample using a QIT-MS^n instrument have been evaluated.[169] The COMPASS algorithm was proven to be reproducible and applied for the genotyping of the polymorphic, Y-chromosomal locus M9 contained in a 62-base pair polymerase chain reaction product.[170]

Another program worth mentioning is the Mongo Oligo Mass Calculater, an excellent tool for calculating and predicting the masses of oligonucleotides and fragments obtained by CID-MS/MS or enzymatic digests. The Mongo Oligo program was originally written by Limbach, Pomerantz, and Rozenski[171] and allowed the mass calculation of oligonucleotides, CID fragments, and endo- and exonuclease digestion. It also contains extra output options and utilities for handling the input sequence and user residue assignment.

3.6. Precise Molecular Weight Determination of PCR Products of the rRNA as a Taxonomic Tool

Bacillus subtilis and *Bacillus atrophaeus* are two closely related species with few distinguishing phenotypic characteristics. *B. subtilis* has recently been subdivided into two subgroups, W23 (type strain, W23) and 168 (type strain, 168). Comparison of sequence variability in the intergenic spacer region (ISR) between the 16S and 23S ribosomal RNA (rRNA) genes has been a useful tool for differentiating between the bacterial species. This ISR region has been shown to vary in size and sequence even within closely related taxonomic groups.

Johnson and co-workers[172] reported the analysis by ESI-MS of the PCR products amplified from the ISR PCR using a primer pair corresponding to conserved sequences of the 16S and 23 rRNA genes, including the 5′-terminal end of the 23S rRNA and a conserved portion of the ISR. These authors found that a 119 or 120 base-pair PCR product was produced for *B. atrophaeus* strains. However, the strains of *B. subtilis* subgroups W23 and 168 each produced 114 base-pair products. The differentiation of *B. subtilis* and *B. atrophaeus* and the genetic similarity of *B. subtilis* subgroups W23 and 168 were confirmed by ESI-MS analysis. Accurate determination of the molecular weight of PCR products from the 16S−23S rRNA intergenic spacer region using ESI-MS showed great potential as a general technique for characterizing closely related bacterial species.

4. Electrospray Ionization MS for Characterization of Covalent and Noncovalent DNA and RNA Complexes

Electrospray ionization mass spectrometry (ESI-MS) has proved to be a useful tool for studying biomolecular structures and noncovalent interactions involving proteins with metals, ligands, peptides, oligonucleotides, and other proteins.[173] Since the first reports in 1991 by Ganem, Li, and Henion,[174,175] the use of conventional ESI-MS for studying noncovalent complexes in solution has been successfully demonstrated and reviewed. Analyses of noncovalent complexes of DNA and RNA by mass spectrometry have been covered briefly in a review by Hofstadler and Griffey.[176]

This technique has been used to determine the stoichiometry and dissociation constants for a variety of biological noncovalent complexes by titration experiments or competition experiments. The advantages of ESI-MS over other techniques for noncovalent binding studies include high sensitivity, speed of analysis, capability of obtaining stoichiometric information, and ability to identify unknown species. Furthermore, using ESI-FTICR-MS^n it is possible to simultaneously monitor interactions between multiple targets and ligands, thereby greatly increasing screening throughput.[176]

4.1. ESI-MS Characterization of Noncovalent DNA Complexes

DNA is capable of binding to many different types of molecule, and MS has become a crucial technique for the study of such relatively weak complexes. The following examples illustrate the potential of ESI-MS as a useful tool for the characterization of specific drug binding to DNA and its application in the many studies of protein−DNA interaction.

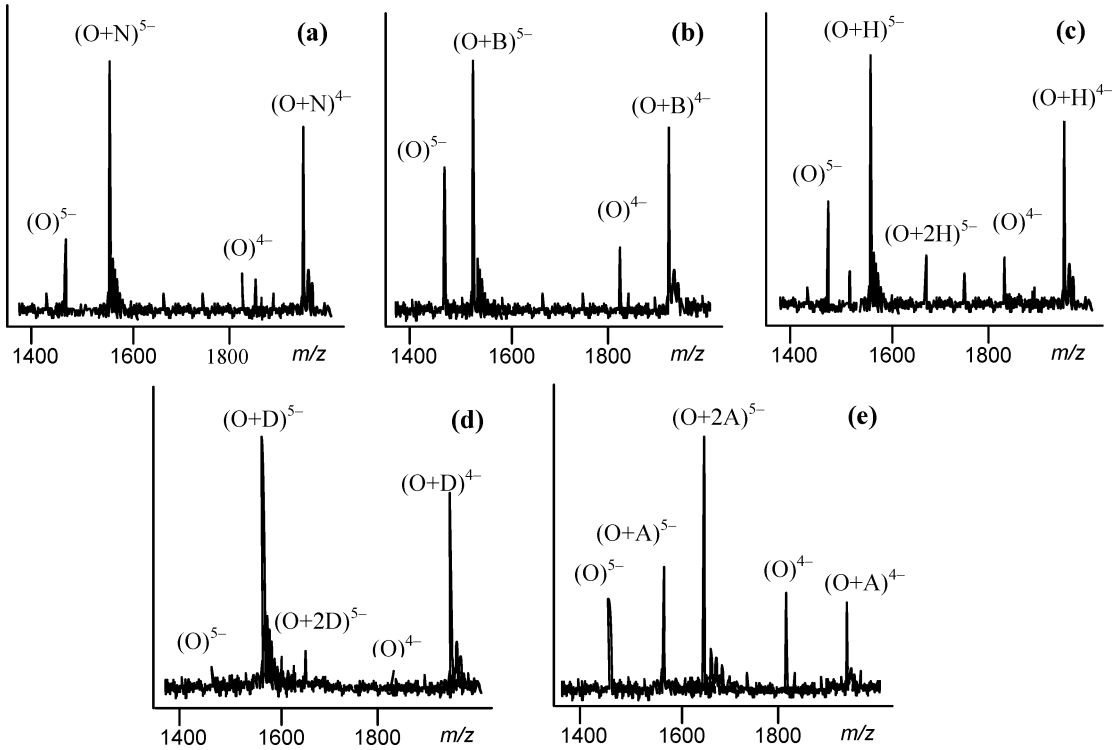


Figure 9. ESI ion-trap mass spectra of equimolar mixtures of a duplex oligonucleotide (O) with (a) netropsin (N), (b) berenil (B), (c) Hoechst 33258 (H), (d) DAPI (D), and (e) Distamyacin A (A). The spectra clearly show the different affinities between drug and duplex and the relative amount of single or double binding of the drug species. (Adapted with permission from ref 177. Copyright 1999 John Wiley and Sons, Ltd.)

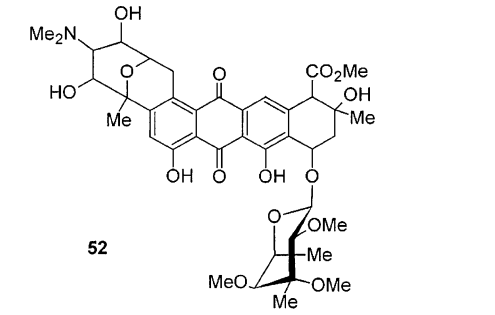
4.1.1. Binding Interaction between Double-Stranded Oligonucleotides and Drug Molecules

The complex formed between double-stranded (ds) oligonucleotides and various antitumor drugs has been investigated recently by Gabelica et al.[177] using ESI-QIT-MS. The ds oligonucleotides were formed by a non-self-complementary oligonucleotide d(GGGGATATGGGG) and the complementary strand d(CCCCATATCCCC). Using two types of antitumor drugs, intercalators (ethidium bromide, amsacrine, and actinomycin D), and minor-groove binders (Hoechst 33258, netropsin, distamycin A, berenil, and DAPI) this study assessed whether the relative intensities in the mass spectra reflect the relative abundances of the species in the solution phase (Figure 9). The full-scan mass spectra suggested nonspecific binding for the intercalators and specific binding for the minor-groove binders. The preferential stoichiometries adopted by each minor-groove binder were determined by studying the influence of the drug concentration on the spectra. The CID product-ion scan of the 1:1 complexes with Hoechst 33258 and netropsin allowed the authors to obtain some structural information, and using competition experiments, the relative binding affinities of the drug for the duplex were determined as netropsin > distamycin A > DAPI > Hoechst 33258 > berenil.[177]

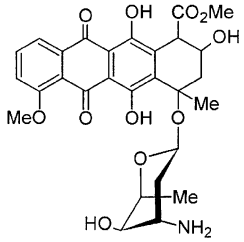
An elegant study of the noncovalent binding of the antitumor drugs nogalamycin (**52**) and daunomycin (**53**) (Chart 9) to duplex DNA has been reported by Sheil and co-workers.[178] If conditions for the formation of drug/duplex complexes were carefully optimized, it was found that for the self-complementary oligonucleotides, 5′-d(GGCTAGCC)-3′ or 5′-d(CGGCGCCG)-3′, the most intense ions generated by ESI corresponded to duplex DNA bound to three molecules of drug with some evidence for binding to four molecules. The assignments of the major ions in the spectra of complexes of **52** with both 8-mer oligonucleotides are given in Table 5. For the larger 12-mer species, 5′-$(TGAGCTAGCTCA)_2$-3′, binding of up to four nogalamycin and six daunomycin molecules could be observed. These data were consistent with the neighbor exclusion principle, whereby intercalation occurred between every other base pair to the extent that up to four bound drugs would be expected for the 8-mer and up to six for the 12-mer.[178] In a later study this group[179] reported that the positive-ion ESI-MS of a 16-mer dsDNA had been obtained with essentially no ions from single-stranded DNA present. Single-stranded DNA formation was minimized by careful choice of DNA sequences, the use of a relatively high salt concentration (0.1 M ammonium acetate, pH 8.5), and a low desolvation temperature (40 °C).[179] These observations contrast with many earlier ESI-MS studies of (ds)DNA and DNA/drug complexes in which ions from (ss)DNA were also normally observed. The ESI-MS of complexes of 16-mer (ds)DNA with cisplatin, daunomycin, and distamycin were obtained under conditions where only negligible amounts of single-stranded DNA were present. The complexes with daunomycin and distamycin were more stable to strand separation in the gas phase than the (ds)DNA alone.

The charged trinuclear platinum compound, BBR3464, is one of the first of a new class of anticancer drugs to enter phase I clinical trials. This species consists of two [*trans*-$PtCl(NH_3)_2$] units linked

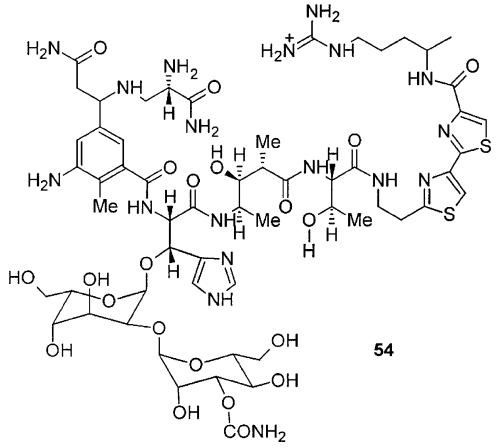
Chart 9



52



53



54

Table 5. Assignments of Major Ions in the Optimized ESI Spectra of Complexes Formed by Mixing Nogalamycin (N) with Duplex DNA 5′-d(GGCTAGCC) (d1) or 5′-d(CGGCGCCG)-3′ (d2) in a Drug/DNA Ratio of 5:1 (multiple binding by nogalamycin is evident)

fragment	expl. mass (d1)/Da	exptl. mass (d2)/Da
N	786.5	786.2
$[M - 5H]^{5-}$	481.4	481.3
$[M - 4H]^{4-}$	601.9	601.7
$[M - 3H]^{3-}$	802.6	802.8
$[M - 2H]^{2-}$		1204.8
$[2M - 5H]^{5-}$		963.0
$[2M + N - 5H]^{5-}$		1120.9
$[2M + N - 4H]^{4-}$		1401.2
$[M + N - 3H]^{3-}/[2M + N - 6H]^{6-}$	1065.1	1065.4
$[2M + 2N - 5H]^{5-}$	1278.4	1278.1
$[M + N - 2H]^{2-}/[2M + 2N - 4H]^{4-}$	1597.8	1598.3
$[2M + 3N - 6H]^{6-}$	1196.4	
$[2M + 3N - 5H]^{5-}$	1436.1[a]	1436.4[a]
$[2M + 3N - 4H]^{4-}$	1795.4	1795.3
$[M + 2N - 3H]^{3-}/[2M + 4N - 6H]^{6-}$	1327.9	1327.9
$[2M + 4N - 5H]^{5-}$	1593.7	1592.9
$[M + 2N - 2H]^{2-}/[2M + 4N - 4H]^{4-}$	1992.2	
$[2M + 5N - 5H]^{5-}$	1751.5	

[a] Most intense ion attributable to the complex.

by a tetraamine [*trans*-$Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2$] unit with a 4+ charge, and the mode of DNA binding is thought to be distinctly different from those of classical mononuclear drugs such as cisplatin, *cis*-$[PtCl_2(NH_3)_2]$. Characterization of the interaction of BBR3464 with (ds)DNA, (ss)DNA, and RNA substrates by ESI-FTICR-MS[180] raised the possibility that the adducts of single-stranded DNA and RNA played an important role in the different antitumor efficacy of this novel drug as compared with cisplatin.

4.1.2. Molecular Characterization of Peptide binding to DNA

The GCN4 peptides are a group of proteins which bind to (ds)DNA through a sequence-specific interaction and are important in the regulation of gene transcription in yeast. In addition to a basic DNA-binding domain, these proteins contain a leucine zipper dimerization domain.[181−183] The protein dimers specifically bind to (ds)DNA containing the binding element 5′-ATGA(C/G)TCAT-3′ to form a tetramolecular noncovalent complex.[184] This noncovalent complex has been detected in the gas phase by ESI-MS. No ions were detected for the peptide dimer itself or for a DNA/peptide monomer complex, indicating that the tetrameric complex is an especially stable unit under the conditions in which it is detected. The observation of these important complexes by MS was extended to several types of (ds)DNA with up to 30 base pairs. The ability to observe these large complexes by mass spectrometry introduces an analytical technique which can probe specific protein/DNA interactions and will surely be applied to other similar structural biological problems including the study of transcription processes.

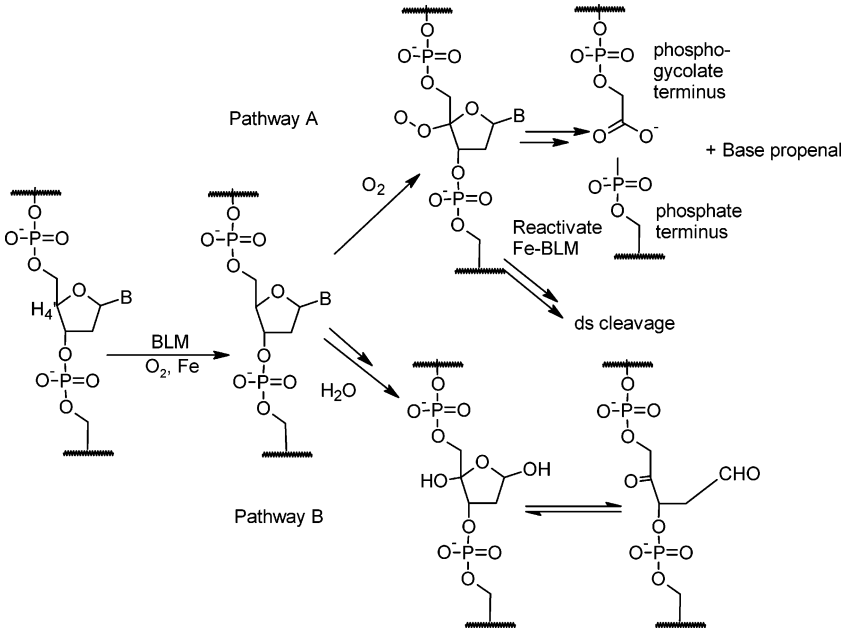
A peptide nucleic acid oligomer (PNA) is a polyamino acid with a DNA base attached to each residue. These compounds bind to complementary (ss)DNA oligonucleotides and are of great interest as DNA analogues. Verheijen and collaborators[181] have shown that attachment to a PNA of a suitable metal-containing ligand such as ferrocene carboxylic acid or a tris(bipyridine)ruthenium(II) derivative provides a convenient marker for biomolecular studies. These novel compounds were characterized by ESI-MS.

The interaction between the DNA-binding domain of the vitamin D receptor (VDR DBD) and the double-stranded DNA sequence containing the vitamin D response element (VDRE) from the mouse osteopontin gene has been studied by ESI-MS.[183] The VDR DBD was shown to bind to the appropriate DNA sequence only when bound to 2 mol of zinc (Zn^{2+}) or cadmium (Cd^{2+}) per mol of protein. Additional binding of Zn^{2+} or Cd^{2+} by the protein caused the protein to dissociate from the (ds)DNA. A μESI-MS methodology for the study of DNA binding to this and other steroid hormone receptors has also been reported.[182]

4.1.3. ESI-MS of Noncovalent Complexes of Ribonuclease A-Cytidylic Acid Substrates

In a recent report Zhang and co-workers[185] described a fully automated, chip-based nanoESI-MS system for investigation of the quantitative noncovalent interactions between ribonuclease A (RNase A, M_r 13 682) and cytidylic acid ligands (2′-CMP, M_r

Scheme 2. Pathway for DNA Cleavage Catalyzed by Bleomycin[a]



[a] The phosphoglycolate- and phosphate-terminated cleaved fragments are detected by ESI-QIT-MS.

323.2 Da; CTP, M_r 483.1 Da). Both titration and competitive binding approaches were performed prior to automated nanoESI-MS analysis with a Q-TOF-MS hybrid instrument. The measured K_d values for the complexes RNase A-2′-CMP and RNase A−CTP were found to be in excellent agreement with the available published values obtained by standard spectroscopic techniques. Several electrospray ionization sources in different commercial mass spectrometers, such as ESI-Q-MS, ESI-TQ-MS/MS, and the Autospec Sector Instrument, were evaluated for use in the study of noncovalent interactions between ribonuclease A and cytidylic acid by Haskins et al.[186] Although signal differences were observed between the various types of source, giving rise to very different spectra, the structural information relating to the complex was identical in all cases. These studies also showed that the noncovalently bonded complexes between ribonuclease A and various cytidylic acid substrates were most stable at lower charge states.

4.1.4. MS Characterization of the Damage and Repair Mechanisms of DNA

Bleomycin (BLM) (**54**) is the major component of the clinically employed antitumor antibiotic Blenoxane (Chart 9). In the presence of its required cofactors Fe(II) and O_2, BLM catalyzes (ss) and (ds) cleavage of DNA. The cleavage mechanism is initiated by removal of a 4′-hydrogen atom from a specific residue and the generated radical partitions between two pathways depending on the availability of O_2 (Scheme

Table 6. Product Ions from Fragmentation Experiments (MS/MS) with the Tethered Duplex Oligonucleotide 5′-CCGTACAA(C_2H_4O)$_6$TTCTACGG-3′ [tO] and Its Ternary Complex with Fe−Bleomycin [FeBLM−tO]

fragments	*m/z*	fragments	*m/z*
oligonucleotide (tO) (*m/z* 1305.5, $z = 4-$)		complex (FeBLM-tO) (*m/z* 1340.1, $z = 5-$)	
$(a_3 - B_3)^-$ and w_2^-	675.4	$(a_3 - B_3)^-$ and w_2^-	675.3
w_5^{2-}	790.4	w_5^{2-}	790.2
$(a_7 - B_7)^{2-}$ and w_6^{2-}	954.9	$(a_7 - B_7)^{2-}$ and w_6^{2-}	954.8
w_3^-	964.4	w_3^-	964.3
$(a_8 - B_8)^{2-}$	1111.3	$(a_8 - B_8)^{2-}$	1111.3
$(a_{12} - B_{12})^{3-}$ and w_{11}^-	1162.5	$(w_{10} + FeBLM)^{4-}$	1163.6
w_{12}^{3-}	1258.9	$(a_{16} - B_{16})^{4-}$ and w_{15}^{4-}	1180.3
$[tO - G]^{4-}$	1267.6	$(w_{11} + FeBLM)^{4-}$ and $(a_{12} - B_{12} + FeBLM)^{4-}$	1241.6
$[tO - A]^{4-}$	1271.7	$(w_{16} + FeBLM)^{5-}$	1298.2
$[tO - C]^{4-}$ and w_4^-	1277.6	$[tO]^{4-}$	1305.5
$(a_5 - B_5)^-$	1308.3	$[FeBLM-tO - G]^{5-}$	1309.6
$(a_{14} - B_{14})^{3-}$	1373.6	$[FeBLM-tO - A]^{5-}$	1313.0
w_9^{2-}	1431.2	$[FeBLM-tO - C]^{5-}$	1317.7
w_{14}^{3-}	1464.5	$[FeBLM-tO - carbamate]^{5-}$	1331.4
$(a_{15} - B_{15})^{3-}$	1477.8	$[FeBLM-tO]^{5-}$	1340.0
$(a_{16} - B_{16})^{3-}$	1574.6	$(a_{14} - B_{14} + FeBLM)^{4-}$	1399.8
w_5^-	1581.3	$(w_{14} + FeBLM)^{4-}$	1468.0
w_{10}^{2-}	1587.8	$(a_{15} - B_{15} + FeBLM)^{4-}$	1478.0
$(a_6 - B_6)^-$	1621.3	$(w_{15} + FeBLM)^{4-}$and $(a_{16} - B_{16} + FeBLM)^{4-}$	1550.4
$(a_{11} - B_{11})^{2-}$	1744.5	$(a_6 - B_6)^-$	1621.3
		$(w_{11} + FeBLM)^{3-}$ and $(a_{12} - B_{12} + FeBLM)^{3-}$	1655.5

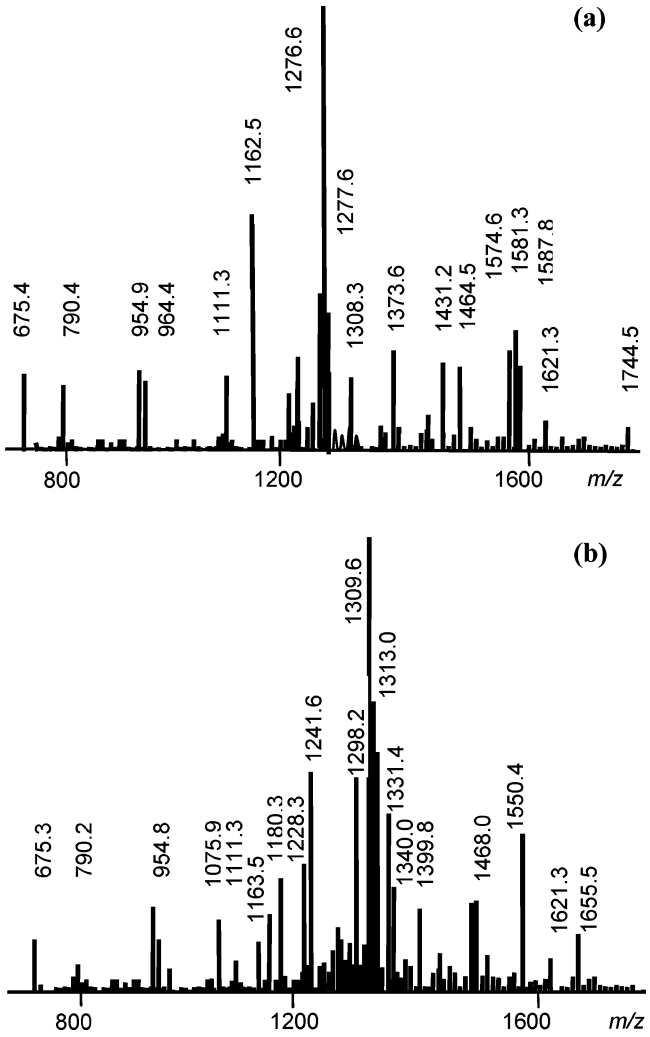


Figure 10. Analysis of the binding of Fe−bleomycin to a tethered duplex oligonucleotide by CID-MS. Full-scan MS/MS of (a) the oligonucleotide ion $[M - 4H]^{4-}$, *m/z* 1305.5, and (b) the complex with Fe−bleomycin, $[M - 5H]^{5-}$. The full list of identified ions is given in Table 6. (Adapted with permission from ref 187. Copyright 2000 Oxford University Press.)

2). These cleavage processes were investigated by ESI-QIT-MS^n by Vouros and co-workers[187] using two duplex oligonucleotides, each containing an 'ethylene oxide' tether between the strands and nucleotide sequences, previously established as specific 'hot spots' for (ss) and (ds) cleavage. This work is an interesting development of a new MS/MS-based methodology which explores the mechanism of the (ds) cleavage process. Not only could the ternary Fe−bleomycin−oligonucleotide complex be detected in the gas phase but also the MS/MS fragmentation profiles were analyzed in detail as shown by the number of identified product ions listed in Table 6 for one of the tethered oligonucleosides and its complex with Fe−BLM. The full-scan MS spectrum of the compound of the tethered duplex oligonucleotide displayed prominent signals at *m/z* 1740.7, 1305.5, 1044.3, and 870.2, reflecting the 3^-, 4^-, 5^-, and 6^- charge states, respectively. CID product-ion scans of the $[M - 4H]^{4-}$ at *m/z* 1305.5 from the tethered duplex oligonucleotide 5′-CCGTACAA$(C_2H_4O)_6$TTCTACGG-3′ and of the $[M - 5H]^{5-}$ at *m/z* 1340, isolated from the noncovalent Fe−BLM complex, are shown in Figure 10.[187]

Nucleotide excision repair (NER) is the process responsible for eliminating most of the damage to DNA from UV radiation and other base alterations caused by a variety of mutagens. The xeroderma pigmentosum group A complementing protein (XPA) is believed to be involved in the early steps of NER by recognizing and binding damaged DNA. Recently, Xu and co-workers[188] reported the use of ESI-FTICR-MS to examine a cisplatin-adducted oligonucleotide and its interaction with the human XPA minimal binding domain (XPA-MBD). High-resolution MS experiments showed the presence of 1:1 noncovalent complexes of XPA-MBD with both damaged and undamaged 20-mer cisplatinum-adducted (ds) oligonucleotides. The damaged (ds) 20-mer also formed a complex with 2:1 binding stoichiometry, but generally the damaged nucleic acid showed only a slight preferential binding of XPA-MBD compared to the undamaged 20-mer. These results demonstrate well the usefulness of ESI-FTICR-MS for probing subtle protein−DNA interactions.

4.2. ESI-MS Characterization of RNA Noncovalent Interactions

RNA plays a key role in a variety of functions within the cell. Messenger RNA (mRNA) is a product of gene transcription, and its translation on ribosomes yields functional proteins. Besides its role as

Table 7. Comparison of Calculated and Measured M_r Values for Selected RNA Oligonucleotides and Their Complexes (asterisk (*) denotes the 5′ terminus of the oligonucleotide)

RNA type		sequence and structure	calcd M_r/Da	exptl. M_r/Da
single strands	RNA1	*CAUCUCAGUCGCACUUAGCUCAGUCAUAGA	9494	9477[a]
				9592[b]
	RNA2	*GUGCGACUGAGAUG	4526	4510[a]
	RNA3	*UCUAUGACUGAGCU	4408	4406[c]
RNA complexes	RNA1 + RNA2	GUAGAGUCAGCGUG*	14 020	13 987[a]
		*CAUCUCAGUCGCACUUAGCUCAGUCAUAGA		14 102[d]
	RNA1 + RNA3	UCGAGUCAGUAUCU*	13 902	13 883[a]
		*CAUCUCAGUCGCACUUAGCUCAGUCAUAGA		13 996[b]
	RNA1 + RNA2 + RNA3	GUAGAGUCAGCGUG*	18 428	18 395[a]
		UCGAGUCAGUAUCU*		18 507[d]
		*CAUCUCAGUCGCACUUAGCUCAGUCAUAGA		

[a] The mass discrepancy is due to replacement of a hydroxyl group by a hydrogen in one or both strands. [b] The mass discrepancy is due to the presence of one *tert*-butyldimethylsilyl group in the RNA1 strand. [c] Within 0.01% mass accuracy for the instrument. [d] The mass discrepancy is due to a combination of the above modifications in the individual strands.

an intermediate code molecule, RNA serves fundamental structural and catalytic roles in many cellular functions.[189,190] These functions include the components (and perhaps the catalysis) of the RNA-splicing apparatus, the ribosome (including its peptidyl transferase activity), the signal recognition particle, RNA editing and other posttranscriptional RNA modifications, X-chromosome inactivation, telomere maintenance, and both natural and engineered regulation of gene expression through sense−antisense interactions.[191,192] RNA participates in noncovalent associations with metals, proteins, and DNA as well as other molecules of RNA. RNA is also the genetic material of many viruses and retroviruses such as HIV and HCV.[72,193]

Hoyne et al.[194] highlighted the increasing role that ESI-MS has played in the study of the noncovalent interactions involved in RNA−RNA complexes and described the application of μESI-MS to this type of base-pairing interaction between RNA oligonucleotides. A set of complementary RNA oligonucleotides (Table 7) was synthesized, and the RNA samples were desalted by sequential steps of gel filtration and cation-exchange chromatography prior to analysis by μESI-MS. The spectra of these desalted oligonucleotides (RNA1, RNA2, RNA3) are shown in Figure 11. Transformation of the multiply charged spectrum of RNA1 shows a prominent species of M_r 9477 Da, which agrees with the calculated M_r of the oligonucleotide if one hydroxyl group is replaced by a proton (Table 7). A second ion response at M_r of 9592 Da probably represents minor contamination by one or more species of RNA oligonucleotide containing a single remaining *tert*-butyldimethylsilyl protecting group at one of the 29 possible 2′-hydroxyl positions in the RNA1 oligonucleotide. For the oligonucleotide RNA2 the measured M_r again suggests an OH→H modification, whereas for oligonucleotide RNA3 excellent agreement between the experimental and calculated M_r values is observed. Appropriate mixtures of these complementary RNA oligonucleotides produced the expected two- and three-component double-helical complexes as confirmed by the measured M_r values (Table 7).[194]

Bacteriophage T4 regA protein, a translational regulator, is a unique member of the RNA-binding protein family. It consists of 122 amino acid residues and regulates the expression of as many as 30 early T4 genes, including the translation of its own mRNA. This is drastically different from the other translational repressors, which generally recognize only one or a few RNA sequences. Liu et al.[46] studied the interactions of bacteriophage T4 regA protein with RNAs of various size and sequence by ESI-FTICR-MS, and using very gentle interface conditions they were able to observe formation of 1:1 regA/RNA complexes for four different RNAs. Each of the RNAs studied contained the sequence for the recognition element for regA, as determined by filter binding experiments.

The high-resolution ESI-FTICR-MS of regA itself gave a charge state distribution that indicated an M_r value 14 618.4 Da, in excellent agreement with a calculated value of 14 618.7 Da. When regA was

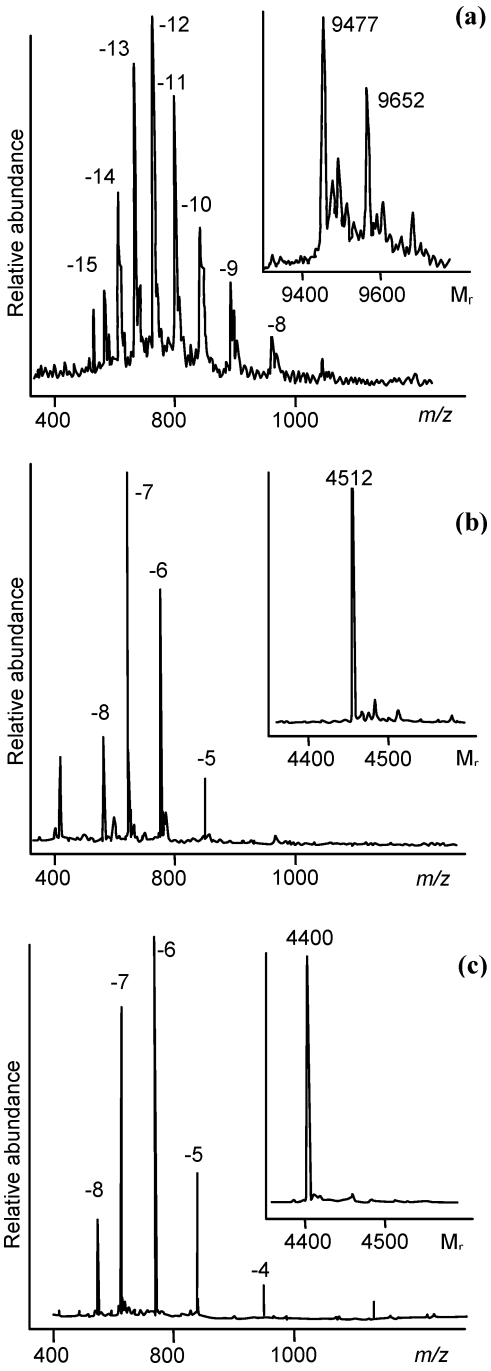


Figure 11. Negative-ion μESI-MS of three RNA oligonucleotides over the *m/z* range 440−2000. Samples were infused in water after desalting offline using a cation-exchange microcartridge. The inserts show transformed spectra and experimental M_r values: (a) RNA 1, (b) RNA 2, (c) RNA 3. (Adapted with permission from ref 194. Copyright 2001 John Wiley and Sons, Ltd.)

mixed with a selected RNA4 at a molar ratio of 1:4 and analyzed by ESI-FTICR, the 1:1 regA/RNA4 complexes (at higher *m/z*) and excess unbound RNA4 (at lower *m/z*) were detected (Figure 12). Numerous additional species were observed in the RNA4 profile corresponding to other complexes and degradation products. Interestingly, the corresponding regA complexes were also observed in many cases in the regA/RNA4 profile, as identified by accurate mass measurements. This correlation between complexed and free species allows insight into the relative binding

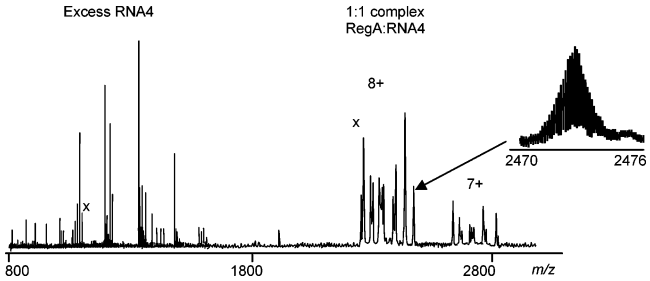


Figure 12. ESI mass spectrum of a mixture of RegA and oligonucleotide RNA4. Two sets of peaks at higher *m/z* correspond to a 1:1 complex with either 8+ or 7+ charge. Uncomplexed excess RNA4 species appear at lower *m/z*. (inset) Resolution of isotopic peaks. The peaks marked x indicate a particular RNA4 and its complex. Several such pairs can be identified. (Adapted with permission from ref 214. Copyright 1998 Academic Press.)

of different species with the protein. For example, loss of a single nucleotide resulted in a dramatic change in binding affinity in some cases. Data from use of sustained off-resonance irradiation for collisionally induced dissociation of a regA/RNA4 complex suggested the potential for directly obtaining information regarding the regA binding domain.[46]

4.3. ESI-MS Characterization of Covalent DNA and RNA Complexes

Almost coincident with the birth of the 21st century has come the evolution of ESI-tandem MS into the analysis of DNA and RNA covalently bound to other species. This is new and exciting work, and the next section details the study of several DNA−enzyme complexes and similar studies.

4.3.1. Characterization of the Binding Site for the Extrahelical Target Base in DNA–Methyltransferase Complexes

DNA methylation is a postreplicative process that is regulated by the DNA methyltransferases (Mtases). This biologically important class of enzymes catalyzes the transfer of the activated methyl group from the cofactor *S*-adenosyl-L-methionine to adenine N-6, cytosine N-4, or cytosine C-5 within their DNA recognition sequences. A particular DNA sequence may exist as its fully methylated, unmethylated, or transient hemimethylated form, and DNA methylation can be regarded as an increase in the information content of DNA, additional information which subsequently serves a wide variety of biological functions. Mtases flip their target adenines or cytosines out of the DNA double helix into a pocket within the cofactor binding domains, where catalysis takes place. Biochemical evidence for a base-flipping mechanism of the N^6-adenine DNA Mtases M.*Eco*RI and M.*Taq*I was obtained using duplex oligodeoxyribonucleotides containing the fluorescent base analogue 2-aminopurine at the target positions.

Krauss and co-workers[195] investigated the site of the covalent coupling of the N^6-adenine DNA Mtases M.*Taq*I and M.*Cvi*BIII with duplex DNA. This was achieved by synthesizing a duplex DNA with the adenine moiety in the recognition sequence 5′-TCGA-3′ replaced by 5-iodouracil, affording species **55**, which was then cross-linked to the enzyme using a high-yield photochemical reaction (Chart 10). Analyses by anion-exchange chromatography revealed that almost all the duplex ODN had reacted, forming the nucleopeptide **56**. The total mixture from the photo-cross-linking reaction from M.*Cvi*BIII was treated with chymotrypsin, and the resulting nucleopeptide was purified by anion-exchange chromatography and analyzed by ESI in the negative-ion mode using a double-focusing sector field instrument.

The first 13 residues of the peptide sequence were found to be DFIVGNPP**X**VVRP (Chart 10), where the residue at position 9 (**X**) was not recognized as one of the 20 natural amino acids. This peptide sequence corresponds to the amino acid residues 114−126 of M.*Cvi*BIII, which contains a tyrosine residue at position 122, strongly suggesting that this is the site of DNA coupling with the intact Mtase. The C-terminal part of the nucleopeptide and its overall structure were verified by ESI-MS. The DNA strands of the covalently linked nucleopeptide underwent dissociation in the transport region of the electrospray interface, and the modified strand was detected as 5- to 9-fold negatively charged ions. A deconvoluted mass spectrum of the modified strand showed that the mass of the nucleopeptide was found to be 5900.5 Da, in excellent agreement with the calculated M_r of 5900.6 Da for a nucleopeptide containing the amino acid residues 114−129 of M.*Cvi*BIII, which has a chymotrypsin cleavage site at its C-terminal end.[195]

Wong and Reich[196] describe a similar highly sensitive strategy combining laser-induced photo-cross-linking and HPLC/ESI-MS for identification of the amino acid residues involved in Mtase−DNA recog-

Chart 10

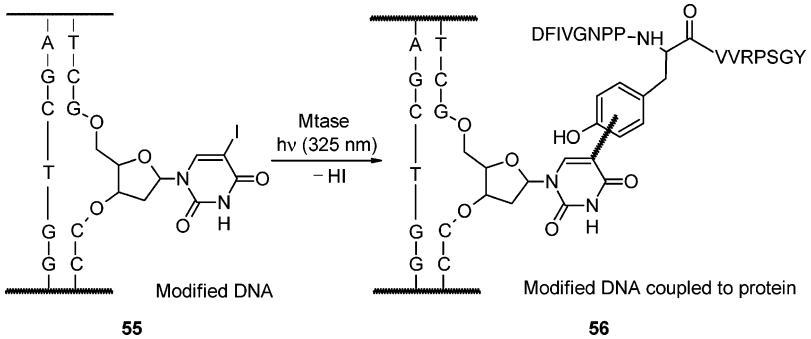


Table 8. ESI-MS Data for Complex of M.*Eco*RI with a DNA Strand

species	calcd mass/Da	exptl. mass/Da
DNA (dGGCG GAATTCGCGG)[a]	4345.1	4344.7 ± 0.43
DNA-I (dGGCG GAA**I**TCGCGG)[b]	4456.1	4455.3 ± 0.08
M.*Eco*RI (325 amino acids)	37913.4	37916.9 ± 30.52
DNA-M·*Eco*RI complex	42241.5	42242.9 ± 21.28

[a] The M.*Eco*RI recognition site in the mer-14 oligonucleotide is underlined. [b] The recognition site is modified by substitution of iodouracil (**I**) for thymine (T).

nition. The covalent linking of hemimethylated DNA duplexes to the enzyme M.*Eco*RI was achieved by photochemical coupling between a 5-iodouracil, incorporated in the recognition site, and the tyrosine 204 residue in the enzyme. Negative ionization ESI-MS analysis was utilized to obtain the accurate masses of these oligonucleotides. As shown in Table 8, the mass data provide unequivocal confirmation of the structure of the component species and the coupled complex.

Although the biological significance of cytosine methylation is not fully understood, there is growing evidence strongly suggesting a link between the development of human cancer and perturbation of the methylation patterns resulting from the infidelity of DNA cytosine Mtase. A novel in vitro assay called mass tagging was developed by Sowers and coworkers,[197] which permitted quantification of the DNA substrate preferences of cytosine Mtases. This approach involved the labeling of target cytosine residues in synthetic DNA duplexes with stable isotopes, such as ^{15}N. Methylation was then measured by the formation of 5-methylcytosine (5mC) by GC-EI-MS, and DNA substrate selectivity could then be determined from the MS by the absence or presence of the label in 5mC. This mass-tagging approach is a powerful tool for examining the substrate selectivity of cytosine DNA methyltransferases and could easily be adapted to the study of other kinds of selectivity.

4.3.2. Mapping of the 5′-(2-Deoxyribose-5-phosphate) Lyase Active Site in Covalently Linked DNA Polymerase β

Mammalian DNA polymerase β (β-pol) is a constitutively expressed enzyme, implicated in DNA base excision repair (BER), probably the major repair pathway protecting cells against single base DNA damage. Some insight into the mechanism of BER has been sought by Deterding et al.,[198] who cross-linked a 5′-(2-deoxyribose-5-phosphate) DNA to the 8 kDa β-pol domain by $NaBH_4$ reduction. The protein−DNA complex was purified and then subjected to peptide mapping and MS sequencing analyses. The ESI-MS of the protein−DNA complex showed a peak at M_r 10 010 with only a small peak for the native 8 kDa protein at M_r 9467, confirming a stable adduct (Figure 13). To determine which amino acid residue was covalently cross-linked to the DNA, the complex was subjected to tryptic digestion followed by MS/MS sequencing. An ion at *m/z* 981.5, corresponding to the doubly charged ion $[M + 2H]^{2+}$ of a DNA-adducted tryptic fragment (amino acid residues 69−81), was selected for tandem MS. The observed fragmentation pattern located the DNA adduct unequivocally at the lysine-72 site (Table 9). This elegant work illustrates the power of MS/MS admirably.

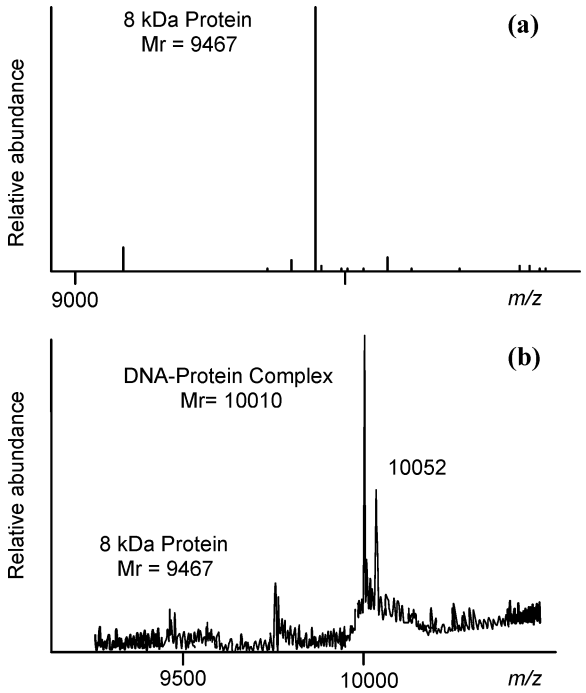
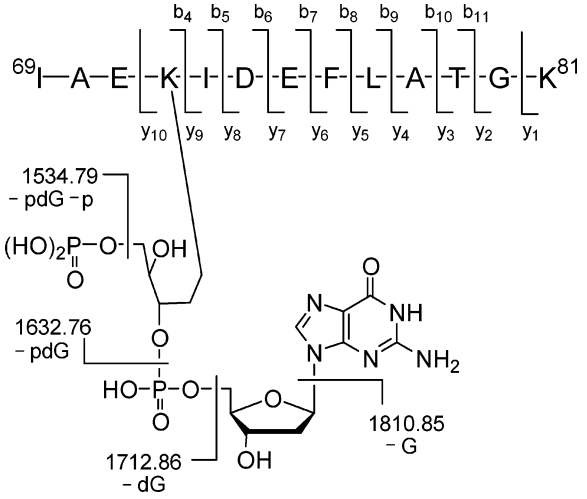


Figure 13. ESI mass spectra of (a) an 8-kDa domain of DNA polymerase β (M_r = 9467) and (b) the HPLC-purified covalent complex of this protein with DNA. The increased mass indicates a DNA fragment of about 543 Da (or less if there is any oxidation of the protein). The ion at *m/z* 10 052 corresponds to the complex solvated with one molecule of acetonitrile. (Adapted with permission from ref 198. Copyright 2000 American Society for Biochemistry and Molecular Biology.)

4.3.3. Covalent Binding of Leukotriene A4 to 2′-Deoxynucleosides and 2′-Deoxynucleotides

Leukotriene A4 (LTA4), a chemically reactive conjugated triene epoxide, is formed by reaction of arachidonic acid with molecular oxygen catalyzed by 5-lipoxygenase. LTA4 is an intermediate in the formation of the biologically active eicosanoids leukotriene B4 and leukotriene C4. Reiber and Murphy[199] applied ESI-MS and MS/MS to the study of the covalent binding of LTA4 with uridine, cytidine, adenosine, and guanosine. Competition reactions with nucleoside mixtures established that guanosine was most reactive toward LTA, yielding five major and at least six minor covalent adduct species. Data from CID-MS/MS of the molecular anion $[M - H]^-$ at *m*/z 600.3 suggested that guanosine attacked LTA4 at either C-12 or C-6 with opening of the epoxide at C-5 to yield a series of adducts. Observation of the ready covalent attachment of guanine to LTA4 raises the possibility that this intermediate of leukotriene biosynthesis formed on or near the cellular nuclear envelope may react with nucleosides and nucleotides present in RNA or DNA.[199]

Table 9. Mass Spectrum Fragmentation Data for the $(M + 2H)^{2+}$ Ion from a Tryptic Digest Fragment of the Covalent Adduct of DNA and DNA Polymerase β



fragmentation	calcd mass	obsd mass
−G	1810.82	1810.85
−dG	1712.78	1712.86
−pdG	1632.81	1632.76
−pdG − p	1534.84	1534.79
y_1	147.11	147.15
y_2	204.13	204.18
y_3	305.18	305.23
y_4	376.22	376.27
y_5	489.30	489.35
y_6	636.37	636.43
y_7	765.41	765.46
y_8	880.44	880.45
y_9	993.53	993.53
y_{10} − dG	1399.62	1399.71
b_4 − dG	720.26	720.36
b_5 − dG	833.35	833.45
b_6 − dG	948.37	948.42
b_7 − dG	1077.72	1077.47
b_8 − dG	1224.48	1224.52
b_9 − dG	1337.57	1337.59
b_{10} − dG	1408.61	1408.62
b_{11} − dG	1509.65	1509.70

5. Matrix-Assisted Laser Desorption/Ionization MS for Characterization of Oligonucleotides

Over the last two decades there has been a huge research effort directed to the MS-based characterization of oligodeoxynucleotides and oligoribonucleotides, including sequencing of DNA by direct MALDI-MS analysis,[200,201] RNA sequencing,[201−203] analysis of DNA tandem repeats,[204] detection of single nucleotide polymorphisms (SNPs) using PNA probes,[205,206] genetic diagnosis, genotyping, mutation detection, and probing virus structures.

Concurrent with the initiation of the human genome-sequencing project, the two research groups of Tanaka and co-workers[40] and Karas and Hillenkamp[39] independently developed MALDI-TOF-MS. Initially this method predominantly found its application in the analysis of proteins and peptides with most studies using ion extraction usually in the positive-ion mode. To extend the MALDI technique to MS sequencing of DNA it became clear that choice of the right matrix and the right laser wavelength[207,208] was crucial. The difficulties encountered in the early application of MALDI mass spectrometry to nucleic acids have been reviewed in detail.[209] In 1995 MALDI instrumentation took a quantum leap when Vestal rediscovered the principle of delayed extraction and integrated it into the MALDI process.[210] This step dramatically improved the resolution of the spectra, and the use of MALDI instruments began to move from the hands of the specialists into the wider research community.

In this section the past decade is reviewed with a special emphasis on the different strategies used in MALDI-MS for sequencing DNAs and RNAs of biological importance. SNP genotyping by MALDI-TOF-MS has been reviewed recently.[211]

5.1. Sample Preparation for MALDI Analysis of Oligodeoxynucleotides

A systematic study of the ionization of the four standard nucleosides in a MALDI-TOF mass spectrometer has been carried out by Zhang and co-workers.[212] Using several common matrixes they confirmed that ionization of DNA in the MALDI procedure was usually dominated by protonation and deprotonation of the bases and does not involve the backbone significantly. Thus, the low ion signal previously reported for DNA in poly-G in MALDI is due to the difficult ionization of guanine. Protonation was dominated by a preprotonation step before laser ablation, and deprotonation was controlled by the thermal reaction, both reactions being strongly structure dependent.[212]

The analysis of oligonucleotides by MALDI-TOF-MS has generally been carried out using negative ionization conditions, usually following ammonium ion-exchange chromatography and the addition of ammonium buffers to the MALDI matrix. The molecular ion region can be very complex as a result of the variable degree of ammoniation of the phosphate backbone, and this causes a decrease in sensitivity (compared with similar size peptides) and ambiguity in the assignment of the relative molecular mass of the sample. Langley and co-workers[213] found that the use of a H^+ ion-exchange resin in situ permitted removal of alkali-metal ions from the oligonucleotide phosphate backbone, and this gave a gain in sensitivity of 1−2 orders of magnitude compared with previous MALDI-TOF methods. In other cases, when DNA was not significantly contaminated, NH_4^+ organic salts as matrix additives were used to replace alkali-metal ions in the polyphosphate backbone chain. To convert oligonucleotides into the ammonium form, ammonium citrate or tartarate salts are usually used.[213] In-line microdialysis has also used for rapid desalting prior to MALDI-MS.[214]

A novel approach to sample preparation, developed by Smirnov and collaborators,[215] is based on the extraction of DNA out of solution onto a solid surface with an attached DNA-binding polymer, such as polyethyleneimine or poly(vinylpyrrolidone). Binding is strong enough to sustain washing, and thus, desalting and concentration can be performed in a single fast step. After removal of the supernatant solution, the addition of the MALDI matrix material releases the DNA from the surface to allow the

required cocrystallization on the support. The mass spectrometric analysis is then performed directly from this support.[215] A similar, fast, efficient method for desalting for oligonucleotides (and peptides) is the use of 96-well solid-phase extraction plates packed with a reverse-phase sorbent that retains the biopolymer analytes while nonretained inorganic ions are washed out with deionized water.[216]

Finally, an interesting study by Bentzley and coworkers revealed variation in the robustness of oligonucleotide strands when subject to repeated freeze/thaw cycles.[217] Using MALDI-MS to monitor purity, these workers have shown that, contrary to general belief, oligonucleotide strands can suffer significant degradation when taken through repeated freeze/thaw cycles. Thus, systematic degradation during the sample preparation and handling procedures is more of a problem than has been appreciated. The extent of degradation is dependent on base composition, solute concentration, strand length, and thawing conditions.

5.2. Matrixes

Acceptable mass resolution and sensitivity for DNA oligomers of increased size has been a constant challenge for MALDI-MS. Routine detection of DNA oligomers larger than 200 bases has usually been problematic as adduct formation and fragmentation are still major limitations, and hence, development of new matrixes and MALDI experimental strategies has received much attention.

A new matrix, 3-hydroxypicolinic acid (HPA), introduced in 1993, showed significant improvement when compared directly with a set of 47 other compounds which included most matrixes used previously.[218] HPA was the best matrix in terms of mass range, signal-to-noise ratio, and ability to analyze mixed-base oligomers up to 67 nucleotides. It has been suggested[219] that picolinic acid is even better than HPA, as evidenced by the detection of oligonucleotides up to 190 bases. Another group of compounds with matrix potential are those with an intramolecular hydrogen bond as in 2-hydroxyacetophenones, 2-hydroxybenzophenones, salicylamide, and related species.[220,221] A unique matrix system, consisting mostly of 4-nitrophenol, was shown by Lin and collaborators[222] to be very effective for the analysis of large DNA oligomers when a cooled sample stage was used to prevent the sublimation of this matrix under vacuum. UV laser desorption from this matrix allowed routine detection of DNA oligomers containing up to about 800 nucleotides at the picomole level. The effectiveness of this matrix was further demonstrated by the observation of a double-stranded DNA oligomer larger than 1000 base pairs, seen as a denatured single-stranded species, with a molecular ion mass exceeding 300 kDa.[222]

Lack of homogeneity in a sample makes it difficult to routinely optimize the laser fluence, and some spots can receive excess energy resulting in loss of sensitivity due to the formation of metastable ions. Shahgholi et al.[223] found that addition of a sugar such as fructose or fucose to the matrix minimizes the effect of excess laser energy and allows detection, for example, of the 9 Da mass difference which characterizes an A/T mutation.

The use of a liquid matrix such as glycerol is known to give better reproducibility, but since glycerol is nonabsorbing in the UV region, a second component must be included. For example, the binary mix of 4-nitroaniline and glycerol is effective for positive- and negative-ion formation for oligonucleotides and other biomolecules.[224] Alternatively, desorption and ionization can be achieved by an IR laser, and Hillenkamp and co-workers[225−227] have shown that IR-MALDI with a glycerol matrix gave excellent results for large double-stranded DNA. Very little fragmentation was observed with an IR laser in the high-mass range compared to UV-MALDI, thus extending the mass limit for IR-MALDI to ca. 500 kDa with a sensitivity in the subpicomole range and a comparable reproducibility.

An ingenious approach to desalting involves the use of 3,4-diaminobenezene as a matrix material dispersed in a sol−gel, the combined material acting as a sample support. Sodium ion adduction is effectively suppressed, allowing detection of a 24-mer oligonucleotide at the 20 fmol level.[228] Similar results are reported[229] for a sol−gel sample substrate doped with a crown ether.

Perhaps the most recent advance in matrix design is the use of a mixture of 3-hydroxypicolinic acid (HPA) and pyrazinecarboxylic acid (PCA). With model synthetic oligonucleotides Deng and co-workers[230] used this matrix to obtain MALDI-TOF mass spectra with isotopic resolution for DNA segments up to 10.5 kDa in mass. Equally impressive is the detection of separate ion peaks for both components of a mixture of two 23-mer DNA segments with a 7 Da difference. With respect to reproducibility, resolution, signal-to-noise ratio, and tolerance to metal salts in DNA analysis, this mixed HPA/PCA matrix is superior to HPA alone.[230]

5.3. Structural Studies of DNA and RNA Using MALDI-TOF-MS

Compared to peptides, the overall sensitivity of DNA analysis by MALDI-TOF-MS is about 100 times lower due to fragmentation, adduct formation, and low ionization efficiency. An improvement in detectability and stability of DNA analysis by MALDI-TOF-MS was recently reported by Berlin and Gut,[231] who modified the oligonucleotides to neutralize all backbone phosphates except one, thus creating a species with a single negative charge. Using the same nonprotonating matrix the enhancement in sensitivity achieved by this relatively simple modification was comparable to that observed in earlier work for DNA carrying a single positive charge, where the required derivatization is more complex. The mechanistic implications of these findings regarding the MALDI process were discussed in detail.

Some recent DNA mapping studies[232] show how technical advances such as time-lag focusing and a carefully selected matrix give excellent results for mixtures of different oligonucleotides. For example, Figure 14 shows the quality of the MALDI-TOF spectra obtained for a mixture of oligonucleotides

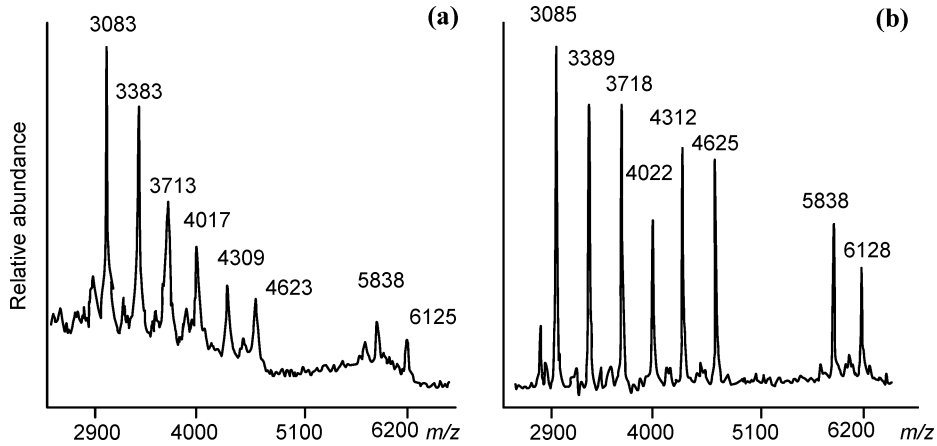


Figure 14. MALDI-TOF-MS spectra of a mixture of species (containing 10, 11, 12, 13, 14, 15, 19, and 20 nucleotides, respectively) derived from plasmid pBR322. Spectra were obtained (a) as negative ions without time-lag focusing and (b) as positive ions with time-lag focusing. (Adapted with permission from ref 232. Copyright 2000 Elsevier Science.)

derived from the plasmid pBR322 (10, 11, 12, 13, 14, 15, 19, 20, and 50 nucleotides long), particularly when time-lag focusing is employed.

Since the early 1980s the method of choice for producing oligonucleotides is solid-phase synthesis (phosphoramidite approach) using, most commonly, controlled-pore glass (CPG) as the solid support. Standard practice has the growing oligonucleotide attached to the solid phase by a succinyl linker group. Vasseur and collaborators[233] tried to evaluate the efficiency of the solid-phase synthesis by MALDI-TOF-MS by employing a photolabile linker group which could be cleaved from the solid support by laser irradiation. Thus, oligonucleotide cleavage and ionization were achieved simultaneously. However, this procedure was less effective for longer oligonucleotides. Analogous MALDI analysis of succinyl-linked oligonucleotides generated fragment ions in either negative or positive mode, which allowed direct access to the nucleotide sequence and identification of the internucleosidic linkage. The Vasseur group[234,235] has shown that the mass ladder generated in the MALDI-TOF spectra of a 3′-CPG-supported cyanoethylphosphotriester oligonucleotide clearly indicated the sequence except for the last two residues at the 5′ end (Figure 15) and readily detected and identified modified residues, nonnucleosides spacers, or abnormal internucleosidic linkages.

Using both static and delayed ion extraction, Hillenkamp and colleagues[225] investigated IR-MALDI-MS with a CO_2 laser (10.6 μm). Compared with an Er:YAG infrared laser ($\lambda = 2.94$ μm, $t = 90$ ns) and a frequency-tripled Nd:YAG ultraviolet laser ($\lambda = 355$ nm, $t = 15$ ns), fewer metastable ions were observed with the CO_2 laser, especially operating in the reflectron mode of the spectrometer.

Thus, mass spectra of large biomolecules up to several hundred kilodaltons could be better analyzed using a reflectron time-of-flight mass spectrometer using CO_2-MALDI-MS. Examples of the use of this technique include the analysis of gel-separated and electroblotted proteins, desorbed directly from a PVDF membrane and a double-stranded DNA of 515 base pairs.[225] Taranenko et al.[236] demonstrated that UV-MALDI-TOF-MS could be used to determine the molecular weight of PCR products of intact 16S rRNA

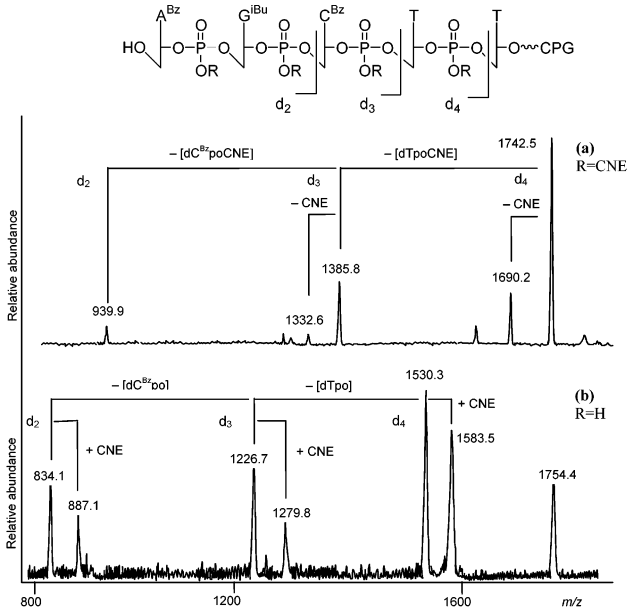


Figure 15. MALDI-TOF mass spectra of negative ions from 3′-CPG-supported oligonucleotides. The spectrum (a) of a cyanoethyl phosphotriester (poCNE) of 5′-AGCTT-3′ shows successive cleavage at the phosphate from the 3′ end with partial loss of CNE. (b) After treatment of the 5-mer with 90 mM DBU solution in THF the CNE group is mostly removed, but the same pattern of fragmentation is observed. (Adapted with permission from ref 233. Copyright 2000 John Wiley and Sons Ltd.)

regions up to 1600 nucleotides in length and profile their restriction digests.

Most established methods for the detection of specific sequences in DNA are based on the use of hybridization probes containing labels that can be directly (radioactivity, fluorescence) or indirectly (hapten, enzyme) detected. In the case of highly multiplexed DNA assays, one important limitation of this approach is the maximum number of different labels that can be simultaneously detected. In the MALDI-TOF-MS DNA multiplex assays the use of a photocleavable peptide−oligonucleotide conjugate has been found to be an attractive method since the peptide portion could serve as a mass marker label with unique mass. The synthesis and characterization of three photocleavable peptide−DNA conjugates

have been described by Olejnik and colleagues.[237] These conjugates were evaluated on the basis that the DNA part acts as the hybridization probe and the peptide a marker for the target DNA sequence. This approach will allow further studies requiring highly multiplexed analysis including the properties of antisense or therapeutic oligonucleotides, such as target binding properties, cellular uptake, or exonuclease stability.

The inefficiency of nucleotide coupling during production-scale synthesis of oligonucleotides can be monitored by MS.[238] The failure species (N-1, N-2, N-3, etc.) are separated from crude synthetic material by solid-phase extraction and analyzed by MALDI-TOF-MS. The sequence information was obtained for a series of failure ions, each of which varied by the molecular mass of the specific nucleotide not coupled.

5.4. Sequencing of DNA by Digestion with Exo- and Endonucleases

DNA sequencing by controlled exonuclease digestion followed by mass spectral analysis of the resulting ladders for DNA sequencing was first demonstrated by Pieles et al.[239] Exonucleases, such as snake venom phosphodiesterase (PDase I) and bovine spleen phosphodiesterase (PDase II), sequentially remove mononucleotides from DNA oligodeoxynucleotides by hydrolyzing the phosphodiester bonds. PDase I attacks DNA at the 3′ end and releases 5′-mononucleotides, and PDase II attacks the oligodeoxynucleotide at the 5′ end and releases 3′-mononucleotides. Both of these phosphodiesterases are nonprocessive enzymes that dissociate from the DNA molecule after removal of each nucleotide and bind DNA again to catalyze the release of the next monomer. Time-controlled exonuclease digestion of DNA thus generates a complete set of oligodeoxynucleotide fragments differing from each other by one mononucleotide, ideal for MS analysis. The introduction of delayed extraction in MALDI has made it possible to expand this approach to longer DNA fragments due to improved resolution and reduced fragmentation. Oligodeoxynucleotides up to 50 bases in length were routinely sequenced using the information from separate digestions with 3′ to 5′ and 5′ to 3′ phosphodiesterases and analysis by MALDI-TOF-MS.

The relative intensities of ladder peaks in the mass spectra of a series of 5-mer and 7-mer oligonucleotides showed that the rate of PDase II digestion was influenced by the sequence of bases in the DNA strand.[240] Thus, 5′-terminal A or G nucleotides are cleaved two to three times faster than sequences terminating in C or T. This sensitivity of PDase II activity to base type extends to at least the third base in the sequence. Reaction rates can be determined quantitatively from the time evolution of digestion as illustrated in Figure 16 for a typical 7-mer oligonucleotide. The initial MALDI spectrum shows the molecular ion before digestion, and the spectra taken at 3 and 6 min show clear evolution of ladder peak intensities for each of the species generated by sequential loss of the 5′ residue.[240]

Recently, it has been found by Wada[241] that the MALDI-TOF-MS of a restriction endonuclease digest

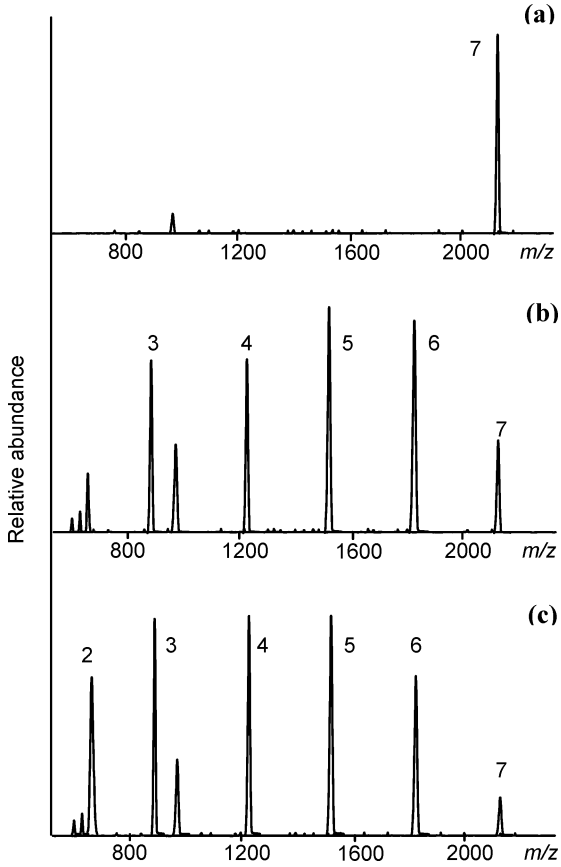


Figure 16. MALDI spectra of the 7-mer oligonucleotide 5′-AAAGTAA-3′ as a function of the time of digestion with PDase. (a) Obtained before digestion, showing only the molecular ion. (b and c) Obtained after 3 and 6 min digestion, respectively, clearly showing development of the sequence ladder by progressive removal of the 5′-nucleotide. (Adapted with permission from ref 240. Copyright 1998 Academic Press.)

facilitated the determination of the molecular mass of a PCR-amplified DNA more easily than the measurement of the undigested DNA. By this method a 664 base-pair region from the FAS gene was analyzed and a two-nucleotide deletion in the L1CAM gene in a restriction fragment of 105 nucleotides was detected. Furthermore, the analysis of smaller fragments allowed separate detection of single-stranded oligonucleotides comprising individual digested fragments. This mixture analysis of restriction enzyme digests improved the resolution, sensitivity, and accuracy of MALDI-TOF-MS of DNA and was thus expected to facilitate its application to genetic diagnosis. The calculated masses for a 664 bp segment of the FAS gene were 205 966/204 780 for sense/antisense strands with dA overhang at the 3′ ends. In the MALDI-TOF-MS a broad signal corresponding to these DNA molecules was detected at *m/z* 210 000 with low intensity (Figure 17a). The PCR product was then digested with restriction endonucleases *Msp*I and *Bam*HI that cleave at positions 296 and 441, respectively. The MALDI-TOF-MS of the digest revealed discrete molecular ion signals for three fragments, although the complementary strands were not resolved in each peak (Figure 17b). The signal for the fragment observed at *m/z* 46 000 was sharp compared with the larger one at *m/z* 93 500,

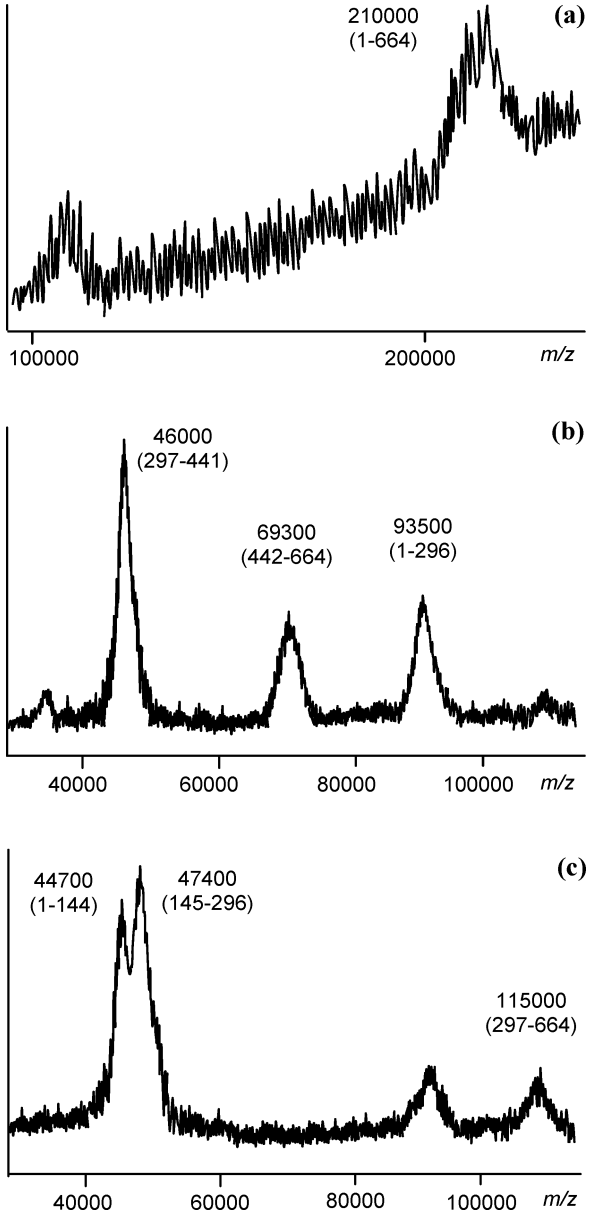


Figure 17. Positive-ion MALDI-TOF mass spectra of a 664bp DNA amplified from the FAS gene. The ion *m/z* values are given with the sequence numbers of the digest fragment in parentheses. (a) The undigested PCR product. The doubly charged ions are observed at *m/z* 110 000. (b) Digest produced by restriction endonucleases *Msp*I and *BamH*I. (c) Digest produced by *Alu*I and *Msp*I. The peak at *m/z* 93 000 is probably uncleaved DNA corresponding to sequence 1-296. (Adapted with permission from ref 241. Copyright 1998 John Wiley and Sons, Ltd.)

and their peak widths at half-height were 2500 and 4000 units, respectively. The peak at *m/z* 93 000 was probably uncleaved DNA corresponding to sequence 1−296 (Figure 17c).[241]

5.5. Sanger Sequencing Ladders Produced by Dideoxynucleotide Chain Termination of PCR Products

Sequencing by the Sanger enzymatic method using gel electrophoresis to read the sequence is relatively slow, especially for genomic research, and the advent of MALDI-TOF-MS methods has been welcomed. Early MS studies were restricted to short oligomers,

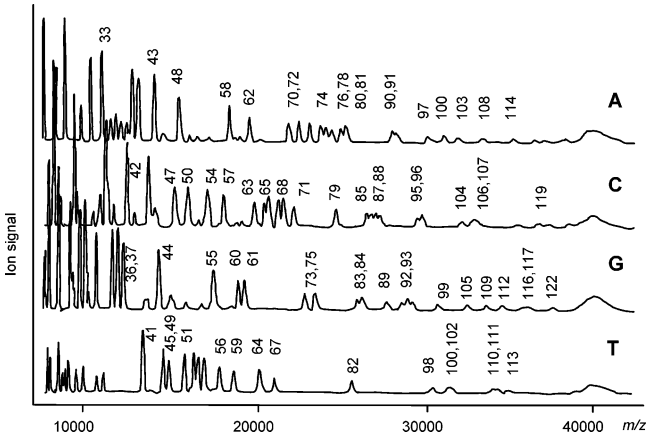


Figure 18. Negative-ion mass spectra of Sanger sequencing ladders produced by dideoxynucleotide chain termination of the PCR product of a double-strand 130 bp template (+ strand) with a 20 nt reverse primer. (Adapted with permission from ref 242. Copyright 1998 Oxford University Press.)

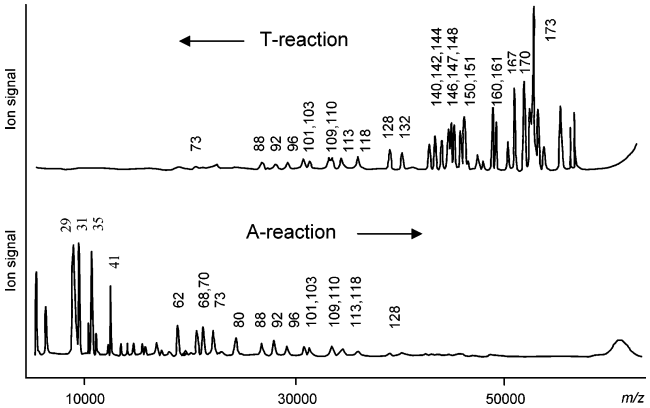


Figure 19. Negative-ion mass spectra of Sanger sequencing ladders produced by dideoxynucleotide chain termination of the PCR product of a double-stranded 200 bp template. The results for the A reaction with reverse primer and T reaction with forward primer are presented. (Adapted with permission from ref 242. Copyright 1998 Oxford University Press.)

but sequencing of longer DNA fragments using MALDI-TOF DNA ladder detection has been demonstrated by Taranenko et al.[242] Using 20-mer primers they successfully obtained DNA ladders from (ds)DNA templates of 130bp (Figure 18). Both forward and reverse primers were used, and the MALDI-TOF-MS of both modes could be combined to give a full 200 bp sequence (Figure 19). With improved resolution and sensitivity mass spectrometric DNA sequencing has thus become a very valuable tool for de novo sequencing, and with the rapid discovery of new genes, mass spectrometry has also evolved as a very valuable method for re-sequencing for mutation detection.

The application of MALDI-TOF-MS to rapid sequencing of short DNA stretches (15−20 nucleotides) was reported by Nordhoff and collaborators[243] using a protocol based on the Sanger enzymatic extension reactions concept with a double-stranded template DNA. All four sequencing reactions were performed simultaneously in one reaction vial. The sequencing products were separated and detected by MALDI-

TOF-MS. The sequence was determined by comparing the measured molecular mass difference with the expected values. One reaction mixture typically includes 300 fmol of (ds)DNA template, 10 pmol of primer, and 200 pmol of each nucleotide monomer. It should be noted that neither the primer nor any of the nucleotide monomers were labeled. Solid-phase purification, concentration, and mass spectrometric sample preparation of the sequencing products were accomplished in a few minutes, and parallel processing of 96 samples was possible. The mass spectrometric analyses and subsequent sequence read-out required only a few seconds per template.[243] This work confirms the increasing trend to high-throughput MS analysis of DNA.

Protonation of the nucleobase leading to base loss and subsequent backbone cleavage is one mechanism of fragmentation which limits mass range and resolution in MALDI-MS analysis of DNA. Smith and colleagues[244] established that replacement of a 2′-hydrogen with a fluorine atom stabilized the N-glycosidic linkage, thus reducing or blocking loss of the nucleobase. The practical usefulness of this approach to improving MALDI-MS sensitivity was demonstrated[245] by the successful incorporation of 2′-fluoronucleoside triphosphates into a growing chain by several DNA polymerases. Furthermore, the presence of these modified oligonucleotides did not affect the incorporation of a dideoxy terminator step in a Sanger sequencing reaction. The UITma DNA polymerase gave the best results in the development of novel 2′-fluoronucleic acids suitable for MALDI analysis using the matrix 2,5-dihydroxybenzoic acid. Fragmentation of these modified nucleic acids during MALDI-TOF-MS was much less when compared to the analogous unmodified species.[245]

5.6. Single Nucleotide Polymorphism

Single nucleotide polymorphisms (SNPs) are the most common type of human genetic variation. Although a number of known inherited diseases are caused by an SNP at a particular locus, many complex disorders such as cancers may result from cumulative effects of several deleterious SNPs. The analysis of such variations is of great interest to the pharmaceutical industry in the quest to design drugs customized for individual patient-specific responses.[246] SNPs are single-base changes that occur at a specific position in a genome, and for these positions different sequence alternatives (alleles) exist in individuals in some populations. By definition it is referred to as a mutation only when the less-frequent allele has an abundance of 1% or greater. On average, one SNP is found in every 500−1000 bases in humans. Only a small portion lies within coding regions, and an even smaller percentage is responsible for amino acid changes in expressed proteins. Tost and Gut[247] recently presented an excellent review on the major contributions in the field of genotyping single nucleotide polymorphism by mass spectrometry. The range of applications in this field is illustrated by the following selected studies.

The use of nuclease selections for genotyping, described by Stoerker et al.,[246] was dependent upon the DNA to be analyzed being treated with oligonucleotide probes which represented known genotypes and with digesting probes that are not complementary to the DNA. With phosphodiesterase I the target-bound, complementary probe was largely refractory to nuclease attack and its peak persists in mass spectra. In optimized assays both alleles of a heterozygote were genotyped with six nanomer DNA probes (>125 fmol each) and asymmetrically amplified DNA from exon 10 of the cystic fibrosis transmembrane regulatory gene (CFTR).

A method for typing SNPs by MALDI-TOF-MS has been described by Fei and Smith[248] in which a mass-tagged dideoxynucleoside triphosphate was employed in a primer extension reaction in place of an unmodified dideoxynucleoside triphosphate (ddNTP). The increased mass difference due to the presence of the mass tag greatly facilitated the accurate identification of the added nucleotide and was particularly useful for typing heterozygous samples. Further validation of the method was demonstrated in the analysis of five single-base mutations in exon IV of the human tyrosinase gene. Single nucleotide variations within 182 base-pair PCR amplicons amplified from three plasmids and three human genomic DNA samples were genotyped at five variable positions with results in 100% concordance with conventional sequencing. Genotypes were determined accurately at five sequence-tagged sites.[148]

Genotyping of short tandem repeat polymorphisms (STRs) has been reported by Seichter and colleagues.[249] For characterization of dinucleotide repeats, essential in animal genome studies, enhanced resolution MALDI-MS is required. This increase in mass resolution in MALDI-TOF-MS was achieved with RNA and modified DNA containing substituents which discourage any intramolecular reactions that provoke fragmentation. RNA transcripts were synthesized enzymatically from PCR products containing a promoter sequence, requiring no specialty reagents or primer labels. Furthermore, the RNA transcripts produced were single-stranded, a prerequisite for high-resolution mass spectrometry of nucleic acids. The resolution of the MALDI-TOF-MS obtained from RNA transcripts was found to be superior to that of DNA molecules of comparable size. For the sake of comparison, it is interesting to note that although the resolution (full-width half-maximum) of DNA dropped significantly with increasing fragment length (50-mer, 330; 75-mer, 115; 100-mer, 40), it remained ~200 for all the RNA sizes observed.[249] More recently these workers characterized STRs in endonuclease-cleaved RNA transcripts by MALDI-TOF-MS.[250]

The measurement of polymorphic trinucleotide repeats in the androgen gene receptor was recently reported by Wada and collaborators.[251] Among the four different triplet sequences known to cause diseases, CAG, CCG, CTG, and GAA, it was established that CAG repeats were responsible for fewer cases of pathological expansion than other types of repeat. The molecular mass of amplified DNA (200 base pairs) from the region containing the CAG repeat of the androgen receptor gene was measured by MALDI-TOF-MS. The single-charged molecular

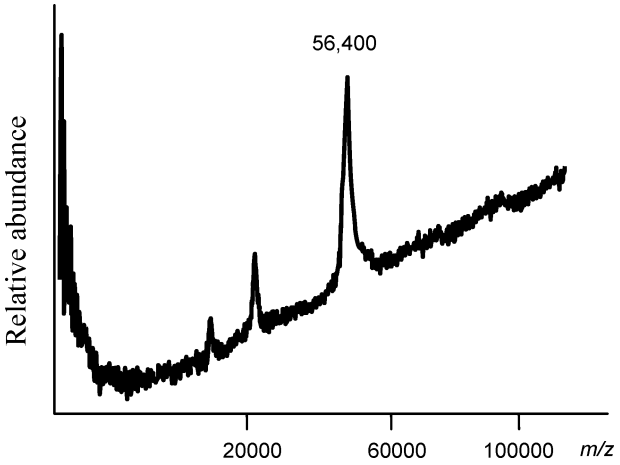


Figure 20. MALDI-TOF mass spectrum of an amplified DNA product from the androgen receptor gene containing 17 CAG repeats. (Adapted with permission from ref 251. Copyright 1999 John Wiley and Sons, Ltd.)

ion was detected using 0.1 pmol of DNA sample, and the number of repeats was determined from the molecular mass (Figure 20). It was established that the length of the CAG repeat sequence in the first exon of the androgen receptor gene was normally polymorphic, and the sequence ranged from 12 to 36 repeats.

A new minisequencing method using MALDI-TOF-MS was developed by Hung et al.[252] for the screening of the 1691G → A substitution in coagulation factor V. This point mutation was found to result in an amino acid substitution of an Arg^{506} residue with a Gln residue in the factor V molecule. This mutation was also defined as factor Vleiden and was responsible for activating protein C (APC) resistance which is the most common genetic risk factor for familial thrombophilia. In this method the authors amplified a fragment of genomic DNA containing the 1691st base, followed by minisequencing in the presence of dGTP and ddATP, ddCTP, and ddTTP. In this manner the primer was extended by one base from one allele and two bases from the other allele. The extended products were analyzed using MALDI-TOF-MS. The base at position 1691 was identified based on the number of nucleotides added.

A novel genotyping method was developed by Wolfe et al.[253] that entailed the incorporation of a chemically labile nucleotide by PCR, followed by specific oxidative chemical cleavage at every site of the resulting amplicon with the modified base. This method was termed incorporation and complete chemical cleavage (ICCC), utilizing modified nucleotides 7-deaza-7-nitro-dATP, 7-deaza-7-nitro-dGTP, 5-hydroxy-dCTP, and 5-hydroxy-dUTP, which have increased chemical reactivity but are able to form standard Watson−Crick base pairs. The identity of the cleaved fragments determined the genotype of the DNA. The MALDI-TOF-MS analysis was used to analyze the molecular masses of the cleaved fragments. Compared with other MS-based genotyping methods that use dideoxynucleotides, the ICCC strategy provided great flexibility and the unique opportunity to generate two useful sets of genotype-specific DNA fragments within a single assay, enabling the possibility of internal confirmation of genotype assignment.[253]

Clinical specimens, such as body fluids, often contain only a small fraction of mutated cells, and assays to detect mutations need to be highly sensitive, specific and amenable to high-throughput automated working to allow large-scale screening. Recently, Sun and co-workers[254]described a screening method, termed PPEM (PNA-directed PCR, primer extension, MALDI-TOF), that effectively addresses the requirements of assays for mutation screening. DNA samples were first amplified using peptide nucleic acid (PNA)-directed PCR clamping reactions in which mutated DNA was preferentially enriched. The PCR-amplified DNA fragments were then sequenced through primer extension to generate diagnostic products. Finally, mutations were identified using MALDI-TOF-MS. This method was shown to detect as few as three copies of mutant alleles in the presence of a 10 000-fold excess of normal alleles in a robust and specific manner. Furthermore, it can adapted for simultaneous detection of multiple mutations and is amenable to high-throughput automation.[254]

5.7. Probing Viruses with Mass Spectrometry

The probing of viruses with mass spectrometry offers a new perspective on the gas-phase and solution properties of these microorganisms, including viral−antibody binding, protein−protein interactions, and protein dynamics. The structures of viral proteins and viral DNA (or RNA) are now well characterized, and identifying a virus based on the mass of the protein and its enzymatic digestion fragments is now relatively routine. Posttranslational modifications of viral DNA have also been characterized by MS.[255] Studies of intact viruses by MS are also possible using charge detection for analyzing megadalton DNA particles.[32] This type of spectrometer measures the total charge z and the m/z value, and Benner and co-workers[256] report that tobacco mosaic virus produces an ion distribution with a maximum corresponding to around 500 positive charges (but ranging up to about 1000 charges). The maximum in the experimental mass distribution was in the range from 39×10^6 to 42×10^6 Da, in good agreement with the calculated mass value of 40.5×10^6 Da. Progress on the mass spectra of whole viruses has been reviewed recently.[257]

RNA species such as viral hepatitis C can exist as quasi-species, dynamic mixtures of mutants having one or more consensus sequences.[193] It is essential that this heterogeneity in the genomic structure of such viral populations be quantitatively assessed, particularly with respect to the quality control of live attenuated viral vaccines, where the presence of even small quantities of mutants or revertants may compromise vaccine safety. Chumakov and co-workers[258] compared a MALDI-TOF-MS protocol for the analysis of genetic variation in live mumps vaccine with the use of mutant analysis by PCR and restriction enzyme cleavage (MAPREC). The results obtained from both methods showed excellent correlation, illustrating the potential utility of MALDI-TOF-MS

for routine quality control of live viral vaccines and for assessment of genetic stability and quantitative monitoring of genetic changes in other RNA viruses of clinical interest.

5.8. SELDI-TOF-MS: A Rapid Method To Capture and Screen for Transcriptions Factors

The need for methods to identify disease biomarkers is underscored by the survival rate of patients diagnosed at early stages of cancer progression. Issaq and collaborators[259] reviewed the technique of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), a novel approach to biomarker discovery that combines two powerful techniques: chromatography and mass spectrometry. SELDI-MS is similar to MALDI-MS inasmuch that the mass of biomacromolecules is determined, based on time-of-flight, after creation of gaseous ions from the analytes in the solid state. Usually a matrix is used as the energy receptacle to assist in the ionization process. However, by using surface-enhanced chips that are compatible with TOF-MS instrumentation, analytes can be captured on chemically modified chip surfaces and analyzed by MS directly from the affinity chips. This technique has been used for biomarker identification as well as the study of protein−protein and protein−DNA interaction. The versatility of SELDI-TOF-MS has allowed its use in projects ranging from the identification of potential diagnostic markers for prostate, bladder, breast, and ovarian cancers and Alzheimer's disease to the study of biomolecular interactions and the characterization of posttranslational modifications.[4,259]

In a recent example of this technique customized affinity DNA chip surfaces were designed to capture the sequence-specific protein/DNA interaction of LacI binding to the *lac* operon promoter sequence. By coupling this unique LacI DNA promoter sequence to a chip surface the chip served to isolate LacI from the total protein lysate of *E. coli*. In addition, the chip surfaces utilized were compatible with physiological buffers used to capture the LacI protein, and hence, binding conditions were optimized on the chips. Accordingly, use of SELDI chip surfaces simplified molecular characterization of captured biomacromolecule analytes by MS.[260]

5.9. MALDI-MS Fragmentation and Sequencing of Oligoribonucleotides

The fragmentation of oligoribonucleotides has been much less studied than that of oligodeoxyribonucleotides, although MALDI investigations have shown that RNA is considerably more stable than DNA. Although RNA has the same ability to form zwitterions as DNA, the increased stability of RNA is due to the presence of the 2′-OH group of the ribose glycone (electron-withdrawing groups at the 2′-position have a strongly stabilizing effect on the N-glycosidic bond, reducing the incidence of its cleavage).[261] Irrespective of the ionization method used to produce the RNA gaseous ions, the major fragmentation pathway for RNA was thought to be the loss of a nucleobase followed predominantly (but not exclusively) by cleavage of the 3′-C−O bond.

Kiperkar and Krogh[261] developed a sensitive method based on MALDI tandem quadrupole/orthogonal TOF mass spectrometer to investigate RNA fragmentation as a means of locating posttranscriptional modifications. The RNA of interest was completely digested with a nucleotide-specific RNase, and the masses of the resulting digestion products were compared to the masses expected from the gene sequence. The product ions generated from MALDI-MS/MS experiments on singly protonated oligoribonucleotides were also studied. They found that complex spectra were obtained reflecting cleavage of the base and of the phosphodiester backbone.

Equal frequency of the loss of cytosine and guanine contrasted with a lower loss of adenine and essentially no cleavage of uracil. It was also found that although the probability of nucleobase loss was most likely the initiating event for oligoribonucleotide fragmentation, this was not that straightforward and depended both upon the ionization method used and the charge state of the ions.[261]

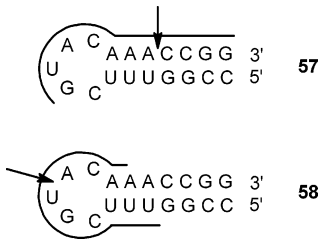
Recent applications of MALDI to RNA studies include the evaluation of in vitro formation of aminacyl 76-mer tRNA[262] and the conclusive demonstration of a disputed modification at C2501 in *E. coli* 23S ribosomal RNA.[263]

5.10. Analysis of RNA−DNA, DNA−Protein, and RNA−Protein Complexes

RNase H is an endonuclease that binds to RNA−DNA complexes and induces cleavage of the RNA strand, but the cleavage is often not selective owing to the possibility of scission at more than one point along the RNA strand, as indicated by the original work of Donis-Keller.[264] This problem was resolved to a large extent by using chimeric oligonucleotides in place of the DNA strand. These chimeras were shown to consist of a DNA tetramer flanked on either side by 2′-*O*-methylribonucleotides, which direct the RNase H cleavage to one position of the RNA backbone. The specificity of RNase H for RNA−DNA hybridization was shown to be organism dependent. To develop a suitable methodology capable of determining the identity of the resulting digestion products it was necessary that the cleavage be directed at the desired position along the RNA strand.

Polo and Limbach[265] described the use of MALDI-TOF-MS for the analysis of products resulting from the digestion of oligoribonucleotides with RNase H. The predicted secondary structure of the oligoribonucleotide hairpins used in this study and binding of chimeras 1 and 2 to the oligoribonucleotide hairpin are shown in Chart 11. This particular sequence of the oligoribonucleotide hairpin was selected to determine the effectiveness of RNase H cleavage on an oligoribonucleotide possessing a known, very stable structure. The two chimeras were designed to induce cleavage at a specific point either in the double-stranded region (**57**) or the single-stranded region (**58**) of the oligoribonucleotide as shown in Chart 11.[265] The RNase H reaction of the hairpin with each of the chimeras was monitored using MALDI-TOF-

Chart 11



MS (Figure 21), which provided a straightforward means of ascertaining that cleavage occurred at the expected location. Mass spectrometric analysis of the products was accomplished in a matter of minutes.

It has been reported that the intermediate filament subunit protein, vimentin, binds to nucleic acids via its non-R-helical head domain. Wang and co-workers[266] identified the amino acid residues responsible for the stable binding of the nucleic acids with vimentin. This was achieved by a combination of enzymatic and chemical ladder sequencing of photocross-linked vimentin−oligodeoxyribonucleotide complexes followed by analysis with MALDI-TOF-MS. Three tryptic peptides of vimentin (vim_{28-35}, vim_{36-49}, and vim_{50-63}) were found to be cross-linked to oligo-$(dGBrdU)_{12}$·dG3′-FITC through a tyrosine residue. The oligo $(dGBrdU)_{12}$·dG3′-FITC was chosen, since dG-rich oligonucleotides were found to bind better to vimentin than dT.

RNA−protein interactions are known to play an important role in different cellular processes, but knowledge of the molecular mechanism of RNA−protein recognition is still limited, although several conserved amino acid sequence motifs for RNA binding have been identified. The structural details of RNA−protein complexes have been traditionally obtained by X-ray crystallography and nuclear magnetic resonance spectroscopy. Recently, Thiede and von Janta-Lipinski[267] used MALDI-TOF-MS (negative-ion mode) to explore the noncovalent interactions between different peptides and ribose nucleic acids (RNAs). The best matrix for this work was 2,4,6-trihydroxyacetophenone, which showed approximately the same peak intensities for free peptides and RNA while allowing detection of RNA−peptide complexes.

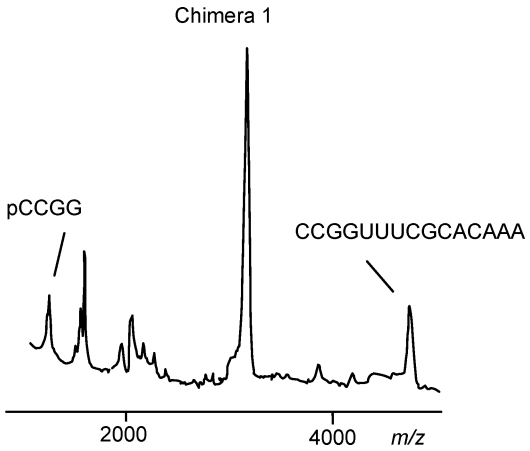


Figure 21. Negative-ion mode MALDI-TOF mass spectrum of the cleavage products of an oligonucleotide hairpin using chimera 1. The two indicated fragments have *m/z* values close to expectation and confirm the clean cleavage at the expected point. (Adapted with permission from ref 265. Copyright 1998 John Wiley and Sons, Ltd.)

6. MS Characterization of DNA Adducts

Since the 1960s it has been known that the mutagenic activity of many substances is linked to the degree to which they covalently bind to DNA. The analysis and measurement of carcinogen−DNA adducts has been proposed as a means of evaluation of the extent of human exposure to specific carcinogens and to monitor the presence and effects of these specific carcinogens in the environment.[56,128,268] DNA adduct analyses are complicated by the low frequency of DNA adduction that occurs in vivo. In experimental animals only about 10−100 pmol of adduct per milligram of DNA are normally formed following treatment with tumorigenic doses of carcinogens. Several techniques, including the use of radiolabeled mutagens or carcinogens, immunoassay, fluorescence, and ^{32}P-postlabeling techniques, have been developed for the analysis of small quantities of carcinogen−DNA adduct. For example, the Randerath enzymatic radiolabeling process is supposed to detect one nucleobase−adduct in 10^8−10^9 normal nucleotides.

Although all of the previous methods are highly sensitive, they do not allow the determination of the molecular structure of the detected adducts. This is a severe limitation when there is uncertainty with regard to the nature and type of carcinogen exposure. Highly sensitive, structurally informative analytical techniques are thus needed for the general characterization of DNA modifications. Mass spectrometry has the potential to provide structural information and, as such, has played an important role in the structural elucidation of covalently modified nucleic acids.

Many modes of ionization have been employed including electron impact, chemical ionization, field desorption, and thermospray ionization, and these have been reviewed by Esmans and co-workers[60] and Appruzzese and Vouros.[268] Although all of the previous techniques provided adequate structural information (especially when coupled with tandem mass spectrometry), they allowed only the analysis of either single nucleobase−adducts or derivatized single nucleobase−adducts. In general, these methods suffered from either lack of the sensitivity necessary for the analysis of trace levels of components derived from small amounts of DNA or the need to use chemical derivatization techniques to increase volatility and decrease thermal lability. Recently, FAB-MS, MALDI-TOF-MS, ESI-FTICR-MS, ESI-MS, ESI-TQ-MS, and ESI-QIT-MS in conjunction with MS/MS have allowed the analysis of nonderivatized adducts of nucleosides and nucleotides, while more recently CE-ESI-MS/MS has also been used for adduct detection.[56,128,268] The following sections illustrate the range of applications of these techniques with selected examples.

Table 10. Selected Applications of GC-MS to the Analysis of Modified Bases Obtained by DNA Hydrolysis Followed by Suitable Derivatization

modified base/method	results
N^2,3-ethenoguanine (**59**), 7-(2-hydroxyethyl)guanine (**60**), GC-EC-NICI-HRMS	High-resolution MS allows the detection of **59** and **60** below the femtomole level. This sensitivity allows analysis at endogenous concentrations in human tissue.[269,270] The GC-MS analysis of **60** was compared with a ^{32}P-labeling protocol using rat DNA. Good agreement was found between the two methods.[271]
1,N^2-ethenoguanine (**61**), GC-EC-NICI-HRMS	Using an immunoaffinity column to trap **60** improved the selectivity of the analysis. Different hydrolysis procedures were compared.[272] Later the assay was improved to permit simultaneous analysis of **60** and its isomer **61**.[273]
3,N^4-ethenocytosine (**62**), GC-NICI-MS	An ultrasensitive assay was developed which detects this etheno adduct at the femtomole level.[274] Compound **62** was detected in the urine of smokers.
5-(hydroxymethyl)uracil (**63**), GC-MS	Extant GS-MS methods for the analysis of **63** in acid hydrolysates of DNA were found to be inadequate, and a modification to the standard protocol was proposed.[275]
5-hydroxy-5,6-dihydrothymine, HPLC with GC-MS	Excision of this base from γ-irradiated DNA by endonuclease III has been monitored.[276]
cis-thymine glycol, GC-MS-SIM	Method for quantification of this species in human placental DNA has been developed.[277]
α-nitrosamino-aldehydes, GC-MS-SIM	Quantitative analysis of in vitro deamination of bases in calf thymus DNA by nitrosation shows that guanine is more reactive than adenine. Reaction rates were measured.[278]
DNA-adducted 4-amino-biphenyl, GC-NICI-MS	Using tissue from bladder tumors, a significant association was found, in high-grade tumors, between adducted 4-aminobiphenyl and a smoking habit in the patient.[279]
8-hydroxyguanine (**64**) and other oxidized bases, GC-MS	Detailed study of modified bases in calf thymus DNA addresses the extent of artifactual formation during derivatization for GC-MS. This study reveals that, contrary to previous claims, only **64** presents a problem that is circumvented by derivatization at room temperature without the use of TFA.[280]

6.1. GC-MS and Tandem MS of DNA Adducts

Traditionally, one basic approach to the analysis of nucleoside and nucleotide adducts has involved trimethylsilylation to make the compounds amenable to analysis by gas chromatography, linked to EI or CI mass spectrometric techniques. These derivatives usually give low-intensity molecular ions and somewhat more intense $[M - 15]^+$ ions. Applications of GC-MS are diminishing, but the selection of work summarized in Table 10 (Chart 12) indicates that with appropriate derivatization useful assays can be designed to have high sensitivity.

6.2. ESI-MS and Tandem MS of Oligonucleotide Adducts

Electrospray ionization (ESI) has proven to be a promising technique for the detection, identification, and quantitation of covalently attached DNA adducts and their derived products. Furthermore, MS/MS analyses readily provide structural characterizations as well as sequence information, permitting rapid and accurate analysis of moderately sized oligonucleotides. This work has been reviewed previously by Esmans et al.,[60] Crain,[128] and Apruzzese and Vouros.[268]

6.2.1. Analysis of Covalently Modified Nucleobases, Nucleosides, and Nucleotides

Radiation or general oxidative damage to DNA is a complex problem, and the development of high-quality assays for damaged nucleosides or bases is of great interest. Cadet and collaborators[281] developed an LC/ESI-MS/MS method in the MRM mode for the assessment of radiation-induced degradation products of DNA, in particular six oxidized 2′-deoxyribonucleosides and two modified purine bases found within both isolated and cellular DNA. Stable isotopically labeled internal standards were prepared and used for isotope dilution mass spectrometry measurements. This methodology was applied to the simultaneous assessment of 8-oxo-7,8-dihydro-2′-deoxyguanosine (**65**), 8-oxo-7,8-dihydro-2′-deoxyadenosine (**66**), 5-hydroxy-2′-deoxyuridine (**67**), 5,6-dihydroxy-5,6-dihydrothymidine (**68**), 5-formyl-2′-deoxyuridine (**69**), and 5-(hydroxymethyl)-2′-deoxyuridine (**70**). This sensitive and specific assay was also applied for the quantification of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (**71**) and 4,6-diamino-5-formamidopyrimidine (**72**) (Chart 12).[281]

Chart 12

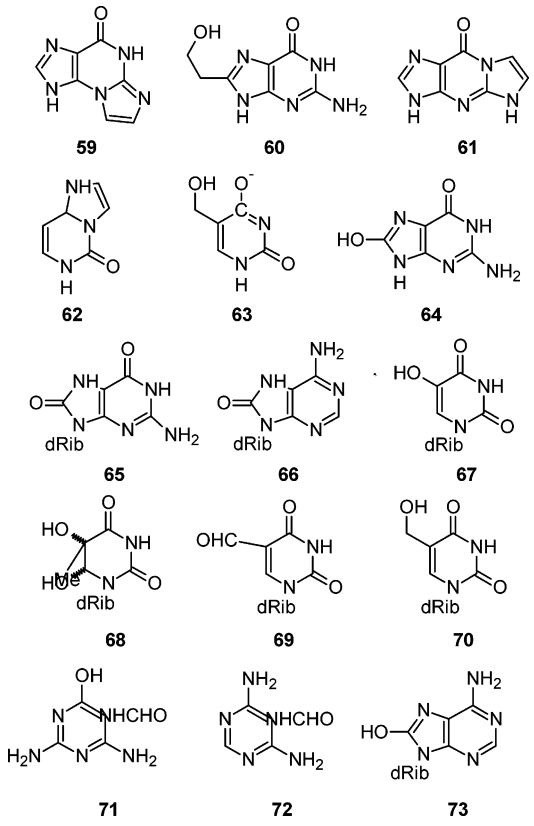
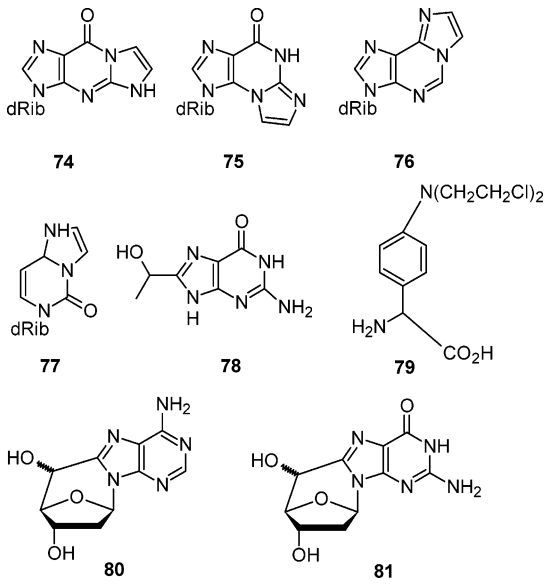


Chart 13



An assay for simultaneous quantitative measurement of **65** and **66**, important biomarkers of oxidative damage to DNA, has also been developed by Podmore and co-workers.[282] Using HPLC coupled to ESI-MS/MS in the MRM mode background levels of these lesions in calf thymus DNA were 85 ± 3 and 7.1 ± 0.2 per 10^6 DNA bases for **65** and **66,** respectively. The method was also successfully applied to the determination of these modified nucleosides in urine.[283] Hydroxyl radical attack at the C-8 position of adenine leads to the formation of 8-hydroxy-2′-deoxyadenosine (**73**), a major DNA lesion. An LC/ESI-MS-SIM protocol for the analysis of **73** in a mixture of nucleosides from DNA hydrolysis allowed quantification at a level of approximately one molecule in 10^6 DNA bases using amounts of DNA as low as 5 μg. Comparison with a GC-MS method indicated similar sensitivity for both methods.[284]

Myeloperoxidase, a heme protein secreted by activated phagocytes, may be involved in the link between chronic inflammation and cancer. Recent studies demonstrated that this enzyme can employ hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) to generate reactive nitrogen species which convert tyrosine to 3-nitrotyrosine. Byun et al.[285] have shown that activated human neutrophils use myeloperoxidase, H_2O_2, and NO_2^- to nitrate 2′-deoxyguanosine. Two major products of this reaction were identified by ESI-MS as 8-nitroguanine and 8-nitro-2′-deoxyguanosine.

The etheno derivatives, 1,N^2- and N^2,3-etheno-2′-deoxyguanosine (**74**, **75**), 1,N^6-etheno-2′-deoxyadenosine (**76**), and 3,N^4-etheno-2′-deoxycytidine (**77**), are promutagenic lesions in DNA (Chart 13). These adducts are produced by either endogenous metabolic processes (e.g., lipid peroxidation) or metabolic activation of exogenous chemicals (e.g., vinyl chloride or urethane). Doerge and collaborators[286] developed a method for the quantification of trace levels of etheno−DNA adducts using on-line sample preparation coupled with liquid chromatography and ESI-MS. They showed that the use of automated solid-phase extraction and stable labeled internal standards allowed the determination of **76** contained in crude DNA hydrolysates from untreated rodent and human tissues at the level of one adduct in 10^8 normal nucleotides from 100 μg of DNA. The method was applied to the analysis of liver DNA from untreated and urethane-treated B6C3F1 mice, untreated rat liver, human placenta, and several commercial DNA preparations.[286] Chen and Chang[287] quantified **76** in human urine using a new assay based on isotope dilution ESI-MS/MS. The detection limit for **76** is 2 pg injected on a column, and accurate quantification is achieved at urinary concentrations of 10 pg mL^{-1}. A similar study[288] of **76** in human urine using atmospheric pressure chemical ionization tandem MS in the MRM mode revealed that a detection limit of 0.7 pmol required about 3 mL of urine. This LC/APCI-MS/MS protocol was applied to the measurement of excreted levels of **76** in 33 men, revealing little correlation with weight, age, or smoking behavior.

The alkylation of DNA by 1-hydroxycarbon radicals produced from photochemical reactions of alcohols has been described since the early 1970s.[289,290] These radicals attack the C8 position of purine bases. Recently, Nakao and Augusto[291] obtained 8-(1-hydroxyethyl)guanine (**78**) and 8-(2-hydroxethyl)guanine (**60**) by incubation of guanine (1.5 mM) with ethanol (75 mM), hydrogen peroxide (4.5 mM), and iron(II) (6 mM) at pH 1 under nitrogen. The structures of these modified bases were confirmed by ESI-MS. They have the same molecular mass (M_r 195), and the spectra showed a similar fragmentation pattern which included the sequential loss of H_2O and NH_2 to produce the product ion at *m/z* 161. However, the product-ion scans obtained for these isomeric species by CID-MS/MS for the protonated molecule $[M + H]^+$ at *m/z* 196 contained distinct diagnostic differences. Adducts **78** and **60** were also obtained from incubations of DNA and RNA. Inevitably other products of DNA base oxidation were also obtained, but this work shows that both 1-hydroxyethyl and 2-hydroxyethyl radicals are likely to be involved in ethanol-mediated toxicity.[291] Melphalan (**79**) is a bifunctional alkylating agent that covalently binds with intracellular nucleophilic sites. A methodology using ESI-MS was developed by Esmans and co-workers[292] to detect and identify DNA adducts, particularly to examine alkylation sites within a nucleotide. In reaction mixtures resulting from the interaction of melphalan with 2′-deoxynucleotides, several base-alkylated adducts were identified. Low-energy CID product-ion scans of the protonated molecules $[M + H]^+$ at *m/z* 616 indicated the presence of different isomeric mono-alkylated adducts of dGMP. Calf thymus DNA was reacted in vitro with melphalan, resulting in alkylated 2′-deoxynucleotides (mainly N-7-alkylated dGMP) and alkylated dinucleotides (pdApdT and pdGpdC). The CID product-ion scan of the $[M + 2H]^{2+}$ ion at *m/z* 453 for the mono-alkylated pdGpdC dinucleotide is shown in Figure 22.[292] The spectrum confirms that alkylation involves a ring-opening reaction. Improved chromatography (nanoLC) led to detection of melphalan adducts at the ~500 fg level and included

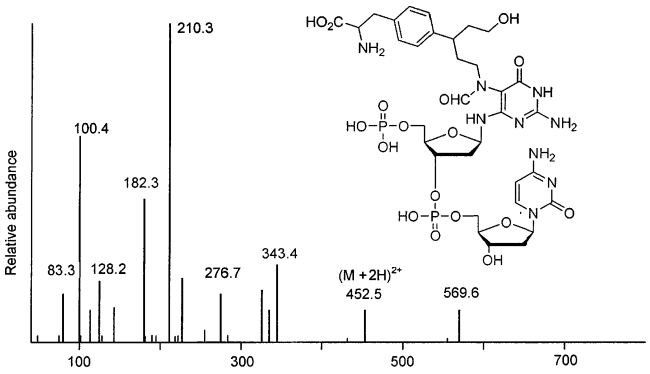


Figure 22. Low-energy CAD product-ion spectrum of $[M + 2H]^{2+}$ (*m/z* 453) of monoalkylated pdGpdC dinucleotides. The assigned structure indicates ring opening of the guanine base. (Adapted with permission from ref 292. Copyright 1999 Elsevier Science.)

several cross-linked adducts previously undetected.[293] Very recently this group applied miniaturized LC/ESI-MS/MS to the study of the specificity of mephalan alkylation of olignucleotides.[294] They showed that the order of reactivity is G > A > C > T and that attack at the 3′ or 5′ end can be distinguished.

Recently, alkylation of DNA at the phosphate group by *N*-ethyl-*N*-nitrosourea has been investigated by the Esmans group.[295] This reagent esterified the free phosphate position with an ethyl group, and after appropriate enzyme digestion a mixture of dinucleotides was obtained. Using miniaturized LC/ESI-MS/MS with column switching permitted selective characterization of 10 ethylated dinucleotides by mass and CAD product-ion spectra. This group also identified estrogen−DNA adducts in breast tumor tissue and healthy breast tissue using nanoLC coupled to nano-ESI-MS/MS. There was no clear difference between the two groups, but in patients using hormone replacement therapy (HRT), such as the hormone drug Premarin, estrogen−DNA adducts of 4-hydroxy-equilenin were detected by CID-MS/MS.[296]

Cyclonucleosides represent another class of tandem lesions in which modification of both the base and 2-deoxyribose has occurred. Romieu et al.[297] successfully incorporated either diastereoisomer of 8,5′-cyclo-2′-deoxyadenosine (**80**) and 8,5′-cyclo-2′-deoxyguanosine (**81**) into a DNA polymer. The modified DNA oligomers were digested with exo- and endonucleases and the base compositions confirmed by ESI-MS. Jaruga's group[298,299] also developed methods for the analysis of **80** and **81**. These workers compared LC-IDMS and GC-MS methods for the identification and quantification of both diastereoisomers of **80** with essentially identical results.[298] The cycloguanosine species **81** was analyzed by electrospray MS in positive-ion mode, and the levels of both diastereoisomers were estimated in calf thymus DNA and DNA from cultured human cells. Background levels were approximately two lesions in 10^6 normal nucleosides. Very recently this group reported on the use of LC/MS to investigate the effectiveness of different

Table 11. Selected Applications of ESI-MS to the Analysis of Modified Nucleosides and Nucleotides

adduct/method	results
2-amino-3-methyl-imidazo[4,5-*f*]quinoline, CLC/ESI-TQMS/MS	Using a highly optimized protocol, two adducts of 2-deoxyguanosine with this food-derived heterocyclic amine were detected in monkey pancreas tissue with detection limits of 1 adduct in 10^9 unmodified bases. Methodology gives results consistent with ^{32}P postlabeling data.[301,302]
2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine, LC/ESI-MS	This food-derived heterocyclic amine attacks 2-deoxyguanosine at C8. LC/MS analysis of the nucleoside was compared with GC-MS of the modified base. Detection limit was 200 pg/500 μg DNA in both methods.[303]
9-hydroxy-benzo[*a*]pyrene, CE-ESI-MS	Novel adduct of 2-deoxyguanosine derived from 9-OH-benzo[*a*]pyrene was synthesized and characterized. This modified nucleoside is thought to come from bioactivation by liver microsomes.[304]
styrene oxide, LC/ICP-HRMS, LC/ESI-MS	Quantitative determination of nucleotides from DNA modified by styrene oxide was achieved using either ICP-MS (phosphorus signal) or ESI-MS. For LC/ESI-MS with single ion monitoring the detection limit for styrene oxide adducts of nucleotides was determined to be 20 pg of absolute or 14 modified residues in 10^8 unmodified nucleotides in a 5 μg DNA sample, similar to the best methods available.[305]
S-[2-(*N*-7-guanyl)ethyl]-glutathione, LC/ESI-MS/MS	Sensitive and specific methods were developed[306] to quantify the level of this guanine adduct in the DNA of animals exposed to 1,2-dihaloethanes. The MRM mode was used to monitor the product ions of the doubly charged molecular ion and the methodology applied to determine adduct levels in rat and fish samples.
aflatoxin, LC/ESI MS/MS	Aflatoxin N^7-guanine and its imidazole ring-opened derivative were detected and quantified in urine from aflatoxin-dosed rats, allowing the efficacy of oltipraz, a chemopreventative agent, to be assessed. Other metabolites derived from the conjugation and/or oxidation of aflatoxin B_1 were also measured.[307]
1,3-butadiene, LC/ESI-MS/MS, CID-MS/MS-MRM	1,3-Butadiene (BD) is a high-volume industrial chemical and probable human carcinogen. Multiple structurally diverse adducts have been detected in vitro (calf thymus DNA and TK6 cell cultures) and in vivo after exposure of animals to BD or its epoxy derivatives.[308]
estradiol-2,3-quinone, LC/MS^n	Estrogen-2,3-quinones were reacted with 2-deoxyadenosine and 2-deoxguanosine to form depurinating adducts. MS/MS and MS^3 experiments carried out by QIT-MS^nallowed establishment of the fragmentation patterns and discrimination of the different isomeric adducts.[309]
7*H*-dibenzo[*c,g*]carbazole-3,4-dione, LC/CAD-MS	Adducts of this dione with a set of nine nucleosides and bases have been characterized. The nature of these species is relevant to the potential carcinogenic activity.[310]
tamoxifen, ESI-MS	Using several synthetic tamoxifen-modified nucleotides for reference, up to nine adducts have been detected in liver samples of mice. The most abundant species occurred at a level of 29 adducts in 10^8 nucleotides.[311]
1-hexanol-1,N^6-etheno-2-deoxyadenosine	Presence and integrity of this modified nucleoside in synthetic oligomers was confirmed by ESI-MS and MALDI-MS.[312]

enzymes in releasing (5′-S)-**80** from DNA and di- or oligonucleotides.[300]

The above section covers only some of the work on nucleic acid adducts, and a further selection of interesting applications is summarized in Table 11.

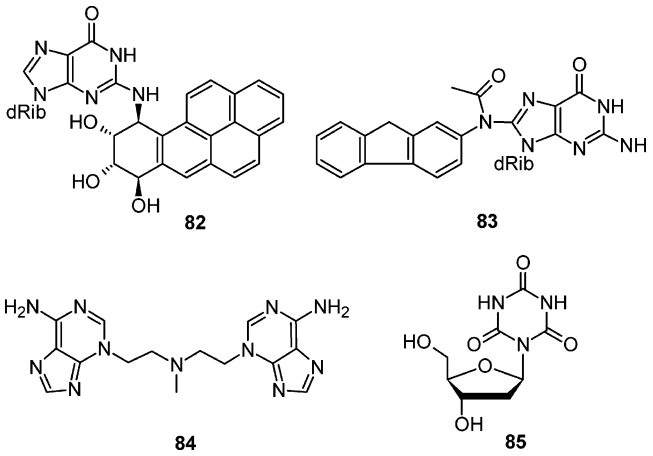
6.2.2. Characterization of Covalently Modified Oligonucleotides

Oxidation of 8-oxoguanine (in DNA as **65**) has been studied extensively. Tretyakova and co-workers[313] treated several synthetic oligonucleotides containing **65** with peroxynitrite ($ONOO^-$), and the products were analyzed by LC/ESI-MS. Generally, the oligonucleotides appeared to be oxidized only at the 8-oxoguanine sites, giving several different products depending on the concentration of the oxidant and the storage time after exposure. Tentative assignment of structures to the modified bases included 3α-hydroxy-5-imino-3,3α,4,5-tetrahydro-1*H*-imidazo[4,5*d*]-imidazol-2-one, 5-iminoimidazolidine-2,4-dione, and its hydrolytic product oxaluric acid. The exact position of the modified base was determined by exonuclease digestion followed by analysis of the fragments by LC/ESI-MS.[313] Similar studies by others[314] verified that in single-strand DNA oxaluric acid is the predominant oxidation product (by MS) in the reaction of **65** with singlet oxygen. Further detailed insight into the mechanism of the oxidation of **65** has come from studies with ^{18}O-labeled singlet oxygen.[315] A water-soluble labeled endoperoxide was shown to act as a clean chemical source of singlet oxygen, and the oxidation products of **65** were examined by HPLC/ESI-MS/MS.

A novel method to map guanine bases in short oligonucleotides has been described by Marzilli et al.[316] Target oligonucleotide strands were subjected to guanine-specific methylation; then the modified strand was analyzed by ESI-QIT-MS^n. The CID product-ion scan of the monomethylated oligonucleotide strand indicated rapid depurination. Further collision (MS^3) of the apurinic oligonucleotide induced preferential cleavage of the backbone at the site of depurination. The mass of the resulting complementary product ions identifies the position of each guanine base in the sequence. This methodology has been demonstrated for oligonucleotide sequences up to 10 bases in length, confirming the utility of this selective fragmentation by QIT-MS^n for sequencing oligonucleotides.[316] Tandem ESI-MS was also applied to determine the position of *p*-benzoquinone adducted to a 7-mer oligonucleotide by Glover and colleagues.[317] CID spectra of ions with different charge states led to ion profiles which allowed full sequencing.

Peroxidation of lipids generates 4-hydroxy-2-nonenal (HNE), a reactive aldehyde known to attack DNA. Recently, Cadet's group[318] investigated by HPLC/MS the pattern of lesions found in DNA under peroxidizing conditions. Under all conditions studied the predominant modification was an adduct of HNE with 1,N^2-propanoguanine, with minor contributions from 1,N^6-ethenoadenine and 1,N^2-ethenoguanine together with their 1,2-dihydroxyheptyl derivatives, all deriving from the reaction of DNA with the 2,3-epoxide of HNE. Similar results were obtained in cultured monocytes incubated with HNE.

Chart 14



The mutagenicity of the tobacco carcinogen, benzo[*a*]pyrene, is believed to result from reactions of a metabolite, benzo[*a*]pyrene diol epoxide (BDE), with DNA. Early studies of the promutagenic lesions have established that the guanine adduct (+)-*trans*,*anti*-7*R*,8*S*,9*S*-trihydroxy-10*S*-(N^2-2′-deoxyguanosinyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**82**) (Chart 14) is implicated. This work depended on the excision of the damaged nucleoside by repair enzymes, a process that is not always effective and does not easily allow the chemical structure and stereochemistry of the lesion to be determined. Tretyakova and her group[319,320] developed an elegant strategy for the direct quantitative analysis of **82** and its stereoisomers, originating from specific guanine nucleobases within DNA sequences derived from the p53 tumor suppressor gene and the K-ras proto-oncogene. Guanosine, substituted with stable isotopes (^{15}N or ^{13}C), was incorporated into specific positions in a set of synthetic DNA strands corresponding to key sequences from these two genes (Table 12). Adduct formation following exposure to BDE was determined by HPLC/ESI-MS/MS by direct comparison of the labeled and unlabeled ions. Adduct **82** was the main product; the reaction occurred preferentially at the guanosine in methylated CG dinucleotides. Small amounts of adduction were also observed from three other stereoisomers of BDE.

Banoub and co-workers[321] prepared a DNA hexamer containing **82** by direct reaction with BDE. They also reacted 14-mer and 18-mer oligomers with *N*-acetoxy-2-acetylaminofluorene and obtained a mixture of 16 products corresponding to the presence of up to four instances of the modified base, N^2-acetyl, N^2-(2-fluorenyl)-2′-deoxyguanosine (**83**). These DNA adducts each exhibited a different series of multiply deprotonated molecular anions having negative charges, mostly in the range of 5^- to 9^-. Component analysis of the different series of the multiply charged deprotonated molecular anions obtained from the 18-mer adducts is shown in Table 13. Fragmentation of these molecular anions was studied by controlled CID, initiated by cone voltage fragmentation. This afforded diagnostic product anions which confirmed the presence and location of the modified guanine

Table 12. Synthetic Oligonucleotides, Incorporating Isotopically Labeled Guanosine and Methylated Cytosine, Used To Locate and Quantify the Adduction of Benzo[*a*]pyrene Diol Epoxide to DNA by HPLC/ESI-MS/MS

sequence	calcd mol. mass	exptl. mol. mass
CC^{Me}C[^{15}*N*3-G]GCACC^{Me}CG^{Me}CGTC^{Me}CG^{Me}CG	5784.7	5785.3
CC^{Me}CGGCACC^{Me}C[^{15}*N*3-G]^{Me}CGTC^{Me}CG^{Me}CG	5784.7	5785.2
CC^{Me}CGGCACC^{Me}CG^{Me}C[^{15}*N*3-G]TC^{Me}CG^{Me}CG	5784.7	5785.3
CC^{Me}CGGCACC^{Me}CG^{Me}CGTC^{Me}C[^{15}*N*3-G]^{Me}CG	5784.7	5785.2
^{Me}CG^{Me}CGGA^{Me}CG^{Me}CGGGTGC^{Me}CGGG	5981.9	5982.9
ATGGG^{Me}C[^{15}*N*3-G]CATGAAC^{Me}CGGAGGCCCA	7773.1	7774.2
ATGGG^{Me}CGGCAT[^{15}*N*3-G]AAC^{Me}CGGAGGCCCA	7773.1	7774.4
ATGGG^{Me}CGGCATGAAC^{Me}C[^{15}*N*3-G]GAGGCCCA	7773.1	7774.1
ATGGG^{Me}CGGCATGAAC^{Me}CG[^{15}*N*3-G]AGGCCCA	7773.1	7774.3
ATGGG^{Me}CGGCATGAAC^{Me}CGGA[^{15}*N*3-G]GCCCA	7773.1	7774.1
TGGGCCTC^{Me}CGGTTCATGC^{Me}CGCCCA	7614.0	7612.0
GCTTT[^{15}*N*3-G]AGGTG^{Me}CGTGTTTGTG	6547.3	6546.8
GCTTTGA[^{15}*N*3-G]GTG^{Me}CGTGTTTGTG	6547.3	5646.8
GCTTTGAG[^{15}*N*3-G]TG^{Me}CGTGTTTGTG	6547.3	6546.8
GCTTTGAGGTG^{Me}C[^{15}*N*3,^{13}C-G]TGTTTGTG	6548.3	6548.5
GCTTTGAGGTG^{Me}C[^{15}*N*5-G]TGTTTGTG	6549.3	6549.4
GCTTTGAGGTG^{Me}CGT[^{15}*N*3-G]TTTGTG	6547.3	6546.7
CACAAACA^{Me}CGCACCTCAAAGC	6336.2	6336.4
ATGGGC[^{15}*N*3,^{13}C-G]GCATGAAC	4646.1	4645.4
ATGGG^{Me}C[^{15}*N*3,^{13}C-G]GCATGAAC	4660.1	4659.5
GTTCATGCCGCCCAT	4504.0	4503.1
GTTCATGC^{Me}CGCCCAT	4518.0	4517.4
CATGAACC[^{15}*N*3-G]GAGGCCCATC	5785.8	5786.8
CATGAAC^{Me}C[^{15}*N*3-G]GAGGCCCATC	5799.8	5800.1
GATGGGCCTCCGGTTCATG	5835.8	5836.3
GATGGGCCTC^{Me}CGGTTCATG	5849.8	5850.3
GCTTTGAGGTGC[^{15}*N*3,^{13}C-G]TGTTTGTG	6534.3	6533.0
CACAAACACGCACCTCAAAGC	6322.2	6321.6

nucleobase adduct and allowed bi-directional sequencing of these DNA−carcinogen adducts. As was the case when using a quadrupole−hexapole−quadrupole instrument, the CID-MS/MS analysis was not as efficient as the analysis obtained from the CID nozzle fragmentation.[321]

The antineoplastic activity of cisplatin [*cis*-diaminedichloroplatinum(II)] is thought to derive from the tendency to form adducts with DNA, especially through coordination with N7 of guanine. Various adducts are formed upon interaction of platinum complexes with nucleotides, but the extent of the contribution of individual adducts to antitumor activity is unknown. The adducts formed in the reaction of cisplatin with various nucleotides have been separated by capillary zone electrophoresis (CZE) and identified by ESI-MS. This CZE-ESI-MS method also allowed the hydrolysis of the adducted platinum complexes to be studied.[322] To investigate the mechanism of the interaction of cisplatin with DNA, outer-sphere association of several 20-mer oligonucleotides with $[Pt(NH_3)_4]^{2+}$ and $[Pt(py)_4]^{2+}$ has been measured by ESI-MS, and affinity constants have been determined for some complexes. The results suggest that electrostatic effects and hydrogen-bonding potential are important factors in outer-sphere complexation.[323]

The interaction of metal ions with DNA has been investigated widely, but there has been little study of chromium despite the fact that chromium(III) complexes are environmentally toxic. Interactions of $[Cr(salprn)(H_2O)_2]ClO_4$ with nucleosides and dinucleotides were studied by Madhusudanan and coworkers[324] using ESI-MS. The nucleosides 2′-deoxycytidine, thymidine, 2′-deoxyadenosine, 2′-deoxyguanosine, cytidine, adenosine, and guanosine form 1:1 and 2:1 adducts with $[Cr(salprn)]^+$, whereas the dinucleotides CpG, GpC, ApT, TpA, and TpC form only the 1:1 adducts. The CID-MS/MS dissociation of these adducts reveals that Cr^+ is linked to the bases in nucleosides and to both the phosphate and base in the nucleotides, especially at cytosine and guanine moieties. The sugar residues appeared to offer no binding sites as elimination of the glycones occurs frequently in the product-ion scans of the nucleoside adducts.

The carcinogenic effect of sunlight is due in part to the formation of dimeric pyrimidine photoproducts induced by short wavelength UV. The Cadet group[325] obtained data for a group of such photodimerization products of the bases, nucleosides, dinucleoside monophosphates, and dinucleotides using ESI-MS/MS (MRM mode). The fragmentation patterns are sensitive to the mechanism of collisional activation (TQ compared to QIT), especially for diastereoisomeric cyclobutane dimers. Similar modified trinucleotides have been identified by LC/ESI-MS/MS in the nuclease P1 digests of DNA irradiated at 254 nm.[326] Tandem cross-linking of thymine and purine bases can be studied in synthetic DNA by incorporation of 5-(phenylthiomethyl)-2′-deoxyuridine in either a 3′ or 5′ position vicinal to adenosine or guanosine residues. UV irradiation induces the cross-link, and a sensitive assay was developed for the four possible linked dinucleoside monophosphates by HPLC/ESI-MS/MS.[327] These four lesions were found in both single-strand oligonucleotides and isolated DNA, subjected to γ irradiation. The tandem G,T lesions were produced more efficiently than the corresponding A,T cross-linked species.

Cross-linking between DNA strands using bifunctional alkylating agents such as nitrogen mustards

Table 13. Component Analysis of the Different Series of Multiple Charge Deprotonated Molecular Anions Obtained from mer-18 Containing One or More Occurrences of the Nucleotide, N^2-acetyl, N^2-(2-fluorenyl)-2′-deoxyguanosine (G′)

18-mer adduct ion	MaxEnt calcd MW	deprotonated anions, *m/z* (%)
[18-mer(G) + Na − H]		A^{9-} = 634.32 (5)
$C_{190}H_{232}N_{66}O_{108}P_{17}Na$	5717.90	A^{8-} = 713.73 (8)
		A^{7-} = 815.84 (10)
		A^{6-} = 951.48 (9)
		A^{5-} = 1142.57 (12)
[18-mer(2G) + Na − H]		B^{9-} = 659.81 (12)
$C_{205}H_{243}N_{67}O_{109}P_{17}Na$	5939.20	B^{8-} = 749.39 (31)
		B^{7-} = 847.45 (43)
		B^{6-} = 988.86 (31)
		B^{5-} = 1186.83 (10)
[18-mer(2G) + 2Na − H]		C^{9-} = 661.36 (8)
$C_{205}H_{242}N_{67}O_{109}P_{17}Na_2$	5961.18	C^{8-} = 744.14 (27)
		C^{7-} = 850.59 (30)
		C^{6-} = 992.52 (19)
		C^{5-} = 1191.23 (16)
[18-mer(3G) + Na − H]		D^{9-} = 683.49 (23)
$C_{220}H_{254}N_{68}O_{110}P_{17}Na$	6160.50	D^{8-} = 769.06 (91)
		D^{7-} = 879.07 (64)
		D^{6-} = 1025.74 (39)
		D^{5-} = 1231.09 (15)
[18-mer(3G) + 2Na − 2H]		E^{9-} = 685.94 (27)
$C_{220}H_{253}N_{68}O_{110}P_{17}Na_2$	6182.48	E^{8-} = 771.80 (40)
		E^{7-} = 882.21 (60)
		E^{6-} = 1029.41 (36)
		E^{5-} = 1235.49 (18)
[18-mer(4G) + Na − H]		F^{9-} = 708.08 (15)
$C_{235}H_{265}N_{69}O_{111}P_{17}Na$	6381.80	F^{8-} = 796.72 (100)
		F^{7-} = 910.68 (79)
		F^{6-} = 1062.63 (38)
		F^{5-} = 1275.36 (11)
[18-mer(4G) + 2Na − 2H]		G^{9-} = 710.53 (9)
$C_{235}H_{264}N_{69}O_{111}P_{17}Na_2$	6403.78	G^{8-} = 799.47 (26)
		G^{7-} = 913.82 (39)
		G^{6-} = 1066.29 (36)
		G^{5-} = 1279.75 (18)

can inhibit replication, leading to apoptosis and suppression of tumor growth. From the reaction of *N*,*N*-bis(2-chloroethyl)methylamine with DNA, Tretyakova and co-workers[328] identified the adenine−adenine interstrand cross-linked species (**84**) and three analogous adenine−guanine cross-linked species by HPLC/ESI-MS/MS. Analogous lesions were also found in DNA treated with 4-(*N*,*N*-bis(2-chloroethyl)aminophenylbutyric acid or L-phenylalanine mustard.

6.3. MALDI-MS of Covalent Modified DNA Adducts

Although most MS analysis of adducted bases or nucleosides has been carried out using ESI tandem MS, several groups have applied MALDI-TOF-MS to the study of oxidative lesions and their stability, the role of repair mechanisms, and other biochemical features.[329]

6.3.1. Measurement of Intact Covalent Modified Oligonucleotides

The Cadet group[330] synthesized oligodeoxyribonucleotides containing the cyanuric nucleoside (Y) (**85**) (Chart 14) by standard phosphoramidite chemistry. The purity and integrity of these synthetic oligonucleotide strands was confirmed by ESI-MS and MALDI-MS. The ability of several nucleases to cleave the phosphodiester linkages between **84** and the normal 2′-deoxyribonucleosides was examined in detail. Enzymatic digestion experiments were performed with the trinucleotide d(TYT) and with a modified 14-mer oligonucleotide using snake venom phosphodiesterase and calf spleen phosphodiesterase in combination with bacterial alkaline phosphatase. Hydrolysis of the DNA strand by the nucleases was monitored by withdrawing aliquots from the digestion mixtures at increasing time intervals. The DNA fragments were then analyzed by MALDI-TOF-MS. The data indicated that these exonucleases failed to release **84** from the oligonucleotide chain.

Tretyakova and co-workers[331] have shown that MALDI-TOF-MS analysis of exonuclease ladders can be used successfully to sequence DNA fragments containing a variety of carcinogen-induced nucleobase adducts. This methodology has been used to determine the sequence of different synthetic 12-mer to 20-mer DNA strands containing O^6-methylguanine, oxidative guanine lesions such as **82** and **84**. These lesions were located without any prior information about the sequence or nature of the adduct. Sequence information was also obtained for DNA containing O^6-pyridyloxoguanine, even although this lesion normally blocks 3′-phosphodiesterase.

D'Ham and co-workers[332] synthesized oligonucleotides that contained modified pyrimidines, such as thymine glycol, 5,6-dihydrothymine, and 5-hydroxycytosine, in order to investigate the substrate specificity and excision mechanism of two *E. coli* repair enzymes, endonuclease III and formamidopyrimidine DNA glycosylase (Fpg). A GC-MS assay with HPLC prepurification was used to quantify the release of the modified base. The results of MALDI-TOF-MS analysis of the molecular masses of the repaired fragments of the synthetic oligonucleotides showed that endonuclease III cleaves the DNA backbone mainly through a hydrolytic process, and no β-elimination product was detected.

Jacutin and collaborators[333] have shown that 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole phosphate (dXP) could be incorporated into a DNA decamer and analyzed by MALDI-TOF-MS. The extent and composition of the various fragment peaks were compared with those of the MALDI-TOF-MS of 10-mer dT4AT5. These authors used two different DNA template assays to attempt to identify DNA replicating enzymes that would incorporate the corresponding triphosphate, i.e., 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole triphosphates (dXTP). Some enzymes were inhibited by dXTP; others could not incorporate it at all. However, both DNA polymerase I Klenow fragment and avian myeloblastosis virus reverse transcriptase incorporated dXTP in place of dATP and then replicated the template overhang in the usual way. The potential of dXTP as a surrogate for dATP in DNA sequencing with MALDI MS analysis was thoroughly discussed.[333]

The adduction of carcinogen **82** has been studied by MALDI-MS using an adduct labeled through the

phosphate group with a fluorescent IMI dye. The MALDI sensitivity was high (300−500 fmol in the laser spot) and the analyses complementary to those carried out by capillary electrophoresis with fluorescence detection[334] and 1,N^6-ethenoadenosine 5′-diphosphoribose has been confirmed as a product of treating adenosine 5′-diphosphoribose with chloroacetaldehyde.[335]

7. Other Original Mass Spectrometric Methods

7.1. Charge Detection MS for Mass Measurement in the Megadalton Range

Fuerstenau and Benner[32] investigated the potential of a novel charge detection ESI-TOF-MS instrument capable of determining the mass of multiply charged electrospray ions generated from samples of macromolecules in the megadalton size range. The instrument utilized a sensitive amplifier that detected the charge on a single ion as it passed through a tube detector. A velocity measurement of an ion with known electrostatic energy provided the value of *m/z* for that ion. Thus, simultaneous determination of *z* and *m/z* for an ion permitted a mass assignment to be made in each case. Electrospray ions of DNA and polymer molecules with masses greater than 1×10^6 Da and charge numbers in excess of $425e^-$ were readily detected in this mass spectrometer. The on-axis single-ion detection configuration provided a duty cycle of nearly 100% and extended the practical application of electrospray mass spectrometry to the analysis of very large molecules with relatively inexpensive instrumentation. Recently, Schultz and co-workers[336] used this CDMS instrument for rapidly analyzing polymerase chain reaction (PCR) products with a minimum of sample cleanup after the PCR reaction. PCR products of 1525, 1982, and 2677 bp were detected and measured. A longer double-stranded, linear DNA sample was also analyzed. Positive-ion mass spectra and ion charge-state distributions were obtained for these electrosprayed DNA samples.

7.2. Inclusion Complexes of Nucleosides and Nucleobases with β-Cyclodextrin: A Study by FAB CID-MS/MS

The nonpolar cavity of the cyclic oligosaccharide β-cyclodextrin (CD) is known to accommodate guest molecules, mainly by hydrophobic interactions. The resulting CD inclusion complexes with drugs are finding widespread application in the pharmaceutical industry as these complexes can improve the solubility, stability, and bioavailability of the guest molecules. Naturally occurring nucleosides and nucleobases form inclusion complexes with β-cyclodextrin, and these host−guest complexes can be detected by fast atom bombardment mass spectrometry.[337] The collision-induced dissociation spectra of the protonated complexes showed mainly ions related to the guest molecules. However, deoxyribonucleoside complexes exhibited the facile elimination of the sugar glycone, whereas this fragmentation was absent in the cases of the ribonucleoside complexes. The deprotonated inclusion complexes underwent collision-induced dissociation to give mainly the deprotonated host molecule. It appears, therefore, that the protonated complexes have protonated guests inside the cavity of the neutral host while the deprotonated complexes have neutral guests in the cavity of the deprotonated host. More recently nucleobase and nucleoside interactions with permethylated CD and permethylated maltoheptose have been investigated[338] by ESI-MS. The ease of guest exchange with gaseous amines is deoxyguanosine > adenine ≫ deoxycitidine > cytosine. Studies with CID-MS/MS show that the preferred dissociation path depends on the structure of both host and guest.

7.3. Modeling Nucleobase Radicals in the Mass Spectrometer Using Neutralization−Reionization Mass Spectrometry

Owing to the importance of DNA damage for physiological processes as diverse as aging, oxidative stress, and radiation damage, the chemistry of DNA damage has been widely studied. It has been generally accepted that DNA is attacked by free radicals formed in the intracellular fluid by radiolysis of water. Among the radiolytic products formed, the hydroxyl radical was particularly reactive and could attack DNA at the nucleobase aglycones to give rise to [DNA + OH]• radical adducts or abstract a hydrogen atom from the nucleobase or sugar moieties to form [DNA − H]• radicals.[339]

Until the neutralization beam technique[340] and its spin-off neutralization−reionization mass spectrometry (NRMS) was reported by Turecek,[341] neutral intermediates could be studied by mass spectrometry only indirectly as products of ion dissociations. NRMS has been a major breakthrough in science which has allowed synthesis of unusual and highly reactive neutral molecules and radicals in the gas phase. It has also permitted the study of their dissociations and analysis of the radicals and their dissociation products 'on-line' by tandem mass spectrometry. Heterocyclic radicals relevant to nucleobases are just one of many structure types that have been studied by NRMS.[342] Therefore, we can say that the mass spectrometric methods based on collisional neutralization of fast ions provide a general strategy for the preparation and study of heterocyclic radicals related to DNA damage. Deuterium labeling, neutral photoexcitation, and variable-time measurements are some of the currently available and powerful methodologies used to investigate the intrinsic properties of DNA radicals in the gas phase. In addition, continuing progress in computer technology allows one to address still larger radical systems at unprecedented levels of ab initio theory. This symbiotic relationship of experiment and theory gives investigators an extremely powerful mass spectrometry tool to probe these interesting and otherwise inaccessible radical systems. It should be noted that preliminary studies of ion−molecule reactions of cation radicals derived from nucleobases have appeared recently, which will further contribute to our knowledge of these fascinating and important species.[342]

7.4. Accelerator Mass Spectrometry

Accelerator mass spectrometry (AMS) is an analytical technique for quantifying rare isotopes with high sensitivity and precision and has been most commonly used to measure ^{14}C in historical artifacts (carbon dating). It has an advantage over traditional methods of dating in that, because the sensitivity is so high, only very small (milligram) samples are required. Only recently has its potential in biomedical research been appreciated. AMS measurement of serially diluted samples containing a ^{3}H-labeled tracer showed a strong correlation with liquid scintillation counting. The sensitivity for AMS detection of the ^{3}H label in tissue, protein, and DNA is 2−4 amol per mg of sample and is comparable to detection limits for ^{14}C-labeled carcinogens using ^{14}C AMS, and it also demonstrates the feasibility of ^{3}H AMS for biomedical studies. Dingley et al.[343] used this extremely sensitive analytical technique to measure DNA adducts in very low doses. Measurements of the levels of ^{3}H-labeled 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and ^{14}C-labeled 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in rat liver tissue and bound to liver DNA and protein were carried out 4.5 h after acute administration of individual or coadministered doses in the range of 4−5100 pmol per kg of body weight. MeIQx−DNA adduct levels were higher than PhIP adduct levels, which is consistent with their respective carcinogenicity in the liver.

The group of Liu in Beijing applied AMS to the study of the DNA adduction of several common chemicals labeled with ^{14}C. One study shows that the adduction of nitrobenzene is suppressed by vitamin C, vitamin E, tea polyphenols, and other dietary substances.[344] Adduction of formic acid to liver and kidney DNA in mice is high even at the lowest dose level (10 μg/kg of body weight).[345] A high-dose level of sodium benzoate (500 μg/kg of body weight) in mice results in higher adduction in the kidney than in the liver. The levels of DNA−benzoate adducts decay quite rapidly initially but persist at a low level which is relevant to chronic use of sodium benzoate.[346] Biomedical applications of AMS have been reviewed recently.[347]

7.5. X-ray Photoelectron Spectroscopy and Time-of-Flight Secondary Ion Mass Spectrometry

There are diverse methods of preparing DNA microarrays, and these have developed into a flexible tool for a plethora of applications including gene mapping, drug discovery, biosensors development, and sequence analysis. The number of immobilized DNA probes in an array can reach tens of thousands, and when this is combined with the number of types of DNA and the number of ways of fixing the probe material to the surface it can be seen that standardization of the analytical methodology which 'reads' the array is extremely important. Castner and his group[348] made a preliminary assessment of the surface techniques of XPS and TOF-SIMS as methods for the analysis of DNA arrays. Elemental composition determined by XPS analysis of bases and nucleosides agreed well with expectation, but the spectra obtained by TOF-SIMS were generally complex. However, a detailed table of all positive and negative ions was built up for all the common DNA components, and this information could then be used to support the analysis of oligomer spectra through the application of principal component analysis. This technique is still in its infancy, and interesting developments will appear in the future.

8. Summary

Nucleic acids and their components play an important role in a variety of fundamental biological processes, and consequently, the study of these species has been at the heart of molecular biology and initiated a wide range of new disciplines over the last two decades. Among these has been the new mass spectrometry techniques for the production of quasi-molecular ions of thermally fragile biomolecules. These have been of such groundbreaking significance that they have led to the awarding of Nobel Prizes in Chemistry (2002) to John Fenn[26] for the development of electrospray ionization and to Koichi Tanaka[27] for matrix-assisted laser desorption/ionization. These novel soft ionization methods are ideally suited to the accurate determination of the molecular weights of the nucleic acid biopolymers and their constituents. They also have provided valuable information on structurally specific biomolecular DNA and RNA interactions. These were essential in establishing direct determinations of stoichiometry for posttranslational modifications for specific DNA−drug, DNA−protein, RNA−protein, and DNA−RNA noncovalent and covalent associations, including DNA and RNA sequencing analysis of single nucleotide polymorphisms, genotyping, mutation detection, genetic diagnosis, and probing of viral structures. In the past decade dramatic progress in the field of MS has established MS as a vital bioanalytical tool in many fields such as functional genomics, proteomics, early drug discovery, and chemical diagnostics. These activities are currently fueled by resources being devoted to drug development and other biologically related activities. Progress in these applications was accelerated with the improved sensitivity, mass accuracy, mass-to-charge, and speed of the newer and exciting developments in availability of the new mass spectrometer systems.[35]

The fundamental biochemical importance of the nucleic acids and their constituents has already been extensively reviewed,[55] and this review has concentrated on the vital contribution made by developments in mass spectrometry and tandem mass spectrometry to the detailed elucidation of structure and biological activity of DNA and its constituents. In the past decade the literature on the recent developments for the characterization of nucleosides, nucleotides, adducts, oligonucleotides, and nucleic acids has been growing exponentially, and consequently, it was not feasible to provide a comprehensive coverage of the published literature in the field. A measure of the rate of progress in the field is the development of mass spectrometry for genotyping assays. In 1998 Ross and co-workers[349] announced their ability to

carry out 12-fold multiplex SNP analysis, but in early 2004 Ju and co-workers[350] reported 30-fold multiplex genotyping of the *p53* gene by MS, a remarkable growth rate with enormous implications for the role of mass spectrometry in the development of routine genomic screening.

9. Acknowledgments

J.H.B. acknowledges the support of his research program by the National Science and Engineering Research Council of Canada in the form of a Discovery Grant and Fisheries and Oceans Canada for financial support. The authors also acknowledge the efforts of Ms. Nancy Barker, Ms. Elizabeth Rowlands, and Mr. Alejandro Cohen from the Biochemistry Department, Memorial University, Mr. Jef Schrooten from the Chemistry Department, Antwerp University, and Mr. Howard Hodder from Fisheries and Oceans Canada for the preparation of this manuscript. J.H.B. would like to thank his wife, Judith, for all the encouragement and love she administered during his illness while writing his part of this manuscript.

10. Note Added after ASAP Publication

This paper was published on the Web on April 23, 2005. A change was made to Chart 2, and the paper was reposted on April 26, 2005.

11. References

(1) Dulbecco, R.; Ginsberg, H. S. In *Microbiology*, 3rd ed.; Davis, B. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., Eds.; Harper and Row: Philadelphia, 1980.
(2) Watson, J. D.; Crick, F. H. C. *Nature* **1953**, *171*, 737.
(3) Jasny, B. R.; Roberts, L. *Science* **2003**, *300*, 277.
(4) Forde, C. E.; McCutchen-Maloney, S. L. *Mass Spectrom. Rev.* **2002**, *21*, 419.
(5) McCloskey, J. A. *Methods in Enzymology*; Academic Press: New York, 1990; Chapter 4.
(6) McCloskey, J. A.; Crain P. F. *Int. J. Mass Spectrom. Ion Processes.* **1992**, *118*−*119*, 593.
(7) Crain, P. F. *Mass Spectrom. Rev*. **1990**, *9*, 505. Crain, P. F.; McCloskey, J. A. *Curr. Opin. Biotechnol*. **1998**, *9*, 25.
(8) Bieman, K.; McCloskey, J. A. *J. Am. Chem. Soc.* **1962**, *84*, 2005.
(9) McCloskey, J. A. In *Basic Principles in Nucleic Acid Chemistry*; Ts'O, P. O. P., Ed.; Academic Press: New York, 1974; p 209.
(10) Wilson, M. S.; McCloskey, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 3436.
(11) Schram, K. H.; McCloskey, J. A. In *GLC and HPLC Determination of Therapeutic Agents*, *Part 3*; Tsuji, K., Ed.; Marcel Dekker Inc.: New York, 1979; p 1149.
(12) Von Minden, D. L.; Stillwell, R. N.; Koenig, W. A.; Lyman, K. J.; McCloskey, J. A. *Anal. Biochem.* **1972**, *50*, 110.
(13) Panzica, R. P.; Townsend, L. B.; von Minden, D. L.; Wilson, M. S.; McCloskey, J. A. *Biochim. Biophys. Acta* **1973**, *331*, 147.
(14) Schulten, H. R. *Int. J. Mass Spectrom. Ion Phys.* **1979**, *32*, 97.
(15) Schulten, H. R.; Schiebel, H. M. *Fresnius' Z. Anal. Chem.* **1976**, *280*, 139.
(16) Schulten, H. R.; Beckey, H. D. *Org. Mass Spectrom.* **1973**, *7*, 861.
(17) McNeal, C. J.; Narang, S. A.; Macfarlane, R. D.; Hsiung, H. M.; Brousseau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 735.
(18) Otake, N.; Ogita, T.; Miyazaki, Y.; Yonehara, H.; Macfarlane, R. D.; McNeal, C. J. *J. Antibiot.* **1981**, *34*, 130.
(19) McNeal, C. J.; Ogilvie, K. K.; Theriault, N. Y.; Nemer, M. J. *J. Am. Chem. Soc.* **1982**, *104*, 981.
(20) Cotter, R. J.; Fenselau, C. *Biomed. Mass Spectrom.* **1979**, *6*, 287.
(21) Esmans, E. L.; Freyne, E. J.; Vanbroeckhoven, J. H.; Alderweireldt, F. C. *Biomed. Mass Spectrom.* **1980**, *7*, 377.
(22) Hunt, D. F.; Shabanowitz, J.; Botz, F. K.; Brent, D. A. *Anal. Chem.* **1977**, *49*, 1160.
(23) Eicke, A.; Sichtermann, W.; Benninghoven, A. *Org. Mass Spectrom.* **1980**, *15*, 289.
(24) Unger, S. E.; Schoen, A. E.; Cooks, R. G.; Ashmorth, D. J.; Gomes, J. D.; Chang, C. J. *J. Org. Chem.* **1981**, *46*, 4765.
(25) Clay, K. L.; Wahlin, L.; Murphy, R. C. *Biomed. Mass Spectrom.* **1983**, *10*, 489.
(26) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
(27) Tanaka, K. *Angew. Chem., Int. Ed.* **2003**, *42*, 3860.
(28) Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675.
(29) Von Brocke, A.; Nicholson, G.; Bayer, E. *Electrophoresis* **2001**, *22*, 1251.
(30) Smith, R. D.; Loo, J. A.; Edmonds, G. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882.
(31) Stults, J. T.; Marsters, J. C. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 359.
(32) Fuerstenau, S. D.; Benner, W. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1528.
(33) Nohmi, T.; Fenn, J. B. *J. Am. Chem. Soc.* **1992**, *114*, 3241.
(34) Yates, J. R. *J. Mass Spectrom.* **1998**, *33*, 1.
(35) McLuckey, S. A.; Wells, M. *Chem. Rev.* **2001**, *101*, 571.
(36) Marziali, A.; Akeson, M. *Annu. Rev. Biomed. Eng.* **2001**, *3*, 195.
(37) Murray, K. K. *J. Mass Spectrom.* **1996**, *31*, 1203.
(38) Monforte, J. A.; Becker, C. H. *Nat. Med.* **1997**, *3*, 360.
(39) Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53.
(40) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151.
(41) Spengler, B. *J. Mass Spectrom.* **1997**, *32*, 1019.
(42) Nordhoff, E.; Kirpekar, F.; Roepstorff, P. *Int. J. Mass Spectrom. Ion Processes* **1996**, *15*, 67.
(43) Spengler, B.; Kirsch, D.; Kaufmann, R.; Jaeger, E. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 105.
(44) (a) *Tandem Mass Spectrometry*; McLafferty, F. W., Ed; John Wiley and Sons, Ltd.: New York, 1983. (b) Busch, K. L.; Glish, G. L.; McLuckey, S. A. *Mass Spectrometry/Mass Spectrometry;* VCH Publishers: Germany, 1988.
(45) Panico, M.; Sindona, G.; Ucella, N. *J. Am. Chem. Soc*. **1983**, *105*, 5607.
(46) Liu, C.; Tolic, L. P.; Hofstadler, S. A.; Harms, A. C.; Smith, R. D.; Kang, C.; Sinha, N. *Anal. Biochem.* **1998**, *262*, 67.
(47) Cerny, R. L.; Gross, M. L.; Grotjahn, L. *Anal. Biochem*. **1986**, *156*, 424.
(48) Phillips, D. R.; McCloskey, J. A. *Int. J. Mass Spectrom. Ion Processes* **1993**, *128*, 61.
(49) Liguori, A.; Sindona, G.; Uccella, N. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 451.
(50) Isern-Flecha, I.; Jiang, X. Y.; Cooks, R. G.; Pfleiderer, W.; Chae, W. G.; Chang, C. J. *Biomed. Environ. Mass Spectrom.* **1987**, *14*, 17.
(51) Neri, N.; Sindona, G.; Uccella, N. *Gazz. Chim. Ital.* **1983**, *113*, 197.
(52) McLuckey, S. A.; Habibi-Goudarzi, S. *J. Am. Chem. Soc.* **1993**, *115*, 12085.
(53) McLuckey, S. A.; Van Berkel, G. J.; Glish, G. L. *J. Am. Soc. Mass Spectrom*. **1992**, *3*, 60.
(54) Medzihradszky, K. F.; Campbell, J. M.; Baldwin, M. A.; Falick, A. M.; Juhasz, P.; Vestal, M. L.; Burlingame, A. L. *Anal. Chem*. **2000**, *71*, 552.
(55) Saenger, N. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(56) Kaneko, T.; Katoh, K.; Fujimoto, M.; Kumagai, M.; Tamaoka, J.; Katayama-Fujimura, Y. *J. Microbiol. Methods* **1986**, *4*, 229.
(57) McCloskey, J. A. In *Proc. 4th International Roundtable on Nucleosides*, *Nucleotides and their Biological Application*; Alderweineldt, F. C., Esmans, E. L.; Eds.; University of Antwerp: Antwerp, 1982; pp 47−67.
(58) McCloskey, J. A. In *Mass Spectrometry in the Health and Life Sciences*; Burlingame, A. L., Castagnoli, N., Eds.; Elsevier: Amsterdam, 1985; pp 521−546 and references therein.
(59) McCloskey, J. A. In *Mass Spectrometry in Biomedical Research*; Gaskell, S. J., Ed.; Wiley: New York, 1986; pp 75−95 and references therein.
(60) Esmans, E. L.; Broes, D.; Hoes, I.; Lemiere, F.; Vanhoutte, K. *J. Chromatogr., A* **1998**, *794*, 109.
(61) Buchanan, J. M.; Hartman, S. C. *Adv. Enzymol*. **1959**, *21*, 199.
(62) Ewing, D. F.; Humble, R. W.; Holý, A.; Mackenzie, G.; Shaw, G.; Votruba, I. *Nucleosides Nucleotides* **1989**, *8*, 1177.
(63) Ewing, D. F.; Holý, A.; Votruba, I.; Humble, R. W.; Mackenzie, G.; Hewedi, F.; Shaw, G. *Carbohydr. Res*. **1991**, *216*, 109.
(64) Liguori, A.; Greco, F. F.; Sindona, G.; Ucella, N. *Org. Mass. Spectrom*. **1990**, *25*, 459.
(65) Crow, F. W.; Tomer, K. B.; Gross, M. L.; McCloskey, J. A.; Bergstrom, D. E. *Anal. Biochem.* **1984**, *139*, 243.
(66) Banoub, J.; MacKenzie, G.; Becchi, M.; Fraisse, D.; Descotes, G. *Spectrosc. Int. J.* **1989**, *7*, 331.
(67) Banoub, J. H.; Becchi, M.; Descotes, G.; Fraisse, D.; Humble, R. W.; Mackenzie, G. *J. Carbohydr. Chem*. **1992**, *11*, 471.

(68) Inou, T.; Kusaba, I.; Takahashi; Sugimot, M.; Kuzohara, Y.; Yamada, Y.; Tamowchi, J.; Otsubo, O. *Transplant Proc.* **1981**, *8*, 315.
(69) Newton, R. P.; Kingston, E. E.; Overton, A. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 729.
(70) Takeda, N.; Yoshizumi, H.; Niwa, T. *J. Chromatogr., B* **2000**, *746*, 51.
(71) Colacino, E.; Giorgi, G.; Liguori, A.; Napoli, A.; Romeo, R.; Salvini, L.; Siciliano, C.; Sindona, G. *J. Mass Spectrom.* **2001**, *36*, 1220.
(72) Gallo, R. C.; Sarin, P. S.; Gelmann, E. P.; Guroff, M. R.; Richardson, E.; Kalyanaraman, V. S.; Mann, D.; Sidhic, G. D.; Stahl, R. E.; ZollaPazner, S.; Leibowitch, J.; Popovic, M. *Science* **1983**, *220*, 865.
(73) Mitsuya, H.; Weinhold, K. J.; Furman, P. A.; St. Clair, M. H.; Lehrman, S. N.; Gallo, R. C.; Bolognesi, D.; Barry, D. W.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7096.
(74) Mitsuya, H.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911.
(75) Matsushita, S.; Mitsuya, H.; Reitz, M. S.; Broder, S. *J. Clin. Invest.* **1987**, *80*, 394.
(76) Montgomery, J. A. *Med. Res. Rev.* **1982**, *2*, 271.
(77) Parks, R. E.; Stoekler, J. D.; Cambor, C.; Savarese, T. M.; Crabtree, G. W.; Chu, S. H. In *Molecular Actions and Targets for Cancer Chemotherapeutic Agents;* Satorelli, A. C., Lazo, J. S., Bertino, J. R., Eds.; Academic Press: New York, 1981; p 229.
(78) Banoub, J.; Gentil, E.; Tber, B.; Fahmi, N. E.; Ronco, G.; Villa, P.; Mackenzie, G. *Spectroscopy* **1994**, *12*, 69.
(79) Walker, M. P.; Appleby, T. C.; Zhong, W.; Lau, J. Y. N.; Hong, Z. *Antiviral Chem. Chemother.* **2003**, *14*, 1.
(80) Shou, Z. W.; Bu, H. Z.; Addison, T.; Jiang, X.; Weng N. D. *J. Pharm. Biomed. Anal.* **2002**, *29*, 83.
(81) Cahours, X.; Dessans, H.; Morin, P.; Dreux, M.; Agrofoglio, L. *J. Chromatogr., A* **2000**, *895*, 101.
(82) Agrofoglio, L. A.; Cahours, X.; Tran, T. T.; Dessans, H.; Kieda, C.; Morin, P. *Nucleosides Nucleotides Nucleic Acids* **2001**, *20*, 375.
(83) Cahours, X.; Morin, P.; Dessans, H.; Agrofoglio, L. A. *Electrophoresis* **2002**, *23*, 88.
(84) Pruvost, A.; Becher, F.; Bardouille, P.; Guerrero, C.; Delfraissy, J. F.; Goujard, C.; Grassi, J.; Benech, H. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1401.
(85) Becher, F.; Pruvost, A.; Goujard, C.; Guerrero, C.; Delfraissy, J. F.; Grassi, J.; Benech, H. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 555.
(86) Daluge, S. M.; Good, S. S.; Faletto, M. B.; Miller, W. H.; St. Clair, M. H.; Boone, L. R.; Tisdale, M.; Parry, N. R.; Reardon, J. E.; Dornsife, R. E.; Averett, D. R.; Krenitsky, T. S. *Antimicrob. Agents Chemother.* **1997**, *41*, 1082.
(87) Faletto, M. B.; Miller, W. H.; Garvey, E. P.; St. Clair, M. H.; Daluge, S. M.; Good, S. S. *Antimicrob. Agents Chemother.* **1997**, *41*, 1099.
(88) Staszewski, S.; Katlama, C.; Harrer, T.; Massip, P.; Yeni, P.; Cutrell, A.; Tortell, S. M.; Harrigan, R. P.; Steel, H.; Lanier, R. E.; Pearce, G. *AIDS* **1998**, *12*, F197.
(89) Fung, E. N.; Cai, Z. W.; Burnette, T. C.; Sinhababu, A. K. *J. Chromatogr., B* **2001**, *754*, 285.
(90) Cai, Z. W.; Fung, E. N.; Sinhababu, A. K. *Electrophoresis* **2003**, *24*, 3160.
(91) St. Claire, R. L. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1625.
(92) Ewing, D. F.; Len, C.; Mackenzie, G.; Ronco, G.; Villa, P. *Tetrahedron* **2000**, *11*, 4995.
(93) Ewing, D. F.; Fahmi, N.-E.; Len, C.; Mackenzie, G.; Ronco, G.; Villa, P.; Shaw, G. *Collect. Czech. Chem. Commun.* **1996**, *61*, S145.
(94) Ewing, D. F.; Fahmi, N.-E.; Len, C.; Mackenzie, G.; Ronco, G.; Villa, P.; Shaw, G. *Nucleosides Nucleotides* **1999**, *18*, 2613.
(95) Ewing, D. F.; Fahmi, N.; Len, C.; Mackenzie, G.; Pranzo, A. *J. Chem. Soc., Perkin Trans. 1* **2000**, *21*, 3561.
(96) Len, C.; Mackenzie, G.; Ewing, D. F.; Sheppard, G.; Banoub, J. *Carbohydr. Res.* **2003**, *338*, 2311.
(97) Richardson, F. C.; Zhang, C.; Lehrman, S. R.; Koc, H.; Swenberg, J. A.; Richardson, K. A.; Bendele, R. A. *Chem. Res. Toxicol.* **2002**, *15*, 922.
(98) Gandhi, V.; Chem, W.; Ayres, M.; Rhie, J. K.; Madden, T. L.; Newman, R. A. *Cancer Chemother. Pharmacol.* **2002**, *50*, 85.
(99) Masuda, M.; Suzuki, T.; Friesen, M. D.; Ravanat, J. L.; Cadet, J.; Pignatelli, B.; Nishino, H.; Ohshima, H. *J. Biol. Chem.* **2001**, *276*, 40486.
(100) Hayatsu, H.; Pan, S.; Ukita, T. *Chem. Pharm. Bull.* **1971**, *19*, 2189.
(101) Abdel-Hamid, M.; Novotny, L.; Hamza, H. *J. Pharm. Biomed. Anal.* **2000**, *22*, 745.
(102) Tuytten, R.; Lemiere, F.; Van Dongen, W.; Esmans, E. L.; Slegers, H. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1205.
(103) Qian, T. X.; Cai, Z. W.; Yang, M. S. *Anal. Biochem.* **2004**, *325*, 77.
(104) Dudley, E.; Lemiere, F.; Van Dongen, W.; Langridge, J. I.; El-Sharkawi, S.; Games, D. E.; Esmans, E. L.; Newton, R. P. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1701.
(105) Dudley, E.; El-Sharkawi, S.; Games, D. E.; Newton, R. P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1200.
(106) Dudley, E.; Lemiere, F.; Van Dongen, W.; Langridge, J. I.; El-Sharkawi, S.; Games, D. E.; Esmans, E. L.; Newton, R. P. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1132.
(107) Dudley, E.; Lemiere, F.; Van Dongen, W.; Esmans, E. L.; El-Sharkawi, S.; Games, D. E.; Brenton, A. G.; Newton, R. P. *Nucleosides Nucleotides* **2003**, *22*, 987.
(108) Lee, S. H.; Jung, B. H.; Kim, B. C.; Chung, B. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 973.
(109) McCloskey, J. A.; Graham, D. E.; Zhou, S. L.; Crain, P. F.; Ibba, M.; Konisky, J.; Soll, D.; Olsen, G. J. *Nucleic Acids Res.* **2001**, *29*, 4699.
(110) Pomerantz, S. C.; McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 796.
(111) Kowalak, J. A.; Bruenger, E.; Crain, P. F.; McCloskey, J. A. *J. Biol. Chem.* **2000**, *275*, 24484.
(112) Limbach, P. A.; Crain, P. F.; McCloskey, J. A. *Nucleic Acids Res.* **1994**, *22*, 2183.
(113) Mengel-Jorgenson, J.; Kirpekar, F. *Nucleic Acids Res.* **2002**, *30*, e135.
(114) Prakash, T. P.; Kawasaki, A. M.; Fraser, A. S.; Vasquez, G.; Manoharan, M. *J. Org. Chem.* **2002**, *37*, 357.
(115) Prakash, T. T.; Kawasaki, A. M.; Lesnik, E. A.; Sioufi, N.; Manoharan, M. *Tetrahedron* **2003**, *59*, 7413.
(116) Fu, H.; Xu, L.; Lu, Q.; Wang, J.-Z.; Xiao, H.-Z.; Zhao, Y.-F. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1813.
(117) Benaiges, M. D.; Lopez-Santin, J.; Sola, C. *Enzyme Microb. Technol.* **1990** *12*, 86.
(118) Todd, B. E. N. In *Proceedings of the 9th Australian Wine Industry Technical Conference*, *Adelaide*; Stockley, C. S., Sas, A. N., Johnson, R. S., Lee, T. H., Eds.; Winetitles: Adelaide, 1996; p 33.
(119) Belem, M. A. F.; Lee, B. H. *Food Sci. Technol. Int.* **1997**, *3*, 437.
(120) Mounolou, J. C. *The Yeasts*; Rose, A. H., Harrison, J. S., Eds.; Academic Press: London, 1971; Vol. 2 (The Physiology and Biochemistry of Yeasts), p 309.
(121) Trevelyan, W. E. *J. Sci. Food Agric.* **1978**, *29*, 903.
(122) Feuillat, M.; Charpentier, C. *Am. J. Enol. Vitic.* **1982**, *33*, 6.
(123) Courtis, K.; Todd, B.; Zhao, J. *Aust. Grapegrower Winemaker* **1998**, *401*, 51.
(124) Charpentier, C.; Feuillat, M. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwood Academic Press: Lausanne, 1993; p 225.
(125) Aussenac, J.; Chassagne, D.; Claparols, C.; Charpentier, M.; Duteurtre, B.; Feuillat, M.; Charpentier, C. *J. Chromatogr., A* **2001**, *907*, 155.
(126) Qiu, F.; McCloskey, J. A. *Nucleic Acids Res.* **1999**, *27*, e20.
(127) Zhu, Y. X.; Wong, P. S. H.; Zhou, Q.; Sotoyama, H.; Kissinger, P. T. *J. Pharm. Biomed. Anal.* **2001**, *26*, 967.
(128) (a) Crain, P. F. In *Electrospray Ionization Mass Spectrometry. Fundamentals*, *Instrumentation and Applications*; Cole, R. B., Ed.; John Wiley and Sons: New York, 1997; p 421. (b) Liguori, A.; Napoli, A.; Siciliano, S. G. *J. Chem. Soc., Perkin Trans. 2* **1994**, 1883. (c) Izatt, R. M.; Christen, J. J.; Rytting, H. *Chem. Rev.* **1971**, *71*, 439.
(129) Gilar, M. *Anal. Biochem.* **2001**, *298*, 196.
(130) Fountain, K. J.; Gilar, M.; Gebler, J. C. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 646.
(131) Fountain, K. J.; Gilar, M.; Gebler, J. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1295.
(132) Cavanagh, J.; Benson, L. M.; Thompson, R.; Naylor, S. *Anal. Chem.* **2002**, *75*, 3281.
(133) Null, A. P.; George, L. T.; Muddiman, D. C. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 338.
(134) Deroussent, A.; LeCaer, J. P.; Rossier, J.; Gouyette, A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1.
(135) Greig, M.; Griffey, R. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 97.
(136) Deguchi, K.; Ishikawa, M.; Yokotura, T.; Ogata, I.; Ito, S.; Mimura, T.; Ostrander, C. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2133.
(137) Van Breemen, R. B.; Tan, Y. C.; Lai, J.; Huang, C. R.; Zhao, X. M. *J. Chromatogr., A* **1998**, *806*, 67.
(138) Janning, P.; Schrader, W.; Linscheid, M. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 1035.
(139) Wu, H. Q.; Aboleneen, H. *Anal. Biochem.* **2001**, *290*, 347.
(140) Null, A. P.; Benson, L. M.; Muddiman, D. C. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2699.
(141) McLuckey, S. A.; Vaidyanathan G.; Habibi-Goudarzi, S. *J. Mass Spectrom.* **1995**, *30*, 1222.
(142) McLuckey, S. A.; Vaidyanathan, G. *Int. J. Mass Spectrom. Ion Proceses* **1997**, *162*, 1.
(143) Doktycz, M. J.; Habibi-Goudarzi, S.; McLuckey, S. A. *Anal. Chem.* **1994**, *66*, 3416.

(144) Doktycz, M. J.; Hurst, G. B.; Habibi-Goudarzi, S.; McLuckey, S.; Tang, K.; Chen, C. H.; Uziel, M.; Jacobson, K. B.; Woychik, R. P.; Buchanan, M. V. *Anal. Biochem.* **1995**, *230*, 205.
(145) Favre, A.; Gonnet, F.; Tabet, J.-C. *Eur. J. Mass Spectrom.* **2000**, *6*, 389.
(146) Little, D. P.; Chorush, R. A.; Speir, J. P.; Senko, M. W.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1994**, *116*, 4893.
(147) Little, D. P.; Speir, J. P.; Senko, M. W.; O'Connor, P. B.; McLafferty, F. W. *Anal. Chem.* **1994**, *66*, 2809.
(148) Little, D. P.; McLafferty, F. W. *J. Am. Chem. Soc.* **1995**, *117*, 6783.
(149) Little, D. P.; Aaserud, D. J.; Valaskovic, G. A.; McLafferty, F. W. *J. Am. Chem. Soc*. **1996**, *118*, 9352.
(150) Little, D. P.; Thannhauser, T. W.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2318.
(151) Sannes-Lowery, K. A.; Hofstadler, S. A. *J. Am. Soc. Mass Spectrom*. **2003**, *14*, 825.
(152) Gentil, E.; Banoub, J. *J. Mass Spectrom.* **1996**, *31*, 83.
(153) Ni, J.; Chan, K. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1600.
(154) Baker, T. R.; Keough, T.; Dobson, R. L. M.; Riley, T. A.; Hasselfield, J. A.; Hesselberth, P. E. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 190.
(155) Stephenson, J. L.; McLuckey, S. A. *J. Mass Spectrom.* **1998**, *33*, 664.
(156) McLuckey, S. A.; Glish, G. L.; Van Berkel, G. J. *Anal. Chem.* **1991**, *63*, 1971.
(157) Ogorzalek-Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Phys. Chem.* **1991**, *95*, 6412.
(158) Stephenson, J. L., Jr.; McLuckey, S. A. *Anal. Chem.* **1998**, *70*, 3533.
(159) Herron, W. J.; Goeringer, D. E.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 529.
(160) McLuckey, S. A.; Wu, J.; Bundy, J. L.; Stephenson, J. L.; Hurst, G. B. *Anal. Chem.* **2002**, *74*, 976.
(161) McLuckey, S. A.; Goeringer, D. E. *Anal. Chem.* **1995**, *67*, 2493.
(162) Josse, J.; Kaiser, A. D.; Kornberg, A. *J. Biol. Chem.* **1961**, *236*, 864.
(163) Rozenski, J.; McCloskey, J. A. *Anal. Chem.* **1999**, *71*, 1454.
(164) Rozenski, J.; McCloskey, J. A. *Nucleosides Nucleotides Nucleic Acids* **1999**, *18*, 1539.
(165) Oberacher, H.; Wellenzohm, B.; Huber, C. G. *Anal. Chem.* **2002**, *74*, 211.
(166) Premstaller, A.; Huber, C. G. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1053.
(167) Ni, J. S.; Pomerantz, S. C.; Rozenski, J.; Zhang, Y.; McCloskey, J. A. *Anal. Chem.* **1996**, *68*, 1989.
(168) Rozenski, J.; McCloskey, J. A. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 200.
(169) Oberacher, H.; Parson, W.; Oefner, P. J.; Mayr, B. M.; Huber, C. G. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 510.
(170) Berger, B.; Hölzl, G.; Oberacher, H.; Niederstätter, H.; Huber, C. G.; Parson, W. *J. Chromatogr., B* **2002**, *782*, 89.
(171) http://medlib.med.utah.edu/masspec/mongohlp.htm, July 2003.
(172) Johnson, Y. A.; Nagpal, M.; Krahmer, M. T.; Fox, K. F.; Fox, A. *J. Microbiol. Methods* **2000**, *40*, 241.
(173) Veenstra, T. D. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 1.
(174) Ganem, B.; Li, Y. T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 6294.
(175) Ganem, B.; Li, Y. T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 7818.
(176) Hofstadler, S. A.; Griffey, R. H. *Chem. Rev.* **2001**, *101*, 377.
(177) Gabelica, V.; De Pauw, E.; Rosu, F. *J. Mass Spectrom.* **1999**, *34*, 1328.
(178) Kapur, A.; Beck, J. L.; Sheil, M. M. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2489.
(179) Gupta, R.; Kapur, A.; Beck, J. L.; Sheil, M. M. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2472.
(180) Kloster, M. B. G.; Hannis, J. C.; Muddiman, D. C.; Farrell, N. *Biochemistry* **1999**, *38*, 14731.
(181) Verheijen, J. C.; van der Marel, G. A.; van Boom, J. H.; Metzler-Nolte, N. *Bioconjugate Chem.* **2000**, *11*, 741.
(182) Craig, T. A.; Benson, L. M.; Tomlinson, A. J.; Veenstra, T. D.; Naylor, S.; Kumar, R. *Nat. Biotechnol.* **1999**, *17*, 1214.
(183) Veentra, T. D.; Benson, L. M.; Craig, T. A.; Tomlinson, A. J.; Kumar, R.; Naylor, S. *Nat. Biotechnol.* **1998**, *16*, 262.
(184) Deterding, L. J.; Kast, J.; Przybylski, M.; Tomer, K. B. *Bioconjugate Chem.* **2000**, *11*, 335.
(185) Zhang, S.; Van Pelt, C. K.; Wilson, D. B. *Anal. Chem.* **2003**, *75*, 3010.
(186) Haskins, N. J.; Ashcroft, A. E.; Phillips, A.; Harrison, M. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 120.
(187) Harsch, A.; Marzilli, L. A.; Bunt, R. C.; Stubbe, J.; Vouros, P. *Nucleic Acids Res.* **2000**, *28*, 1978.
(188) Xu, N. X.; Pásа-Tolić, L.; Smith, R. D.; Ni, S. S.; Thrall, B. D. *Anal. Biochem.* **1999**, *272*, 26.
(189) Hope, I. A.; Struhl, K. *Cell* **1985**, *43*, 177.
(190) Hope, I. A.; Struhl, K. *Cell* **1986**, *46*, 885.
(191) Hope, I. A.; Struhl, K. *EMBO J.* **1987**, *6*, 2781.
(192) Talanian, R. V.; McKnight, C. J.; Kim, P. S. *Science* **1990**, *249*, 769.
(193) Morice, Y.; Roulot, D.; Grando, V.; Stirneman, J.; Gault, E.; Jeantils, V.; Bentata, M.; Jarousse, B.; Lortholary, M.; Pallier, C.; Deny, P. *J. Gen. Virol*. **2001**, *82*, 1001.
(194) Hoyne, P. R.; Benson, L. M.; Veenstra, T. D.; Maher, L. J.; Naylor, S. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1539.
(195) Holz, B.; Dank, N.; Eickhoff, J. E.; Lipps, G.; Krauss, G.; Weinhold, E. *J. Biol. Chem.* **1999**, *274*, 15066.
(196) Wong, D. L.; Reich, N. O. *Biochemistry* **2000**, *39*, 15410.
(197) Rusmintratip, V.; Riggs, A. D.; Sowers, L. C. *Nucleic Acids Res.* **2000**, *28*, 3594.
(198) Deterding, L. J.; Prasad, R.; Mullen, G. P.; Wilson, S. H.; Tomer, K. B. *J. Biol. Chem.* **2000**, *275*, 10463.
(199) Reiber, D. C.; Murphy, R. C. *Arch. Biochem. Biophys.* **2000**, *379*, 119.
(200) Köster, H.; Tang, K.; Fu, D. J.; Braun, A.; Van den Boom, D.; Smith, C. L.; Cotter, R. J.; Cantor, C. R. *Nat. Biotechnol.* **1996**, *14*, 1123.
(201) Fu, D. J.; Tang, K.; Braun, A.; Reuter, D.; Darnhofer-Demar, B.; Little, D. P.; O'Donnell, M. J.; Cantor, C. R.; Köster, H. *Nat. Biotechnol.* **1998**, *16*, 381.
(202) Hahner, S.; Lüdemann, H. C.; Kirpekar, F.; Nordhoff, E.; Roepstorff, P.; Galla, H. J.; Hillenkamp, F. *Nucleic Acids Res.* **1997**, *25*, 1957.
(203) Faulstich, K.; Wörner, K.; Brill, H.; Engels, W. J. *Anal. Chem.* **1997**, *69*, 4349.
(204) Ross, P. L.; Belgrader, P. *Anal. Chem.* **1997**, *69*, 3966.
(205) Ross, P. L.; Lee, K.; Belgrader, P. *Anal. Chem.* **1997**, *69*, 4197.
(206) Griffin, J. T.; Tang, W.; Smith, L. M. *Nat. Biotechnol.* **1997**, *15*, 1368.
(207) Nordhoff, E.; Ingendoh, A.; Cramer, R.; Overberg, A.; Stahl, B.; Karas, M.; Hillenkamp, F.; Crain, P. F. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 771.
(208) Nordhoff, E.; Kipekar, F.; Karas, M.; Cramer, R.; Hahner, S.; Hillenkamp, F.; Kristiansen, K.; Roepstroff, P.; Lezius, A. *Nucleic Acids Res.* **1994**, *22*, 2460.
(209) Nordhoff, E.; Kirpekar, F.; Roepstorff, P. *Mass Spectrom. Rev.* **1996**, *15*, 67.
(210) Vestal, M. L.; Juhasz, P.; Martin, S. A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1044.
(211) Gut, I. G. *Hum. Mutat.* **2004**, *23*, 437.
(212) Kong, Y.; Zhu, Y.; Zhang, J.-Y. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 57.
(213) Langley, G. J.; Herniman, J. M.; Davies, N. L.; Brown, T. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1717.
(214) Liu, C.; Wu, Q.; Harms, A. C.; Smith, R. D. *Anal. Chem.* **1996**, *68*, 3295.
(215) Smirnov, I. P.; Hall, L. R.; Ross, P. L.; Haff, L. A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1427.
(216) Gilar, M.; Belenky, A.; Wang, B. H. *J. Chromatogr., A* **2001**, *921*, 3.
(217) Davis, D. L.; O'Brien, E. P.; Bentzley, C. M. *Anal. Chem.* **2000**, *72*, 5092.
(218) Wu, K. J.; Steding, A.; Becker, C. H. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 142.
(219) Tang, K.; Taranenko, N. I.; Allman, S. L.; Chen, C. H.; Chang, L. Y.; Jacobson, K. B. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 673.
(220) Krause, J.; Stoeckli, M.; Schlunegger, U. P. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1927.
(221) Zhu, Y. F.; Chung, C. N.; Taranenko, N. I.; Allman, S. L.; Martin, S. A.; Haff, L.; Chen, C. L. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 383.
(222) Lin, H.; Hunter, J. M.; Becker, C. H. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2335.
(223) Shahgholi, M.; Garcia, B. A.; Chiu, N. H. L.; Heaney, P. J.; Tang, K. *Nucleic Acids Res*. **2001**, *29*, e91.
(224) Williams, T. L.; Fenselau, C. *Eur. J. Mass Spectrom.* **1998**, *4*, 379.
(225) Menzel, C.; Berkenkamp, S.; Hillenkamp, F. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 26.
(226) Kirpekar, F.; Berkenkamp, S.; Hillenkamp, F. *Anal. Chem.* **1999**, *71*, 2334.
(227) Berkenkamp, S.; Menzel, C.; Karas, M.; Hillenkamp, F. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1399.
(228) Chen, W. Y.; Chen, Y. C. *Anal. Chem.* **2003**, *75*, 4223.
(229) Weng, M. F.; Chen, Y. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1421.
(230) Zhou, L. H.; Deng, H. M.; Deng, Q. Y.; Zhao, S. K. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 787.
(231) Berlin, K.; Gut, I. G. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1739.
(232) Lichtenwalter, K. G.; Apffel, A.; Bai, J.; Chakel, J. A.; Dai, Y.; Hahnenberger, K. M.; Li, L.; Hancock, W. S. *J. Chromatogr., B* **2000**, *745*, 231.
(233) Meyer, A.; Spinelli, N.; Imbach, J.-L.; Vasseur, J.-J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 234.

(234) Meyer, A.; Spinelli, N.; Brès, J.-C.; Dell'Aquila, C.; Morvan, F.; Lefebvre, I.; Rayner, B.; Imbach, J.-L.; Vasseur, J.-J. *Nucleosides Nucleotides Nucleic Acids* **2001**, *20*, 963.
(235) Guerlavais. T.; Meyer, A.; Depart, F.; Imbach, J.-L.; Morvan, F.; Vasseur, J.-J. *Anal. Bioanal. Chem.* **2002**, *374*, 57.
(236) Taranenko, N. I.; Hurt, R.; Zhou, J. Z.; Isola, N. R.; Huang, H.; Lee, S. H.; Chen, C. H. *J. Microbiol. Methods* **2002**, *48*, 101.
(237) Olejnik, J.; Ludemann, H.; Krzymanska-Olejnik, E.; Berkenkamp, S.; Hillenkamp, F.; Rothschild, K. J. *Nucleic Acids Res.* **1999**, *27*, 4626.
(238) Alazard, D.; Filipowsky, M.; Raeside, J.; Clarke, M.; Majlessi, M.; Russell, J.; Weisburg, W. *Anal. Biochem.* **2002**, *301*, 57.
(239) Pieles, U.; Zurcher, W.; Schar, M.; Moser, H. E. *Nucleic Acids Res*. **1993**, *21*, 3191.
(240) Bentzley, C. M.; Johnston, M. V.; Larsen, B. S. *Anal*. *Biochem*. **1998**, *258*, 31.
(241) Wada, Y. *J. Mass Spectrom.* **1998**, *33*, 187.
(242) Taranenko, N. I.; Allman, S. L.; Golovlev, V. V.; Taranenko, N. V.; Isola, N. R.; Chen, C. H. *Nucleic Acids Res.* **1998**, *26*, 2488.
(243) Nordhoff, E.; Leubbert, C.; Thiele, G.; Heiser, V.; Lehrach, H. *Nucleic Acids Res.* **2000**, *28*, e86.
(244) Tang, W.; Scalf, M.; Smith, L. M. *Anal. Chem.* **1997**, *69*, 302.
(245) Ono, T.; Scalf, M.; Smith, L. M. *Nucleic Acids Res.* **1997**, *25*, 4581.
(246) Stoerker, J.; Mayo, J. D.; Tetzlaff, C. N.; Sarracino, D. A.; Schwope, I.; Richert, C. *Nat. Biotechnol.* **2000**, *18*, 1213.
(247) Tost, J.; Gut, I. G. *Mass Spectrom. Rev.* **2002**, *21*, 389.
(248) Fei, Z.; Smith, L. M. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 950.
(249) Krebs, S.; Seichter, D.; Forster, M. *Nat. Biotechnol.* **2001**, *19*, 877.
(250) Seichter, D.; Krebs, S.; Forster, M. *Nucleic Acids Res.* **2004**, *32*, e16.
(251) Wada, Y.; Mitsumori, K.; Terachi, T.; Ogawa, O. *J. Mass Spectrom.* **1999**, *34*, 885.
(252) Hung, K.; Sun, H.; Ding, M.; Kalafatis, P.; Simioni, P.; Guo, B. *Blood Coagul. Fibrinolysis* **2002**, *13*, 117.
(253) Wolfe, J. L.; Kawate, T.; Sarracino, D. A.; Zillmann, M.; Olson, J.; Stanton, V. P., Jr.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11073.
(254) Sun, X. Y.; Hung, K.; Wu, L.; Sidransky, D.; Guo, B. B. *Nat. Biotechnol.* **2002**, *20*, 186.
(255) Siuzdak, G. *J. Mass Spectrom.* **1998**, *33*, 203.
(256) Fuerstenau, S. D.; Benner, W. H.; Thomass, J. J.; Brugidou, C.; Bothner, B.; Siuzdak, G. *Angew. Chem., Int. Ed.* **2001**, *40*, 542.
(257) Bothner, B.; Siuzdak, G. *Chembiochem* **2004**, 5, 258.
(258) Amexis, G.; Oeth, P.; Abel, K.; Ivshina, A.; Pelloquin, F.; Cantor, C. R.; Brau, A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12097.
(259) Issaq, H. J.; Veenstra, T. D.; Conrads, T. P.; Felschow, D. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 587.
(260) Forde, C. E.; Gonzales, A. D.; Smessaert, J. M.; Murphy, G. A.; Shields, S. J.; Fitch, J. P.; McCutchen-Maloney, S. L. *Biochem. Biophys. Res. Commun*. **2002**, *290*, 1328.
(261) Kirpekar, F.; Krogh, T. N. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 8.
(262) Petersson, E. J.; Shahgholi, M.; Lester, H. A.; Dougherty, D. A. *RNA −Publ. RNA Soc.* **2002**, *8*, 542.
(263) Andersen, T. E.; Porse, B. T.; Kirpekar, F. *RNA −Publ. RNA Soc.* **2004**, *10*, 907.
(264) Donis-Keller, H. *Nucleic Acids Res.* **1979**, *7*, 179.
(265) Polo, L. M.; Limbach, P. A. *J. Mass Spectrom.* **1998**, *33*, 1226.
(266) Wang, Q.; Shoeman, R.; Traub, P. *Biochemistry* **2000**, *39*, 6645.
(267) Thiede, B.; Von Janta-Lipinski, M. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1889.
(268) Apruzzese, W. A.; Vouros, P. *J. Chromatogr., A* **1998**, *794*, 97.
(269) Ranasinghe, A.; Scheller, N.; Wu, K. Y.; Upton, P. B.; Swenberg, J. A. *Chem. Res. Toxicol.* **1998**, *11*, 520.
(270) Wu, K. Y.; Scheller, N.; Ranasinghe, A.; Yen, T. Y.; Sangaiah, R.; Giese, R.; Swenberg, J. A. *Chem. Res. Toxicol.* **1999**, *12*, 722.
(271) Eide, I.; Zhao, C.; Kumar, R.; Hemminki, K.; Wu, K. Y.; Swenberg, J. A. *Chem. Res. Toxicol*. **1999**, *12*, 979.
(272) Ham, A. J. L.; Ranasinghe, A.; Morinello, E. J.; Nakamura, J.; Upton, P. B.; Johnson, F.; Swenberg, J. A. *Chem. Res. Toxicol.* **1999**, *12*, 1240.
(273) Morinello, E. J.; Ham, A. J. L.; Ranasinghe, A.; Sangaiah, R.; Swenberg, J. A. *Chem. Res. Toxicol.* **2001**, *14*, 327.
(274) Chen, H. J. C.; Lin, T. C.; Hong, C. L.; Chiang, L. C. *Chem. Res. Toxicol*. **2001**, *14*, 1612.
(275) LaFrancois, C. J.; Yu, K.; Sowers, L. C. *Chem. Res. Toxicol.* **1998**, *11*, 786.
(276) D'Ham, C.; Ravanat, J. L.; Cadet, J. *J. Chromatogr., B* **1998**, *710*, 67.
(277) Farooq, S.; Bailey, E.; Farmer, P. B.; Jukes, R.; Lamb, J. H.; Hernandez, H.; Sram, R.; Topinka, J. *J. Chromatogr., B* **1997**, *702*, 49.
(278) Park, M.; Loeppky, R. N. *Chem. Res. Toxicol.* **2000**, *13*, 72.
(279) Airoldi, L.; Orsi, F.; Magagnotti, C.; Coda, R.; Randone, D.; Casetta, G.; Peluso, M.; Hautefeuille, A.; Malaveille, C.; Vineis, P. *Carcinogenesis* **2002**, *23*, 861.
(280) Senturker, S.; Dizdaroglu, M. *Free Radical Biol. Med*. **1999**, *27*, 370.
(281) Frelon, S.; Douki, T.; Ravanat, J. L.; Pouget, J. P.; Torabene, C.; Cadet, J. *Chem. Res. Toxicol.* **2000**, *13*, 1002.
(282) Podmore, I. D.; Cooper, D.; Evans, M. D.; Wood, M.; Lunec, J. *Biochem. Biophys. Res. Commun.* **2000**, *277*, 764.
(283) Weimann, A.; Belling, D.; Poulsen, H. E. *Free Radical Biol. Med.* **2001**, *30*, 757.
(284) Jaruga, P.; Rodriguez, H.; Dizdaroglu, M. *Free Radical Biol. Med.* **2001**, *31*, 336.
(285) Byun, J.; Henderson, J. P.; Mueller, D. M.; Heinecke, J. W. *Biochemistry* **1999**, *38*, 2590.
(286) Doerge, D. R.; Churchwell, M. I.; Fang, J. L.; Beland, F. A. *Chem. Res. Toxicol.* **2000**, *13*, 1259.
(287) Chen, H.-J. C.; Chang, C.-M. *Chem. Res. Toxicol*. **2004**, *17*, 963.
(288) Hillestrom, P. R.; Hoberg, A. M.; Weimann, A.; Poulsen, H. E. *Free Radical Biol. Med*. **2004**, *36*, 1383.
(289) Salomon, J.; Elad, D. *J. Org. Chem.* **1973**, *38*, 3420.
(290) Livneh, Z.; Livneh, E.; Sperling, J. *Photochem. Photobiol*. **1980**, *32*, 131.
(291) Nakao, L. S.; Augusto, O. *Chem. Res. Toxicol.* **1998**, *11*, 888.
(292) Hoes, I.; Lemiere, F.; Van Dongen, W.; Vanhoutte, K.; Esmans, E. L.; Van Bockstaele, D.; Berneman, Z. N.; Deforce, D.; Van den Eeckhout, E. G. *J. Chromatogr., B* **1999**, *736*, 43.
(293) Hoes, I.; Van Dongen, W.; Lemiere, F.; Esmans, E. L.; Van Bockstaele, D.; Berneman, Z. N. *J. Chromatogr., B* **2000**, *748*, 197.
(294) Van den Driessche, B.; Lemiere, F.; Van Dongen, W.; Esmans, E. L. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 568.
(295) Haglund, J.; Van Dongen, W.; Lemiere, F.; Esmans, E. L. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 596.
(296) Embrechts, J.; Lemiere, F.; Van Dongen, W.; Esmans, E. L.; Buytaert, P.; Van Marck, E.; Kockx, M.; Makar, A. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 482.
(297) Romieu, A.; Gasparutto, D.; Cadet, J. *Chem Res. Toxicol.* **1999**, *12*, 412.
(298) Rodriguez, H.; Jaruga, P.; Dizdaroglu, M. *Free Radical Biol. Med*. **2001**, *30*, 774.
(299) Jaruga, P.; Birincioglu, M.; Rodriguez, H.; Dizdaroglu, M. *Biochemistry.* **2002**, *41*, 3703.
(300) Jaruga, P.; Theravathu, J.; Dizdaroglu, M.; Brooks, P. J. *Nucleic Acids Res.* **2004**, *32*, e87.
(301) Gangl, E. T.; Turesky, R. J.; Vouros, P. *Chem. Res. Toxicol*. **1999**, *12*, 1019.
(302) Gangl, E. T.; Turesky, R. J.; Vouros, P. *Anal. Chem.* **2001**, *73*, 2397.
(303) Crosbie, S. J.; Murray, S.; Boobis, A. R.; Gooderham, N. J. *J. Chromatogr., B* **2000**, *744*, 55.
(304) Fang, A. H.; Smith, W. A.; Vouros, P.; Gupta, R. C. *Biochem. Biophys. Res. Commun.* **2001**, *281*, 383.
(305) Siethoff, C.; Feldmann, I.; Jakubowski, N.; Linscheid, M. *J. Mass Spectrom.* **1999**, *34*, 421.
(306) Huang, H.; Jemal, A.; David, C.; Barker, S. A.; Swenson, D. H.; Means, J. C. *Anal. Biochem.* **1998**, *265*, 139.
(307) Walton, M.; Egner, P.; Scholl, P. F.; Walker, J.; Kensler, T.; Groopman, J. D. *Chem. Res. Toxicol.* **2001**, *14*, 919.
(308) Treyakova, N. Y.; Chiang, S. Y.; Walker, V. E.; Swenberg, J. A. *J. Mass Spectrom.* **1998**, *33*, 363.
(309) Convert, O.; Van Aerden, C.; Debrauwer, L.; Rathahao, E.; Molines, H.; Fournier, F.; Tabet, J.-C.; Paris, A. *Chem. Res. Toxicol.* **2002**, *15*, 754.
(310) Xue, W. L.; Siner, A.; Rance, M.; Jayasimhulu, K.; Talaska, G.; Warshawsky, D. *Chem. Res. Toxicol.* **2002**, *15*, 915.
(311) Umemoto, A.; Monden, Y.; Suwa, M.; Kanno, Y.; Suzuki, M.; Lin, C. X.; Ueyama, Y.; Momen, M. A.; Ravindernath, A.; Shibutani, S.; Komaki, K. *Carcinogenesis* **2000**, *21*, 1737.
(312) Carvalho, V. M.; Gasparutto, D.; Di Mascio, P.; Medeiros, M. H. G.; Cadet, J. *Biorg. Med. Chem.* **2003**, *11*, 2445.
(313) Tretyakova, N. Y.; Niles, J. C.; Burney, S.; Wichnok, J. S.; Tannenbaum, S. R. *Chem. Res. Toxicol*. **1999**, *12*, 459.
(314) Duarte, V.; Gasparutto, D.; Yamaguchi, L. F.; Ravanat, J. L.; Martinez, J. R.; Medeiros, M. H. G.; Di Mascio, P.; Cadet, J. *J. Am. Chem. Soc.* **2000**, *122*, 12622.
(315) Martinez, G. R.; Medeiros, M. H. G.; Ravanat, J.-L.; Cadet, J.; Di Mascio, P. *Biol. Chem.* **2002**, *383*, 607.
(316) Marzilli, L. A.; Barry, J. P.; Sells, T.; Law, S. J.; Vouros, P.; Harsch, A. *J. Mass Spectrom.* **1999**, *34*, 276.
(317) Glover, R. P.; Lamb, J. H.; Farmer, P. B. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 368.
(318) Douki, T.; Odin, F.; Caillat, S.; Favier, A.; Cadet, J. *Free Radical Biol. Med*. **2004**, *37*, 62.
(319) Tretyakova, N.; Matter, R.; Jones, R.; Shallop, A. *Biochemistry* **2002**, *41*, 9535.
(320) Matter, R.; Wang, G.; Jones, R.; Tretyakova, N. *Chem. Res. Toxicol.* **2004**, *17*, 731.
(321) Banoub, J.; Combden, S.; Miller-Banoub, J.; Sheppard, G.; Hodder, H. *Nucleosides Nucleotides* **1999**, *18*, 2751.

(322) Warnke, U.; Gysler, J.; Hofte, B.; Tjaden, U. R.; van der Greer, J.; Kloft, C.; Schunack, W.; Jaehde, U. *Electrophoresis* **2001**, *22*, 97.
(323) Carte, N.; Legendre, F.; Leize, E.; Potier, N.; Reeder, F.; Chottard, J. C.; Dorsselaer, A. V. *Anal. Biochem.* **2000**, *284*, 77.
(324) Madhusudanan, K. P.; Katti, S. B.; Vijayakshmi, R.; Nair, B. U. *J. Mass Spectrom.* **1999**, *34*, 880.
(325) Douki, T.; Court, M.; Cadet, J. *J. Photochem. Photobiol., B: Biol.* **2000**, *54*, 145.
(326) Wang, Y. S.; Taylor, J. S.; Gross, M. L. *Chem. Res. Toxicol*. **1999**, *12*, 1077.
(327) Bellon, S.; Ravanat, J. L.; Gasparutto, D.; Cadet, J. *Chem. Res. Toxicol.* **2002**, *15*, 598.
(328) Balcome, S.; Park, S.; Dorr, D. R. Q.; Hafner, L.; Phillips, L.; Tretyakova, N. *Chem. Res. Toxicol.* **2004**, *17*, 950.
(329) Gasparutto, D.; Saint-Pierre, C.; Jaquinod, M.; Cadet, J. *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 1583.
(330) Gasparutto, D.; Da Cruz, S.; Bourdat, A. G.; Jaquinod, M.; Cadet, J. *Chem. Res. Toxicol.* **1999**, *12*, 630.
(331) Tretyakova, N.; Matter, B.; Ogdie, A.; Wishnok, J. S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **2001**, *14*, 1058.
(332) D'Ham, C.; Romieu, A.; Jaquinod, M.; Gasparutto, D.; Cadet, J. *Biochemistry* **1999**, *38*, 3335.
(333) Jacutin, S.; Zhang, A. J.; Russell, D. H.; Gibbs, R. A.; Burgess, K. *Nucleic Acids Res*. **1997**, *25*, 5072.
(334) Lan, Z. H.; Wang, P.; Giese, R. W. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1454.
(335) Bobalova, J.; Bobal, P.; Mutafova-Yambolieva, V. N. *Anal. Biochem.* **2002**, *305*, 269.
(336) Schultz, J. C.; Hack, C. A.; Benner, W. H. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 15.
(337) Madhusudanan, K. P.; Katti, S. B.; Dwivedi, A. K. *J. Mass Spectrom.* **1998**, *33*, 1017.
(338) Vrkic, A. K.; O'Hair, R. A. J.; Lebrilla, C. B. *Eur. J. Mass Spectrom.* **2003**, *9*, 563.
(339) Von Sonntag, C. In *Physical and Chemical Mechanism in Molecular Radiation Biology*; Glass, W. A., Varma, M. N., Eds.; Plenum Press: New York, 1991; pp 287−321.
(340) Gellene, G. I.; Porter, R. F. *Acc. Chem. Res*. **1983**, *16*, 200.
(341) Turecek, F. *Org. Mass Spectrom*. **1992**, *27*, 1087.
(342) Turecek, F. *J. Mass Spectrom.* **1998**, *33*, 779.
(343) Dingley, K. H.; Roberts, M. L.; Velsko, C. A.; Turteltaub, K. W. *Chem. Res. Toxicol*. **1998**, *11*, 1217.
(344) Li, H.; Cheng, Y.; Wang, H.; Sun, H. F.; Liu, Y. F.; Liu, K. X.; Peng, S. X. *Appl. Radiat. Isot.* **2003**, *58*, 291.
(345) Wang, H. F.; Xu, L. H.; Sun, H. F.; Xue, B.; Liu, Y. F.; Peng, S. X.; Liu, K. X.; Ma, H. J.; Guo, Z. Y. *Nucl. Instrum. Methods Phys. Res., Sect. B:Beam Interact. Mater. Atoms* **2004**, *223*−*24*, 745.
(346) Xue, B.; Wang, H. F.; Xu, L. H.; Sun, H. F.; Liu, Y. F.; Peng, S. X.; Liu, K. X.; Guo, Z. Y. *Nucl. Instrum. Methods Phys. Res., Sect. B: Beam Interact. Mater. Atoms* **2004**, *223*−*24*, 765.
(347) Lappin, G.; Garner, R. C. *Anal. Bioanal. Chem.* **2004**, *378*, 356.
(348) May, C, J.; Canavan, H. E.; Castner, D. G. *Anal. Chem.* **2004**, *76*, 1114.
(349) Ross, P.; Hall, L.; Smirnov, I.; Haff, L. *Nat. Biotechnol.* **1998**, *16*, 1347.
(350) Kim, S.; Ulz, M. E.; Nguyen, T.; Li, C.-M.; Sato, T.; Tycko, B.; Ju, J. *Genomics* **2004**, *83*, 924.

CR030040W