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Mass spectrometry of DNA constituents

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Mass Spectrometry of DNA Constituents

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Other publications

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"Primary Structure of Trypsin Inhibitors from *Sicyos australis*." Randolph I. Mar, John A. Carver, Margaret M.Sheil, Jacqueline Boschenok, Shanlin Fu and Denis C. Shaw, Phytochemistry (1996) 41(5), p1265-1274.

"Electrospray Ionisation Mass Spectrometry of Covalent Ligand-Oligonucleotide Adducts: Evidence for Specific Duplex Ion Formation." Geoffrey Wickham, Paula Iannitti, Jacqueline Boschenok and Margaret M. Sheil, Journal of Mass Spectrometry and Rapid Communications in Mass Spectrometry (1995) (special conference ed.), pS194-S203.

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Abstract

This study explores the utility of mass spectrometry for the characterisation of DNA constituents.

The tandem mass spectra (MS/MS) following electrospray ionisation (ESI) of nucleotides (from 2-12 mers) were examined under a range of collision and ionisation conditions. In general, abundant peaks due to the free bases and phosphate groups were observed along with a limited number of sequence ions. A number of strategies for increasing the extent of the observed fragmentation were investigated. Increasing the skimmer potential, and thereby increasing the initial internal energy of the incident ions, did not significantly enhance the sequence-specific fragmentation, although the relative intensities of fragment ions increased. In the ESI-MS/MS spectra of different adducts (i.e. $[M+H]^+$, $[M-H]^-$) of dinucleotides, the intensity of the ions due to the bases (BH_2^+ or B^-) is dependent on both the base position and the nature of the adduct ion. These data show that the MS/MS spectra of $[M-H]^-$ ions of dinucleotides generated by ESI may provide as much structural information as the MS/MS spectra obtained following fast atom bombardment. With $[M+Li]^+$ ions, more sequence ions were observed compared to the corresponding protonated and deprotonated species. For oligonucleotide hexamers, MS/MS spectra of the $[M-2H]^{2-}$ ions show fragment ions due to the loss of bases followed by cleavage of the 3′ C-O bond in the sugar from which the base is lost (i.e. w ions); however, a strong preference for loss of any particular base is not observed. Finally, MS/MS spectra of 5′-ApCpC-3′, 5′-d(AGGCCT)-3′ and 5'-d(CCGTAATTACGG)-3' obtained on a magnetic sector orthogonal acceleration time-of-flight mass spectrometer demonstrate the potential utility of this instrumental configuration for MS/MS analysis of oligonucleotides.

The reaction between the antitumour agent pyrindamycin A (PyA) and the oligonucleotides 5'-CGGTAATTACCG-3' and 5'-CGCGAATTCGCG-3' was studied by HPLC and ESI-MS. Pyrindamycin A is a minor groove binding agent that alkylates the N3 position of adenine. A range of experimental conditions were examined where the pH, reaction time and the reaction buffer were varied.

Formation of the PyA adduct of the oligonucleotide was optimised when the reaction was buffered to pH 8.0 with sodium phosphate for 11 days. During HPLC purification of the reaction mixture, two peaks were generally observed due to the unreacted oligonucleotide and the adduct respectively. ESI-MS spectra of the adduct showed the presence of two different species, namely the oligonucleotide less base A and the $[(PyA\text{-}HCl)+A]^-$ adduct. These were thought to be decomposition products of the PyA adduct of the oligonucleotide occurring during the ESI process. This suggests that the adducts of pyrindamycin A are more labile than adducts formed between other alkylating agents such as those that alkylate guanine (for which it has been shown that intact adducts can be observed). While only one adduct was observed for the 5'-CGGTAATTACCG-3' sequence based on the HPLC profiles, two adducts were evident for 5'-CGCGAATTCGCG-3'. This is thought to arise because the first oligonucleotide has a highly preferred binding site at A9, whereas in the second sequence no adenines are contained within a preferred binding site and the binding is therefore less specific.

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1. Introduction

Deoxyribonucleic acid (DNA) is a polynucleotide which serves as the carrier for genetic information. The primary structure of DNA is a specific sequence of deoxyribonucleotides. These deoxyribonucleotides are comprised of a base, a sugar moiety, and a phosphate group. The base of the DNA is responsible for carrying the genetic information, while the sugar and phosphate groups perform a structural role. There are four different bases, each of which can exist in a different tautomeric form. They are adenine (A), thymine (T), guanine (G) and cytosine (C), and their predominant tautomeric forms are shown in Figure 1.1. (1)

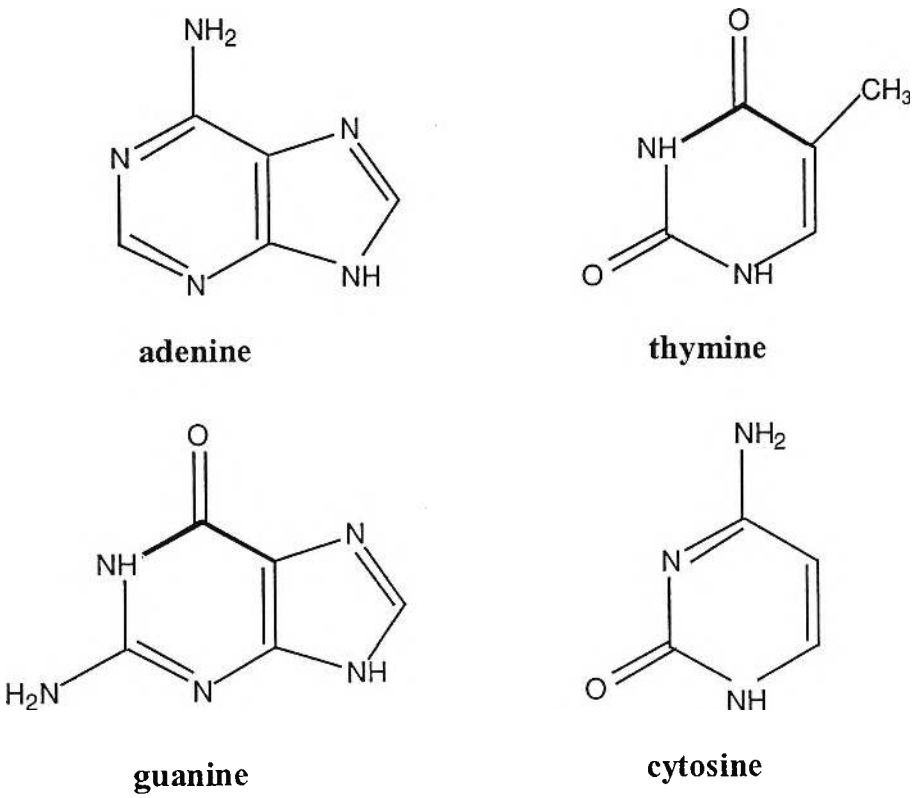


Figure 1.1: Structures of the DNA bases

The secondary structure of DNA is normally a double helix, which is also referred to as a duplex. The two sequence strands are held together through hydrogen bonding of the base pairs A:T and G:C.(1) The difficulty of using DNA as a target for drug interactions stem from its biological role and the risk of disrupting a normal function. For this

reason only serious diseases, such as cancer, have been treated with drugs that interact with nucleic acids. The development of more effective, less toxic, antitumour agents require that the techniques utilised for assessing the binding of the ligand to DNA become very specific.(2) Techniques such as nuclear magnetic resonance spectroscopy (NMR) and x-ray crystallography have previously been used for the characterisation of ligand-DNA interactions, however, large amounts of sample are required for these analyses. For this reason mass spectrometry is rapidly becoming an established tool for the analysis of drug-DNA interactions since only very low concentrations of sample (in the order of pmol/μL) are required. Mass spectrometry can be used to determine the molecular weight of the ligand-DNA complexes, and tandem mass spectrometry (MS/MS) experiments can provide structural information.

1.1. Mass Spectrometry Instrumentation

Mass spectrometry is a technique that is used to determine the molecular weight of a molecule. A mass spectrometer is normally comprised of an ionisation source, in which ions are introduced into the gas phase; a mass analyser, which is responsible for separating the ions according to their mass-to-charge ratio or velocity; and a detector and computer data system, by which the data is recorded and processed. In addition, samples must be analysed under high vacuum. Many different molecules have been analysed by mass spectrometry, including peptides, proteins, oligonucleotides, sugars and low molecular weight organic and inorganic compounds.

1.1.1. Ionisation Techniques

The method by which molecules are ionised for mass spectrometry depends on the volatility of the sample to be analysed. Electron ionisation (EI) and chemical ionisation (CI) are the commonly used ionisation techniques for volatile compounds. With EI, ionisation occurs when organic molecules in the gas phase are bombarded by energetic electrons. In CI, the molecules are ionised by ion-molecule reactions with a positively or negatively charged reagent ion that may itself be formed as a result of EI or subsequent ion-molecule reactions.(3)

Techniques with the capability for ionisation of thermally labile involatile compounds have been under development for nearly thirty years. Fast-atom bombardment (FAB), plasma desorption (PD), matrix-assisted laser desorption/ionisation (MALDI), field desorption (FD), electrospray ionisation (ESI), thermospray (TS) and aerospray (AS) are techniques that have been developed during this period for the ionisation of involatile compounds. All of these techniques are commonly referred to as 'soft' ionisation techniques. This means that they are capable of producing at least some intact ions of the involatile parent species after ionisation.(4)

Early techniques for the ionisation of involatile compounds were not suitable for the analysis of long sequences of oligonucleotides. FD was introduced in 1969 but never became widely used because it was technically very difficult to implement. In the mid 1970's PD was a favoured technique but it was mainly used for proteins and generally had poor resolution. In 1981, the introduction of FAB initiated the widespread use of mass spectrometry for biomolecules but it was still not very successful for the analysis of DNA, as only strands up to 13 bases could be analysed. The introduction of MALDI and ESI in the mid to late 1980's allowed the analysis of oligonucleotides and intact DNA to be performed routinely for the first time.

1.1.1.a. Matrix-Assisted Laser Desorption / Ionisation (MALDI)

The MALDI technique was developed by Karas and Hillenkamp in 1988, (5)(6) when they reported the use of MALDI with a UV laser for molecular weight determination of proteins up to 67 kDa. MALDI was based on the LD (laser desorption) technique(7), with the introduction of a matrix to reduce the radiation damage observed in LD. Because of this damage, LD was limited to molecules of up to approximately 1000 Da. MALDI involves isolating the analyte molecules in an appropriate UV absorbing matrix, and irradiating the sample with a high intensity pulsed laser beam tuned to an absorption band of the matrix. Mass analysis of the resulting ions is most often carried out by time-of-flight mass spectrometry.(8)(9)

MALDI has been used in the analysis of a wide range of biomolecules where it has the advantage of high sensitivity (pmol) and rapid routine analysis.

1.1.1.b. Electrospray Ionisation (ESI)

Electrospray was developed in 1968 by Dole and coworkers who published their report on "Molecular Beams of Macroions"(10) in which they concluded that the electrospray technique could be used to produce distinct macroions of defined mass-to-charge ratio (m/z) in the gas phase. These macroions were detected in a Faraday cage, after concentration in a supersonic jet (with energy analysis for mass determination). Despite some errors in the assumptions made by Dole, several groups including that of Fenn, used the basic principles of the study as a starting point for the continued study and development of the electrospray technique.(4) Fenn conducted the majority of early electrospray studies(4)(11-14) and obtained results similar to Dole, but could not solve two major experimental problems, namely:

i. the retarding potential method used for estimating m/z was unsatisfactory for determining the mass of very large ions, and no alternatives were readily available;

ii. the gains that electron multipliers normally provide were denied because large macroions did not produce secondary electrons when they struck the first dynode of the multiplier.

Due to these difficulties, Fenn abandoned work on macroion beams for a number of years. His interest was later rekindled as a potential means of introducing complex, relatively involatile molecules into the gas-phase for infrared spectroscopic studies without decomposition.(11) These new ESI studies analysed low molecular weight solute species so a quadrupole mass filter could be employed for mass analysis. Arginine and polyethylene glycol (PEG), with an average molecular weight in the range 200-17500 Da were two of the first involatile solutes that Fenn examined by ESI. (13)(14)

There are several different electrospray sources in common use. Figure 1.2 shows a schematic diagram of the electrospray source on one of the instruments used in this study.

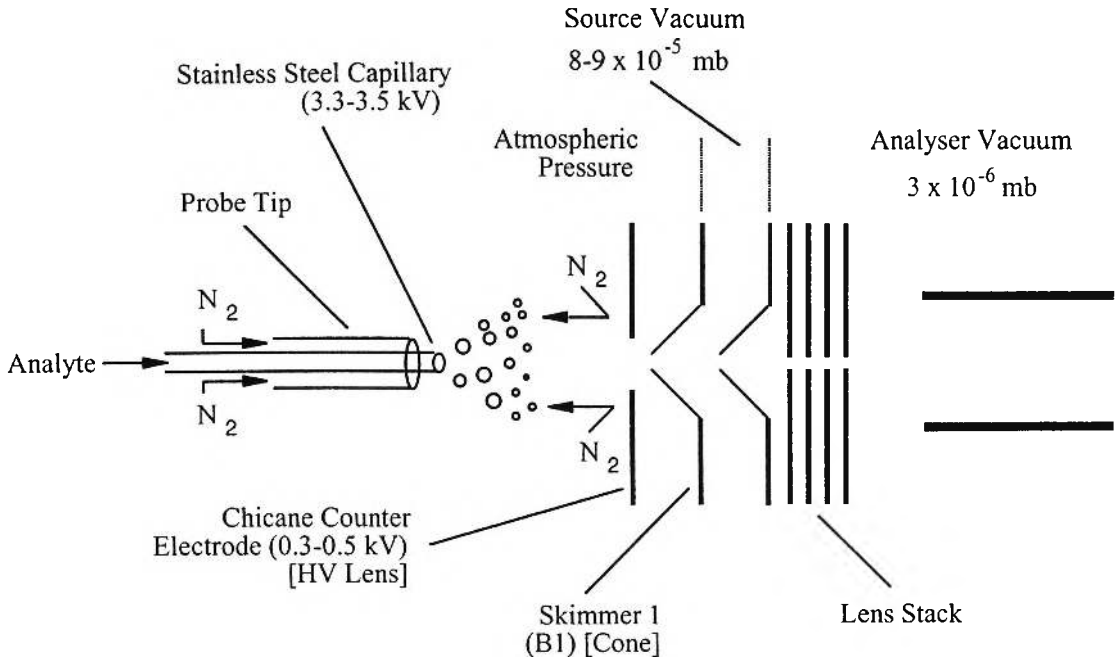


Figure 1.2: Electrospray Ionisation Source

Each different source invokes different ion optics and / or desolvation methods, however the mechanism for ion formation is fundamentally the same as that used by Fenn and coworkers. Fenn used the model displayed in Figure 1.3a to describe the mechanism of ESI, which was based on the mechanism proposed originally by Iribarne and Thompson(15). The solution is passed through a capillary at a potential of several kilovolts relative to the surrounding chamber walls, thus depositing charge onto the surface of the emerging liquid. The consequent coloumbic forces are sufficient to overcome the surface tension so that the liquid is dispersed in a fine spray of charged droplets. These droplets rapidly evaporate in the bath gas. As the droplets decrease in size, the increase in surface charge density and the decrease in radius of curvature result in electric fields sufficiently strong to desorb solute ions. Part of the resulting dispersion of ions in the bath gas passes through a small orifice into an evacuated region, through the skimmer orifice, and then finally on to the mass analyser.(11)(12)(14)

There are two advantages of spraying into a bath gas instead of a vacuum. The energy of the ions entering the mass analyser can be more readily controlled using the bath gas, as it prevents the acceleration of ions to corresponding high energies. It also stops the fouling of the high-vacuum portion of the apparatus with nonvolatile solvent material.

An alternative ESI mechanism was proposed by Rollgen and coworkers(14), who favoured a variation to the charged-residue model originally proposed by Dole *et al.*(10) displayed in Figure 1.3b. They found that Fenns' desorption model did not produce ions with sufficient velocity to account for the observed ion currents. Their mechanism proposed that ESI dispersion of a sufficiently dilute solution would produce charged droplets containing a single solute molecule. This molecule would retain some or all of the droplet charge as the last of the solvent evaporated. Fenn had disagreed with this mechanism as it did not seem able to account for the marked effect of cosolutes on the population of cluster ions in the gas-phase, the studies of which had supported the ion desorption mechanism(14).

Since 1988, there have been many reports (13)(16-26) discussing the two mechanisms, however, none have been conclusive and the mechanism of ESI is still the subject of debate.

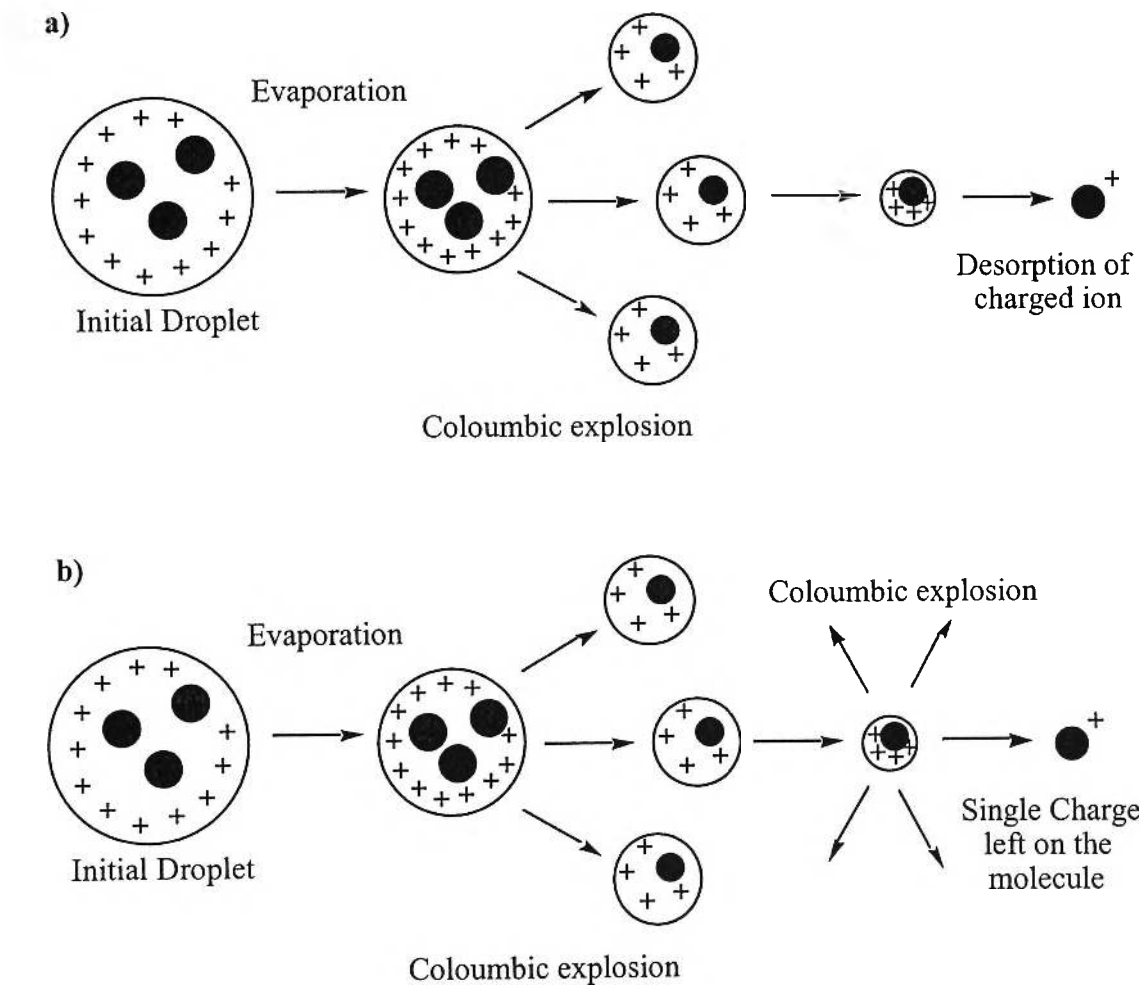


Figure 1.3: a) the ESI ion desorption mechanism **b)** the ESI charge residue mechanism.

A mechanism for the production of multiply charged molecular ion species has yet to be proposed. ESI-MS generally imparts multiple charges to larger analyte molecules. The extent of this charging increases in near proportion with the molecular weight of the molecule.(25) Multiple charging generates multiple experimental data points which improve the mass accuracy and precision of molecular weight measurement.(24) If the charge states are not known and adjacent peaks differ by a charge of one, the molecular weight can be determined from the m/z values(23) using the averaging algorithm described by Equation 1.1 and 1.2.(27)

Equation 1.1: $$Ki = \frac{(M+ima)}{i} = \frac{M}{i} + ma$$

where:

$M = M_r$ = molecular mass of the molecule

i = number of charges

ma = mass of the adduct, if the ions are protonated ma=1

Ki = apparent value of m/z for peak position Ki' = Ki-ma

therefore, using charge states i and i+j (j>0)

Equation 1.2: $$i = \frac{j\,(Ki+j')}{Ki'-Ki+j'}$$

where Ki and Ki+j are determined directly from the ESI mass spectrum

By rearranging the above equations, M, ma, Ki or Ki+j can also be determined. When this algorithm is used to determine M a number of values are obtained and then averaged to give the best estimate of the molecular mass. Most commercial ESI mass spectrometers incorporate computer packages that enable these calculations to be readily performed.

Cation adduction and resolved isotopic peaks can also provide mass and charge assignment for electrosprayed molecules.(28)

1. 1. 1. b. a. Electrospray of Peptides and Proteins

The majority of early ESI-MS studies concerned peptides and proteins. ESI-MS was used mainly for sequence confirmation(26) and to detect changes to proteins such as

post-translational modifications. Conformational changes in proteins have also been probed by ESI(29)(30). Structural information was gained through ESI-MS/MS studies(26)(31)(32) allowing sequence databases to be established(33), and a common nomenclature for fragmentation to be developed.(34) It was also shown that when disulphide bonds were reduced, increased multiple charging was observed, and that a correlation existed between the maximum positive charge observed in the spectrum and the number of basic residues in the sequence when no disulphide bonds were present.(35)

1. 1. 1. b. b. Recent Developments in Electrospray

The most recent ESI-MS development has been that of nano- or micro-electrospray. A number of different systems have been developed, producing similar results. These techniques increase the sensitivity of the conventional electrospray source, mainly through the use of lower solvent flow rates in the nL / min range. The reduced flow results in smaller droplets being formed, which allows a larger proportion of analyte molecules to be available for desorption, longer measurement times, and rapid evaporation of solvent. This allows the tip of the capillary to be positioned closer to the orifice of the vacuum system, allowing a larger fraction of the ESI generated analyte ions to be drawn into the analyser with less solvent than in a conventional ESI source.(36)(37)

Solutions containing up to 0.1 M salt content have been shown to produce spectra successfully using nano/micro-ESI/MS. This increase in salt tolerance indicates that spectra can be obtained from near physiological concentrations of buffer.(37)(38)

Nano/micro-ESI/MS has been applied to a range of biological systems. These studies include:

i. resolution of individual glycoforms of a glycoprotein with the resolution only limited by the mass analyser(37)
ii. enzymatic C-terminal amino acid sequencing of a 39 kDa protein(37); and
iii. MS/MS of methionine enkephalin from a 500 amol injection.(36)

Nano/micro-ESI/MS has also recently been demonstrated for oligonucleotides and their

noncovalent complexes. The ratio of duplex : single stranded DNA was 100 fold greater for nano/micro-ESI compared to a conventional ESI source. This may be due to low-flow rate sources generating less collision-induced dissociation in the transport region of the source.(38)

Reduced detection limits for proteins, in the attomole range, have also been obtained using capillary electrophoresis (CE) coupled to ESI/MS.(39) The sensitivity of this technique is greatly improved with the use of smaller internal diameter (i.d.) capillaries. The efficiency of the process and the solute sensitivity are also increased with decreasing capillary i.d. The mass spectra obtained for a 600 amol injection of a protein were of sufficient quality to allow determination of their individual molecular weights to approximately 0.03%.(39)

1.1.2. Mass Analysers

Electrospray ionisation can be used with quadrupole, time-of-flight, ion trap, magnetic sector, and Fourier transform mass analysers, all of which separate ions according to their mass-to-charge (m/z) ratio. Of these, only the quadrupole and magnetic sector analysers were used in this study and are described here.

1.1.2.a. Quadrupoles

Quadrupole mass filters are one of the most commonly used mass analysers due to their relative low cost and ease of use. A typical quadrupole consists of four conductive parallel rods, symmetrical to the horizontal axis, with the opposite rods being electrically connected. These rods shape an electrodynamic field which is a combination of radio frequency (RF) and direct current (DC). The ion trajectories in this field are determined by the amplitude of the electric field. With the correct amount of RF and DC voltage applied to the quadrupole, an ion of a particular selected m/z emerges from the opposite end of the analyser. Ions with other m/z ratios will oscillate further and further from the axial path until they strike or pass between the rods. The higher the RF and DC amplitude the higher the m/z selected, and vice versa. By scanning RF and DC voltages from zero to their maximum value, ions will be transmitted sequentially in order of their m/z with a constant m/z window.(7)(40)

1.1.2.b. Magnetic Sector

Magnetic sector mass analysers are more expensive than quadrupoles, but have high mass resolution (when combined with electric sectors) and a generally higher mass range. Magnetic sector analysers use a magnetic field to separate ions of different m/z ratios. The ions are accelerated from the source (with energy V_{acc}) and through the magnetic field (with field strength B). This combination of magnetic field and the forward velocity of the ion cause it to follow a curved path. The radius (r) of the curve is dependent on the m/z ratio of the ion, magnetic field strength and the charge and initial energy of the ion. For a given field strength, only a single m/z value will curve at the radius needed to reach the detector. The m/z determination is given in equation 1.3.

Equation 1.3: $m/z = B^2r^2 / 2V_{acc}$

To overcome the spread in ion energies, double focussing magnetic sector instruments are used, in which an electrostatic sector is combined with the magnetic sector to give energy focussing and thus enable higher mass resolution.(7)(40)(41)(42)

1.1.2.c. Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS), has been used for over a decade to gain structural information on a variety of compounds. As the name suggests there are two stages of mass analysis. The first is the mass selection of the precursor ion. Nonselected m/z values are in effect filtered out. The precursor ion then undergoes the reaction:

$$Mp^+ \rightarrow Md^+ + Mn$$

where,

Mp^+ is the precursor ion mass

Md^+ is the product ion mass

Mn is the neutral fragment mass

Before this reaction can occur the activation barrier must be overcome. The energy required to do so can come from one of two sources:

i. metastable decomposition, in which excess energy is deposited on the precursor ion during the ionisation process. An ion with the appropriate dissociation rate versus internal energy content may survive long enough to be extracted from the ion source before it fragments, but may then fragment before detection;

ii. collision-induced dissociation (CID), in which the addition of internal energy to the precursor ion occurs in a reaction region or collision cell. A gas is admitted to the reaction region to induce dissociation following activation of the precursor ion by collision with the neutral target gas.

The second mass analysis stage displays the spectrum of product ions resulting from the fragmentation of the precursor ion.(3)

1.2. Mass Spectrometry of DNA

The use of mass spectrometry for the investigation of nucleosides and nucleotides is well established.(43) EI and CI have only been used for very small species that are either volatile or have been derivatised.(44) FD has also been used to provide molecular weight and structural information for nucleosides and nucleotides.(45)(46)

Oligomers have been studied by PD and FAB. Longer strands of DNA could only be analysed after they had been enzymatically digested into smaller fragments. PD has been successfully used to observe protected oligonucleotides up to 12 bases in length(47), whereas FAB has been used to analyse an unprotected oligonucleotide of 13 bases.(48) These were the largest oligonucleotides studied prior to the development and application of the ESI and MALDI techniques.

ESI and MALDI are relatively new techniques that have revolutionised mass spectrometry of DNA constituents by allowing much larger oligonucleotides to be analysed. The only major problem with these techniques is their inability to deal with high concentrations of salt. Oligonucleotides are sensitive to the presence of salt due to their polyanionic phosphodiester backbone.(49) In addition to affecting the quality of the ESI mass spectrum, the presence of salt can also influence ligand-DNA binding.(50) This is because salt dependent electrostatic effects play a central role in the inter-molecular interactions involving nucleic acids. It has been found that univalent ions

substantially destabilise charged ligand-DNA complexes at physiological salt concentrations due to a large unfavourable change in electrostatic ion-molecule interactions.(50)

HPLC and CE have both been successfully coupled with ESI-MS. Bleicher and Bayer were the first to report the analysis of oligonucleotides using HPLC coupled with ESI-MS(51), which has subsequently been used to characterise DNA adducts.(52) CE-ESI-MS preceded by enzymatic digestion of DNA has been used to analyse large DNA strands and their adducts.(53)

1.2.1. Matrix-Assisted Laser Desorption / Ionisation of Oligonucleotides

As with ESI-MS, early studies using MALDI concentrated mainly on peptides and proteins.(9)(54-57) MALDI has now been used to successfully characterise and sequence both natural and modified oligonucleotides.

Initially, most MALDI analyses of oligonucleotides used matrices that had been found to be suitable for the analysis of proteins. While using these matrices, only very small oligonucleotides were detected successfully. Since then a range of MALDI matrices specifically for oligonucleotides have been examined.(58)(59) 3-Hydroxypicolinic acid was proven to produce the best mass spectra for poly A, poly G, poly C and mixed base oligonucleotides.(58) The matrix used, as well as the concentration of the analyte in the matrix solution and the substrate, have also been shown to influence the formation of either singly or multiply charged ions.(60)

Addition of salts has also been shown to have an effect on the sensitivity, reproducibility and accessible mass range in the MALDI analysis of oligonucleotides. Of the salts tested, only ammonium acetate has been shown to improve MALDI spectra.(61) Other studies have determined the tolerance levels for some of the more common impurities such as sodium chloride.(62)

Molecular weight information for a variety of both modified and unmodified oligonucleotides has been obtained by MALDI with an accuracy of up to ±0.06%. Chemically-modified oligonucleotides have been shown to behave similarly to their

unmodified counterparts. The mass of the modification moiety can be deduced through comparison of the molecular weight of the oligonucleotide before and after modification.(63-65)

A major problem with the MALDI analysis of oligonucleotides is the base loss induced by the relatively high energy imparted during ionisation. Loss of bases occurs through protonation of A, G and C bases, which then destabilises the N-glycosidic bond during the desorption process.(66) This base loss appears to be the major cause of the decrease in mass spectrometer response for mixed base oligonucleotides containing >20-30 nucleotides.(67) Modification of the bases, such as conversion of guanine to the 7-deaza form, offers higher stability and increased sensitivity during MALDI analysis.(68)(69)

DNA segments as large as 500 nucleotides have been successfully detected by MALDI, however, the resolution is still too low for sequencing strategies to be employed for oligonucleotides of this length.(70)(71) Double stranded DNA has also been observed using MALDI-TOF.(72)(73)

Some studies of oligonucleotide adducts have also been performed by MALDI. These include protein-nucleic acid complexes(74), adducts of polycyclic aromatic hydrocarbons (PAH's)(75) and of the antitumour agent cisplatin.(76)

Studies of the fragmentation of oligonucleotides(77)(78) observed in MALDI spectra have shown that the major fragmentation pathway is similar to that observed in ESI-MS. This fragmentation is the loss of a base followed by the backbone cleavage of the 3′ C-O bond of the corresponding deoxyribose. Sequencing strategies using MALDI have been demonstrated with both modified and unmodified oligonucleotides as models for DNA.(79-84)

Initially, the mass resolution of MALDI was insufficient to enable the sequencing of very large oligonucleotides. Recent studies by MALDI have concentrated on attempting to increase its resolution.(85-87) The biggest advance to date has been the development of delayed extraction (DE) MALDI TOF-MS which provided greater sensitivity and resolution. This has allowed oligonucleotides up to 50 bases to be routinely sequenced.(88-90)

1. 2. 2. Electrospray Ionisation Mass Spectrometry of DNA Constituents

ESI-MS of an oligonucleotide was first reported by Covey and coworkers(91) in 1988 for a 14 mer with a molecular weight of 4262.8 Da. In this study, in the negative ion mode, charge states ranging from the $[M-6H]^{6-}$ to $[M-11H]^{11-}$ were observed.

Oligonucleotides up to 80 bases can now be routinely studied by ESI-MS,(92) although considerably higher mass species (up to a 133 mer) have been analysed with careful attention to sample preparation. ESI-MS has been used to confirm the sequences of synthetic oligonucleotides(93), detect modifications(94)(95), and to identify products of unstable oligomer degradation in the analysis of oligonucleotides and antisense oligonucleotides(96). Duplex DNA, DNA complexes (covalent and non covalent) and megadalton DNA have also been studied by ESI-MS.

ESI-MS is used to verify synthetic oligonucleotide sequences because it is a rapid and accurate technique requiring only very small (10-50 pmol) amounts of sample for analysis.(93) Potier *et al.*(92) reported the accuracy of molecular weight determination of oligonucleotides by ESI-MS to be 0.01% for oligonucleotides up to 80 mers, and that a 132 mer could also be measured successfully, albeit with lower accuracy. Little and coworkers (97) also used ESI-MS in conjunction with FT-MS for sequence verification by molecular weight for oligonucleotides up to a 100 mer with an accuracy of 0.003%.

ESI-MS has also been used for the analysis of modified oligonucleotides from simple monomers to oligomers containing up to 50 bases.(93) Modifications such as the incorporation of stable isotopes(94), modified bases(98), and phosphorothioate linkages(94) have all been successfully verified by ESI-MS.

Antisense oligonucleotides have also been studied by ESI-MS.(96)(99) Interest has increased recently in this area because they are being used as potential therapeutic agents as antiviral drugs (in particular against HIV.) They are complementary to specific RNA sequences and bind to messenger RNA (m-RNA), thereby inhibiting the synthesis of specific proteins or the expression of the targeted gene.(96)(99) Deroussent and coworkers used ESI-MS to determine the masses of antisense oligonucleotides and to characterise the purity of the oligonucleotide being tested for pharmacological

studies.(96) The method was optimised with phosphodiester oligonucleotides and then applied to antisense oligonucleotides.

1.2.2.a. ESI-MS of Megadalton DNA

Megadalton DNA has been successfully ionised and the molecular weight determined by ESI-MS.(100)(101) Early studies(102) were unable to confirm the actual molecular weight because the individual charge states were unresolved, so it was assumed that the m/z value of the band maximum corresponded to the most probable charge state of the most abundant oligomer.

Fuerstenau and Benner have also used TOF-MS to detect the electrosprayed ions from large DNA.(100) The charge of the ion was determined in a Faraday cage charge detector. The entrance and exit signals from the passage of each ion provide the ion velocity which allowed for the calculation of the m/z for each ion and the mass could then be determined.

Cheng and coworkers reported on FT trapping of individual highly charged plasmid DNA(101). Charge state shifting was accomplished by creating multiple peaks from the same ion, so that molecular weights could be calculated. Before this FT-MS method for measuring megadalton size molecules, this group measured large coliphage T4 DNA ions up to 108 kDa based on the measurement of the induced current and therefore the charge. This approach, however, showed much less precision.(100)(101)(103)

1.2.2.b. Charge-State Reduction

Charge-state reduction is the shift of charge states normally observed by ESI-MS to a higher m/z ratio to simplify analysis of complex mixtures. This is normally achieved through the addition of acids, or the addition of organic bases such as diamine.(104-106)

When $d(pT)_{12}$ was analysed in negative ESI-MS, the charge state distribution showed a maximum at $[M-9H]^{9-}$, but after charge state reduction the $[M-5H]^{5-}$ ion was the maximum charge state observed. This was thought to occur due to the shielding of charge sites on the oligonucleotide by ammonium ions. Addition of increasing amounts

of either acetic or formic acid shifted the charge state distribution progressively lower to the charge states $[M-4H]^{4-}$ and finally $[M-3H]^{3-}$.(105)

Charge state reduction can also be used to more efficiently analyse oligonucleotide mixtures. A mixture containing equal concentrations of $d(pT)_{12}$, $d(pC)_{12}$, and $d(pG)_{10}$ was analysed by ESI-MS. Each component gave several charge states, and many of the charge states overlapped. Following charge state reduction, the spectrum consisted of one charge state for each component, thereby reducing the mass spectral complexity of the mixture. All components were then observed to be well separated and easily identifiable.(105)

1.2.2.c. Oligonucleotide Desalting Techniques for ESI-MS Analysis

The quality of the electrospray spectra is greatly affected by the presence of involatile salts. The spectra may show low signal intensity and clustering with salt ions, making molecular weight determination difficult. In severe cases it can even preclude spectral interpretation. Oligonucleotides are intrinsically more sensitive than proteins to the presence of salt(107) owing to their polyanionic phosphodiester backbone(49). Common salt species observed include Na^+ and K^+.(108)(109)

Common desalting techniques often rely upon centrifugation or off-line micro-dialysis. Centrifugation has been used in conjunction with size exclusion chromatography media using both Bio P-2 gel (1800 Da cutoff) and Sephadex G-10 gel (700 Da cutoff) to desalt carbohydrates for FAB analysis. This technique should also be applicable for ESI-MS of oligonucleotides.(110)(111) The P-2 gel was found to be sufficient to permit FAB mass analysis without any additional treatment of the sample. The G-10 gel displayed a much lower desalting capacity and molecular ion species were only obtained for sequences up to a 5 mer, however when the G-10 gel was used with permethylation the sensitivity and the number of structurally informative peaks increased.(111) Centrifugal size-exclusion chromatography is highly effective, extremely easy and very rapid method for desalting carbohydrate samples. The number of samples that can be desalted simultaneously is limited only by the size of the centrifuge used.(111)

Wu *et al.*(109) developed an online desalting system for ESI-MS. In this approach they infused the solution to be sprayed inside the dialysis tube that was contained inside the

probe tip, which caused the exchange of small molecules or ions (such as Na^+) with more volatile components (such as NH_4^+). The buffer flow rate is important for this technique because if it is too low or too high, incomplete desalting occurs. Results obtained at neutral pH also suggest that native protein conformations and noncovalent complexes should be preserved through this rapid desalting ESI process.(109)

Ammonium precipitation has also been another common desalting method used on oligonucleotides. This method uses precipitation of the oligonucleotide in ammonium acetate to replace Na^+ ions with NH_4^+ ions. In a report by Stults and Marsters this desalting technique was applied to a 30 mer.(112) The resulting spectra displayed much sharper peaks, with the largest peak containing no sodium ions. Some Na^+ adducts were still observed but these had much lower abundance. This technique is not quantitative, however, as the effectiveness is a function of the sequence length, and loss of analyte during precipitation occurs.(49)

Addition of organic solvents has also been used to suppress adduct ion formation and to increase the ESI response for the molecule of interest. Changes in the solvent composition using various percentages of acetonitrile did not substantially alter the ESI mass spectra. Completely aqueous solutions gave similar spectra, although the electrospray was more difficult to stabilise.(112) When strong bases, such as triethylamine (TEA) or piperidine were added, the best cation adduction suppression was obtained. The level of cation adduction could be reduced up to 100-fold. With the co-addition of imidazole, an additional increase in the sensitivity was also observed.(49) Besides the pH and the nature of the solvent, base composition of the oligonucleotide has an impact on the signal intensity. When Bleicher and Bayer studied the sequences $d(A)_{10}$, $d(G)_{10}$, $d(C)_{10}$ and T_{10}, all of the oligomers behaved similarly except T_{10}.(108) This sequence displayed a greater signal intensity than the others, which they suggested was because it is the only monomer that contains an acidic N-H bond.

1. 2. 2. d. Noncovalent Complexes By ESI-MS

ESI-MS is a very gentle ionisation technique and can therefore be used to study the noncovalent interactions that occur in solution, as it allows experiments to be performed under near physiological conditions.(113) ESI-MS has advantages over other techniques

used to study noncovalent interactions, because it is more sensitive and more specific than UV or fluorescence assays.

To date noncovalent biomolecular complexes studied include:
enzyme-substrate, (114)
protein-ligand, (115-122)
multimeric proteins, (123-125)
DNA duplexes,(126-129) and
DNA-ligand complexes (130-136)

There has been much discussion over whether the observation of noncovalent complexes between biomolecules by ESI-MS necessarily reflect specific solution interactions.(137) Evidence both for and against such observations has been published. In studies supporting the existence of noncovalent complexes in ESI-MS, Ganem(120) used MS/MS to prove that the interactions between a receptor (FKBP) and two ligands (rapamycin and FK506) were in fact specific noncovalent interactions. The receptor binds to both ligands, but when the receptor was denatured by lowering the pH all complex ions disappeared from the spectra, indicating that the binding was specific. MS/MS was then used to support the notion that the binding was noncovalent, by demonstrating that the energy required to dissociate the FK506 ligand from the receptor was one tenth of that required to break a covalent amide bond within the receptor.

Ogorzalek-Loo and coworkers(113) showed that when ribonuclease-S is enzymatically cleaved to yield S-peptide and S-protein, there are selected conditions where they can reform ribonuclease-S. No homodimers were observed, only heterodimers, indicating specific binding rather than random aggregation in solution or during the ESI process. When the pH of the solution was lowered, no complex peaks were observed, as was expected if the binding were specific.

Suizdak and coworkers(138) have demonstrated, for the first time, that very large noncovalent complexes can survive the ESI-MS process in their native conformation. They passed a live virus, tobacco mosaic virus, through a mass spectrometer and collected the electrosprayed virus on a plate before the detector. Transmission electron microscopy revealed that the tobacco mosaic virus retained its rod-like structure. Confirmation that the virus was still intact was provided when the virus was observed to

be still capable of infecting the tobacco plant, which shows that the noncovalent biomolecular complex was unaltered through the ESI-MS process.

Cunniff and Vouros(139) discovered in studies using cyclodextrin, that not only do aromatic amino acids and peptides containing aromatic moieties form complexes as expected, but that any amino acid or oligopeptide will produce peaks in ESI mass spectra, which correspond to a complex with cyclodextrin. The results from these studies also suggested that cyclodextrin-amino acid complexes detected by ESI-MS may not be inclusion complexes but rather electrostatic adducts.

In studies by Ding and Anderegg(140) specific and nonspecific noncovalent dimer ions of oligonucleotides were observed. The appearance of these nonspecific dimers appeared to be concentration dependent. When the concentration of the two strands was reduced by a factor of ten, ions from all the dimers disappeared.

In recent studies by Chillier and coworkers(141) it has been shown that under certain conditions ESI-MS does not reflect equilibrium ion abundances in the electrosprayed solution. One such case is that of the free-base octaethylporphyrin, which in solution is predominantly the doubly-charged species, yet when it is electrosprayed only the singly charged species is observed. The conversion from dication to monocation appears to occur fairly late in the ESI process, after the dications are sampled into the atmosphere / vacuum interface and before mass analysis.

Results presented by Aplin *et al.*(137) indicated that complexes observed between peptides and porcine pancreatic elastase (PPE) do not always fully reflect structurally specific interactions. They concluded that at this point in their research it was impossible to give a general answer to whether ions observed in the gas phase are indicative of what is occurring in solution.

Smith and Light-Wahl conducted studies(142) to determine if it was possible for ion-neutral or ion-ion processes to produce gas-phase concentrations of noncovalent complexes. Models for the ESI-MS process imply that after the production of individual molecular species, the concentration of neutral molecules should be zero or very small in the gas-phase. Ion-molecule reactions are thought to have no effect since they only become important at gas-phase neutral concentrations far greater than can be produced

for solutes from electrosprayed solutions. Ion-ion processes are unlikely to result in the formation of the same species, owing to repulsive long range coulombic forces. Hence it was suggested that noncovalent complexes must be maintained or formed prior to completion of the evaporative loss of solvent by the ESI process. For example, with higher order protein structures coulombic considerations dictate that the loss of such structure will almost always be irreversible in the gas-phase.

Smith *et al.*(142) concluded that while nonspecific associations are not likely to form in the gas-phase, each case has to be independently proven. Proof of noncovalent interactions is normally provided by negative data, i.e., the complex disappears on denaturation of the host, replacement of the host, alteration of pH, or on desalting of the sample. Some of these tests support their theory but are inconclusive because the changing of the parameters may also change the conditions required to form the aggregate. A better control would be to substitute the "guest". MS/MS is also an effective method to indicate if the interaction is noncovalent. A good indication of aggregates, especially for DNA duplexes, is if peaks corresponding to the homodimer are observed as well as the heterodimer.

These studies show that noncovalent interactions can be monitored by ESI-MS, but that it is impossible to give a general answer to whether it is indicative of what is happening in solution. Each individual case has to be studied separately and proven accordingly.

1. 2. 2. e. Duplexes

Duplexes are helical double-stranded DNA formed by Watson-Crick base-pairing, i.e., A-T and G-C. The observation of duplex DNA by ESI-MS was first reported in early 1993 by Light-Wahl *et al.*(129) when they acquired an ESI mass spectrum after annealing two complementary 20-mer strands of DNA. This was closely followed in the same year by a report by Ganem *et al.*(128), who studied the duplex formation of self complementary DNA strands as well as a pair of complementary sequences. Doktycz *et al.*(127) showed that duplexes could also be observed when ESI-MS was performed with an ion-trap mass analyser. MS/MS was performed on the 10-mer duplex $[M-5H]^{5-}$ charge state. It was found to dissociate only into each of the individual strands, as was previously demonstrated with a quadrupole mass analyser.(128) Studies by Bayer *et*

al.(126) concentrated on hybridisation of mixed double strands of natural and chemically modified oligonucleotides by ESI-MS. This is thought to be very important because it is this hybridisation from an antisense oligonucleotide which is expected to act in the target cells to prevent translation and inhibit gene expression. The signal intensity of the duplex increased with the length of the complementary strand.

In addition to duplexes, ions corresponding to a noncovalent, four stranded oligonucleotide $d(CGCG4GCG)_4$ have been observed by ESI-MS.(143) These structures were stabilised by the cations and when the solution was desalted no quadruplex was observed by ESI-MS.

1.3. Tandem Mass Spectrometry (MS/MS) of DNA Constituents

1.3.1. Fast-atom Bombardment (FAB) MS/MS

Fast-atom bombardment (FAB) MS/MS has been used extensively to obtain structural information on nucleosides, nucleotides, small oligonucleotides and some of their adducts. Analysis of nucleosides by FAB-MS/MS was first reported in 1983. These molecules were found on fragmentation to form the sugar and BH_2^+ ions and to a lesser extent specific bases and fragmentation across the sugar group.(144-147)

The earliest report on FAB-MS/MS studies of nucleotides was in 1984. Nucleotides fragmented to form mainly BH_2^+ in the positive ion mode and B^-, PO_3^- and $H_2PO_4^-$ in the negative ion mode. Other fragmentation products observed to a lesser extent, in both the positive and negative ion modes, were fragmentation across the sugar group, and the loss of H_2O and CH_2O from the protonated or deprotonated molecular ions.(144-146)

The first study of oligonucleotides by FAB-MS/MS was made in 1983 when the oligomers GGTA and TACC were shown to yield sequence information in the negative ion mode.(144) Phillips and McCloskey(148), and Gross and coworkers(145), utilised FAB-MS/MS for the extensive characterisation of dinucleotides as they are the smallest subunit of nucleic acids that contain sequence information. Ions representing the bases were generally amongst the most abundant fragments in both positive and negative ion

modes. The $[M-H]^-$ ion fragments were observed to produce ions from the 5′ terminus more readily than from the 3′ terminus. Therefore, the abundance of the 5′ base of the dinucleotide is greater than that for the 3′ base. The reverse is observed for fragmentation of $[M+H]^+$ as the 3′ base is of greater abundance. The only exception to this observation is when thymidine (T) is the 3′ base, as no peak for it is observed in the positive ion MS/MS spectrum. In ribodinucleotides the same observation was made for Uracil (U). The absence of protonated T was attributed to the greater stability of the thymidine glycosidic bonds. Isomeric dinucleotides can be distinguished on the basis of this fragmentation. The preferred loss of the 5′ base to the 3′ base in negative ions becomes less significant for higher oligonucleotides so it cannot be used unequivocally for the assignment of the 5′ base.

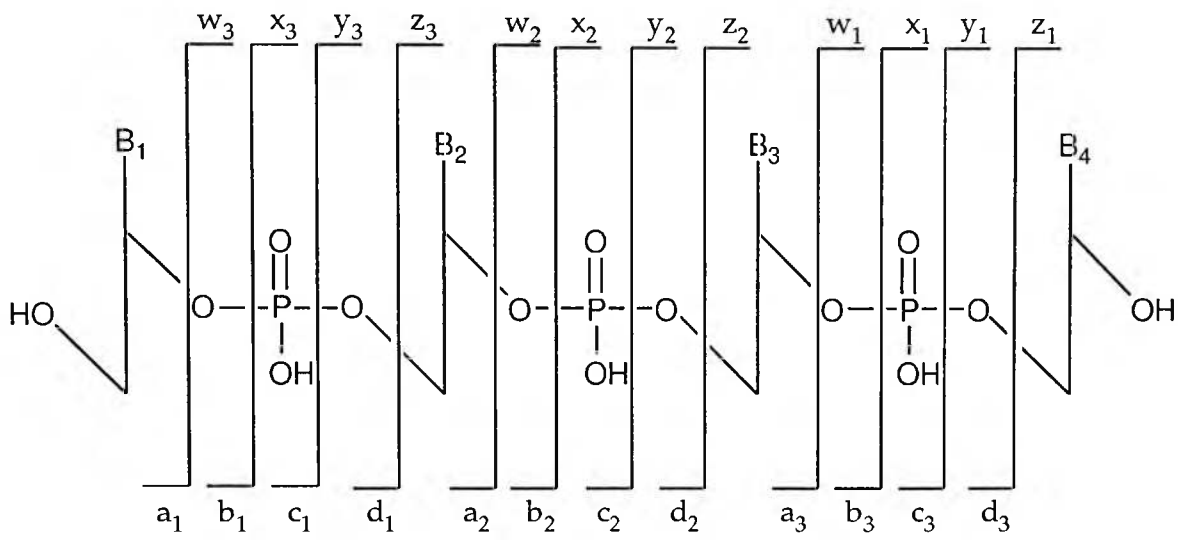
Studies of nucleoside and nucleotide adducts with genotoxic compounds have also been analysed by FAB-MS/MS. Tomer *et al.*(149) studied 12 modified nucleoside and nucleotide adducts bonded to various pyrrolizine alkaloid metabolites by FAB-MS/MS. Sites of base, sugar and alkaloid modification were identified, and in some cases information as to the location of the alkaloid-base bonding was observed. The most informative product ion spectra were those obtained from the MS/MS of the $[M+H]^+$ ion, whereas fragmentation of $[M+K]^+$ and $[M-H]^-$ provided mainly confirmatory information.

1.3.2. Electrospray (ESI) Tandem Mass Spectrometry (MS/MS)

There have been relatively few reports concerning ESI-MS/MS of oligonucleotides, which may in part be because of the difficulty associated with assigning the charge states to the product ions produced from multiply-charged precursor ions.(150)

Scheme 1.1 shows the nomenclature for the fragmentation of oligonucleotides based on the system used for peptides and proteins(34), which was proposed by McLuckey *et al.*(151) and has now been widely adopted. This scheme is illustrated with a triply charged tetra-nucleotide, bases B1→B4. They developed this new nomenclature scheme

because the original scheme proposed by Cerny *et al.*(152) could not fully account for all of the product ions formed in the MS/MS spectra of oligonucleotides.



Scheme 1.1: Nomenclature for ESI-MS/MS fragmentation of oligonucleotides

McLuckey and coworkers(151)(153)(154) studied both modified and unmodified small oligonucleotides (up to 12 mer), by ESI-MS/MS with a quadrupole ion trap. All ions studied showed structurally informative fragmentation. They applied a third mass spectrometry analysis step to provide complete sequence information. Complementary fragment ions were normally observed in these spectra. Complementary ions result from the single cleavage reactions of the precursor ion, with the charge being shared among the two product ions. Both these ions are normally observed in the spectrum. If the parent ion charge state is known, and one of a set of complementary peaks is also known, the pair can be identified in the spectra by charge separation ratios.

McLuckey and coworkers established a number of rules for the MS/MS of multiply charged oligonucleotide anions. They were:

- for highly charged anions, charged bases are lost in preference to neutral bases;
- charged bases are lost in the order of preference $A^- > T^- > G^-, C^-$ independent of chain position (first decomposition step);
- in the absence of a preference for base loss, the 3′ residue is the least likely to be lost;

- the second decomposition step is the cleavage of the 3′ C-O bond of the sugar from which the base is lost;
- loss of the PO_3^- group from phosphorylated ions can compete with base loss;
- complementary ions are normally observed, provided both are stored in the ion trap;
- the maximum charge state for anions is equal to the number of bases - 1 or the number of phosphodiester linkages;
- the lower the total charge state, the lower the number of mass analysis stages that can be performed;
- these rules also apply for modified oligonucleotides.

Gentil and Banoub performed a study of MS/MS of oligonucleotides on a triple quadrupole mass spectrometer, comparing these results with the results obtained by McLuckey.(151)(153)(154) To try and obtain similar conditions to those in the ion trap they changed the collision gas from argon to helium.(150) This appeared to have little impact because the ESI-MS/MS spectra obtained using both collision gases were identical. They were unable to obtain similar results and concluded that the difference in the fundamental behaviour between the two mass analysers could be due to much higher energy being imparted to these oligonucleotide molecules in the triple quadrupole experiments.

ESI-MS/MS has also been used to differentiate between isomeric DNA.(150) Two series were analysed by ESI-MS and displayed an identical series of multiply charged molecular ions. MS/MS studies produced a complete or very near complete set of singly or doubly-charged w series ions. Each hexamer in the first series was unable to be completely sequenced, however those in the second series had their sequences fully confirmed.

MS/MS studies have been performed on antisense oligonucleotides.(99) The exact sequence of these molecules is very important, since altering a sequence by as little as one base is enough to potentially change the action of these therapeutic agents. Baker *et al.*(99) used MS/MS to verify the sequence of some synthetically prepared methylphosphonate oligodeoxyribonucleotides. MS/MS of the doubly and triply charged molecular ions of this sequence provided little structural information as little

fragmentation was evident. Peaks owing to part of the Y series were identified, but most fragmentation peaks were independent of the sequence. The best result was obtained from MS/MS of the $[M+4H]^{4+}$ molecular ion for which considerable information was obtained, confirming the expected sequence.

Sequence information for an 8mer to a 25mer were obtained by Little *et al.* using an ESI-FTMS system.(155) Fragmentation was obtained by MS/MS in the ICR cell and by nozzle skimmer dissociation, with similar fragmentations being obtained in each case. For the sequences 8-14 bases in length, full sequence information was obtained. In most cases the total sequence was determined from the w series alone. The $(a_n\text{-}B_n)^{x-}$ series was also instrumental in the sequence determination. For the larger sequences, 21mer and 25mer, only partial sequence information was obtained.

McLuckey and coworkers(156) have studied the competition between the loss of the nucleobase as a neutral and the loss of the base as an anion. They used a series of oligonucleotides, monomers through to hexamers, that only contained adenine for this study. ESI-MS/MS of the $[M-H]^-$ to $[M-5H]^{5-}$ molecular ions were compared. The likelihood of the loss of a charged versus neutral adenine was found to be strongly dependent on the charge state of the precursor ion, as might be expected. Increasing the charge state was found to favour charged base loss.

1.4. DNA-Ligand Complexes

DNA can act as a receptor for a number of different types of ligands. These include proteins, peptides, metals, metal complexes, and alkylating agents. Ligands can bind to either the major or minor groove of DNA by intercalation, groove binding, alkylation, crosslinking, electrostatic forces, or some combination of these. The binding can be either reversible (noncovalent) or irreversible (covalent). Figure 1.4 displays some of the more common forms of DNA-ligand binding.

Intercalation occurs when a planar or approximately planar aromatic ring system is inserted between DNA base-pairs. Groove binding is when the ligand twists so it can lie within either the major or minor groove. External electrostatic interactions are when the ligand binds along the exterior of the helix through interactions which are generally non-

specific and primarily electrostatic in origin. Crosslinking occurs when the ligand binds in at least two places, normally on two separate DNA strands, linking them together.(1)

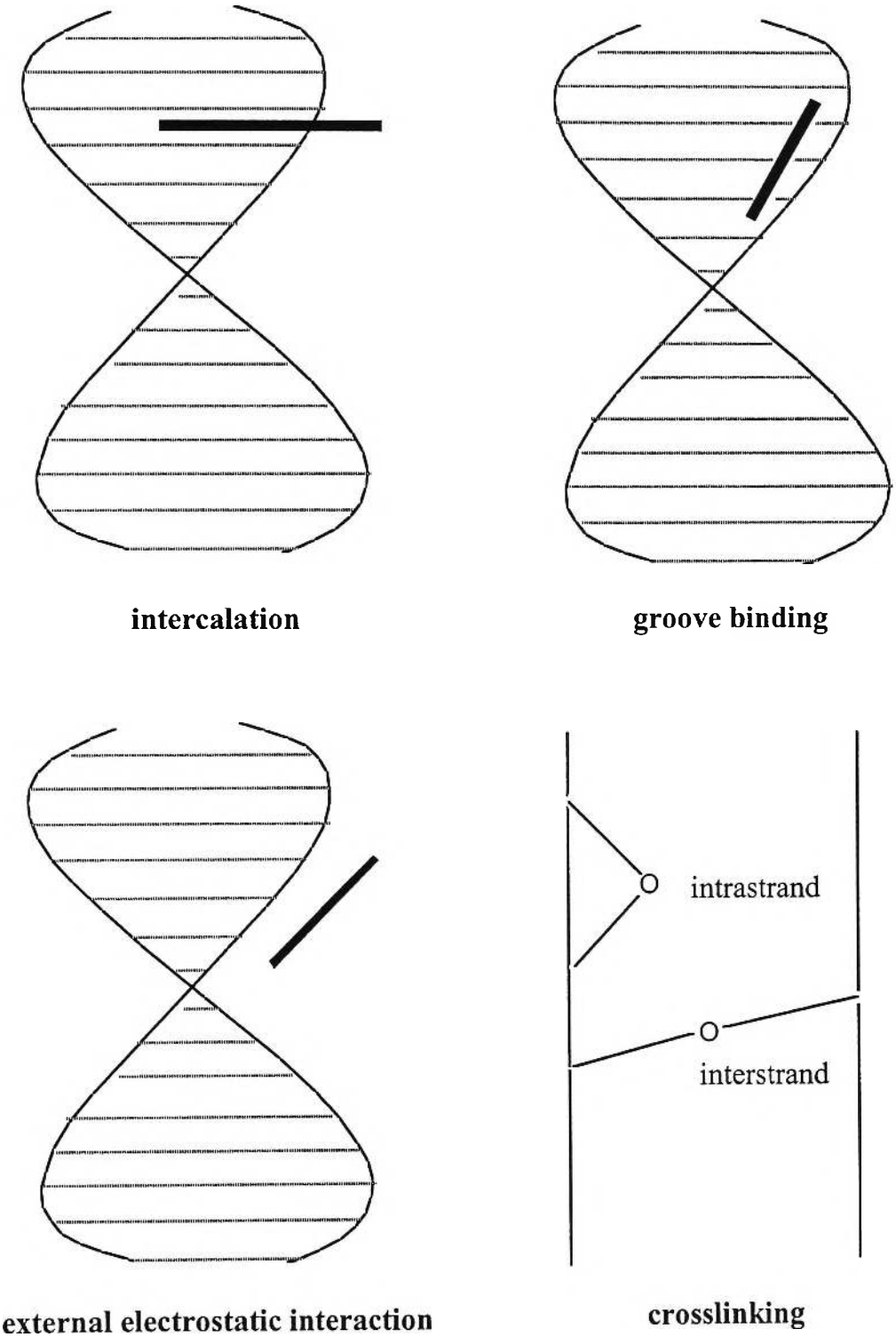


Figure 1.4: Some of the types of DNA-ligand binding.(1)

DNA-ligand interactions have been studied extensively to aid in the design of more effective antitumour agents.(157) Structural studies of DNA-ligand interactions have

been performed mainly by NMR spectroscopy(158) and x-ray crystallography(159), and to a lesser extent by circular dichroism (CD)(160) and fluorescence spectroscopy.(161) The sequence selectivity of antitumour agents is normally determined by methods based on molecular biology.(162) Mass spectrometry is increasingly being used for the characterisation and identification of DNA-ligand interactions since it offers advantages in terms of speed and sensitivity of analyses compared to other structural techniques.

One of the most extensively studied antitumour agents is cisplatin. It is primarily used to treat testicular cancer, and to a lesser extent other cancers such as ovarian cancer. Cisplatin is a bidentate ligand and as such can bind to two adjacent guanines on the same DNA strand (intrastrand binding), two guanines separated by another nucleotide on the same chain, or two guanines on different DNA strands (interstrand binding).(163)

Early studies of cisplatin complexes with nucleoside analogs were observed using EI-MS.(164)(165) The limitations of this technique (volatility and mass range) resulted in only neutral complexes with single bases being analysed. Pyrolysis mass spectrometry with EI was later used(166) to analyse a series of cisplatin-DNA complexes in the 100 to 300 Da range from the fragmentation of the cisplatin-salmon sperm DNA complex. FAB-MS, and more recently MALDI and ESI-MS, have been used for the study of DNA-drug binding. Martin *et al.* (163) published the first FAB mass spectrum of a platinated oligonucleotide which showed cisplatin bound to a tetramer. This study showed that the platinum (Pt) was bound to bases in adjacent nucleotides, with one base usually being guanine. FAB-MS/MS, in the positive ion mode, produced fragment ions consisting of Pt-base, Pt-nucleoside, or Pt-nucleotide complexes, whereas the fragments produced in the negative ion mode were mainly nonplatinated sequence ions. Further studies by Costello *et al.*(164) confirmed this.

Prior to the development of ESI-MS and MALDI, studies involving larger oligonucleotides were limited as FAB was only capable of measuring small (up to 13 mer) oligonucleotides. ESI-MS and MALDI made it possible to obtain spectra of higher molecular weight DNA-ligand complexes. In 1991 Poon *et al.*(167) published the first report on the application of ESI-MS to cisplatin. They gained structural information from the ESI-MS/MS of cisplatin, but never applied it to cisplatin-DNA

complexes. The first report of the cisplatin-DNA complex detected by ESI-MS was that by Wickham *et al.*(107) in 1995.

The use of MALDI to study cisplatin was reported by Costello *et al.*(76) It was concluded that MALDI provided fairly accurate molecular weight information for cisplatin adducts of oligonucleotides as well as their unplatinated analogs. The best sensitivity was obtained in the negative ion mode, using IR irradiation and sinapinic acid.

Gale *et al.*(131) were the first to report the use of ESI-MS for detection of a noncovalent duplex DNA-minor groove binder complex. They studied the interaction between the self complementary DNA strand 5′-d(CGCAAATTTGCG)-3′ and the antibiotic distamycin A. Distamycin binds noncovalently in the minor groove of the AATT or ATTT duplex region. Hydrogen bonding, van der Waals forces, and electrostatic interactions account for the sequence selectivity of this molecule. They later expanded this study to include the minor groove binding molecules pentamidine and Hoechst 33258.(132)(133) Product ion spectra of the duplex-ligand noncovalent complexes were obtained. The dissociation behaviour of all three complexes was similar. This may have been due to the absence of significant binding differences between the three drugs. It may also have been caused by the dissociation mainly being influenced by the duplex, which was constant for these experiments.

Other ESI-MS investigations of noncovalent complexes include those performed by Gao *et al.*(136) and Hsieh *et al.*(130) Gao and coworkers(136) studied the neocarzinostatin drug-bulged DNA complex. Hsieh and coworkers(130) studied actinomycin D and single stranded DNA complexes. Both groups utilised ESI-MS and ESI-MS/MS to observe the complex and prove the specificity of the interaction.

Wickham *et al.*(168) published the first report of a covalent ligand-duplex DNA adduct by ESI-MS. They studied the antitumour antibiotic hedamycin and its binding to the self complementary oligonucleotide 5′-d(CACGTG)-3′. The specificity of the base-paired duplex was proven by a titration experiment involving alkylated and nonalkylated complementary strands. No peaks due to duplex formation for the sterically hindered alkylated DNA were observed, as was expected. On further addition of free DNA, a

duplex was formed. Further studies by this group(107) also examined the covalent adduct formed between cisplatin and DNA.

Other covalent complexes studied by ESI-MS include work by Sigurdsson *et al.*(169) and Chaudhary *et al.*(52) Sigurdsson *et al.*(169) used ESI-MS to determine the mass of the complex formed by an agent, 2,3-bis-(hydroxymethyl)-pyrrole linked to distamycin and crosslinked to DNA. This agent contains both covalent and noncovalent binding regions that contribute to the specificity of the interaction. MALDI has also been used to study covalently crosslinked agent-DNA complexes.(170) ESI-MS has also been linked with liquid chromatography (LC) to characterise nucleoside adducts. Chaudhary *et al.*(52) used LC-ESI-MS and LC-ESI-MS/MS to study the endogenous adduct of malondialdehyde with 2′-deoxyguanosine.

For antitumour drug design it is important for the exact nature of the interaction between the ligand (drug) and DNA to be determined. This requires the identification of DNA binding characteristics, which leads to the design of effective analogues. ESI-MS and ESI-MS/MS have, and will continue, to expand studies in this area.

1. 5. Pyrindamycins

Pyrindamycins A and B are two potent antitumour antibiotics that derive their biological properties through sequence selective DNA minor groove alkylation. The pyrindamycins are known also as duocarmycin C2 and C1 respectively*. The different names arose because two different Japanese research teams discovered these compounds simultaneously. Ichimura *et al.* published their findings on duocarmycin C1(171) and duocarmycin C2(172) in September 1988. This was closely followed in a report by Ohba *et al.*(173) of the structure of both pyrindamycins in October 1988. Both groups isolated the antibiotics from the culture broth of a streptomycete which had been isolated from soil samples.

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* NOTE: this group, known as the duocarmycins, also contains A, SA, B1 and B2.

The structures of pyrindamycin A and B, displayed in Figure 1.5, were verified by a combination of mass spectrometry (EI-MS), high resolution EI-MS, NMR, UV/VIS, IR and X-ray crystallographic analyses.(171)(173)(174)

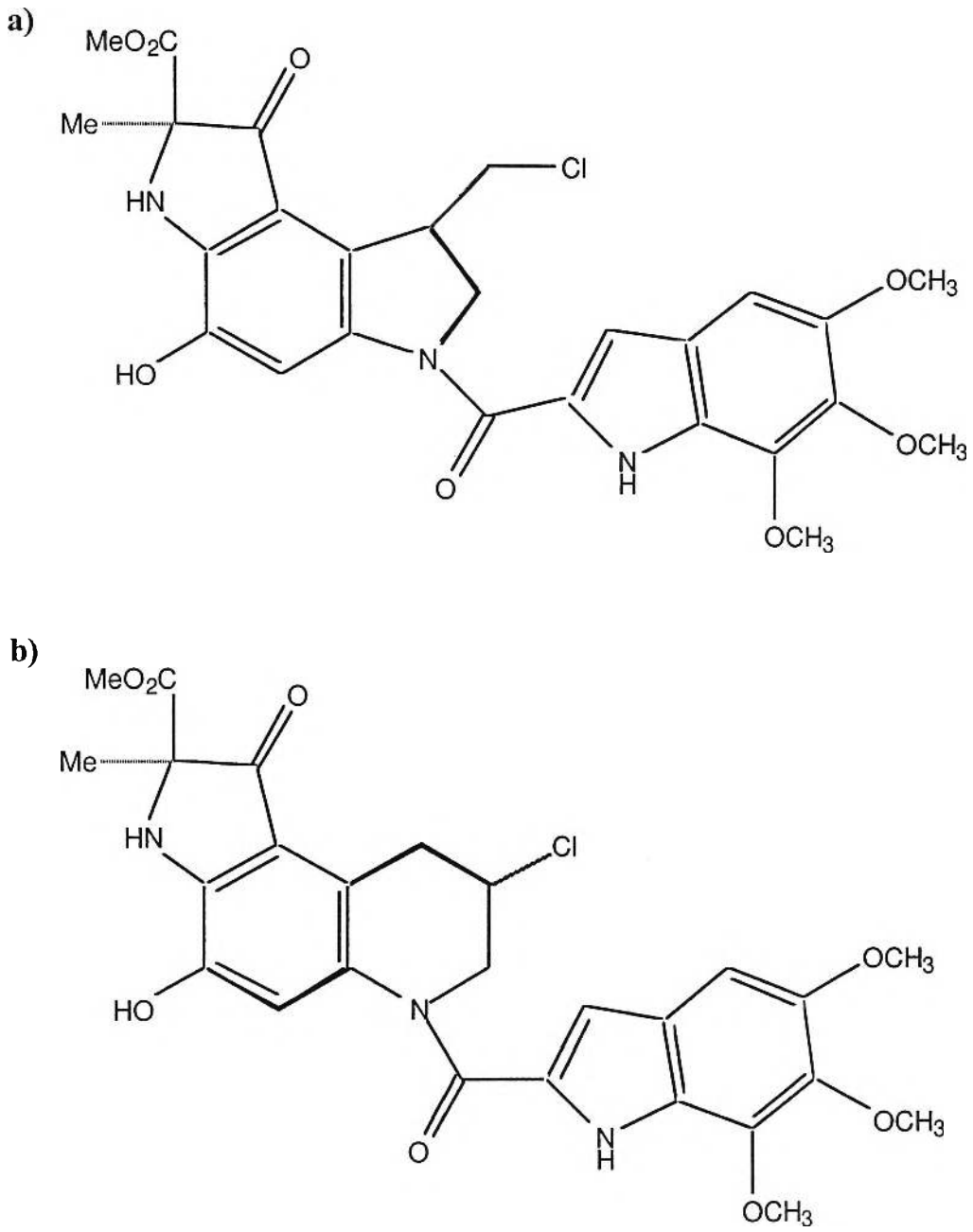


Figure 1.5: The structures of **a)** Pyrindamycin A, and **b)** Pyrindamycin B

Pyrindamycin A and B were found to be active against Gram-positive and Gram-negative bacteria, and exhibit strong therapeutic effects against both drug-sensitive and drug-resistant P388 leukemia cells in mice. They were not, however, effective towards solid tumours.(171)(173-175) These antibiotics also strongly inhibit

DNA synthesis, but have little effect on RNA or protein synthesis.(175) Pyrindamycin A and B possess a pharmacophore similar to that of CC-1065 shown in Figure 1.6.(176)(177)

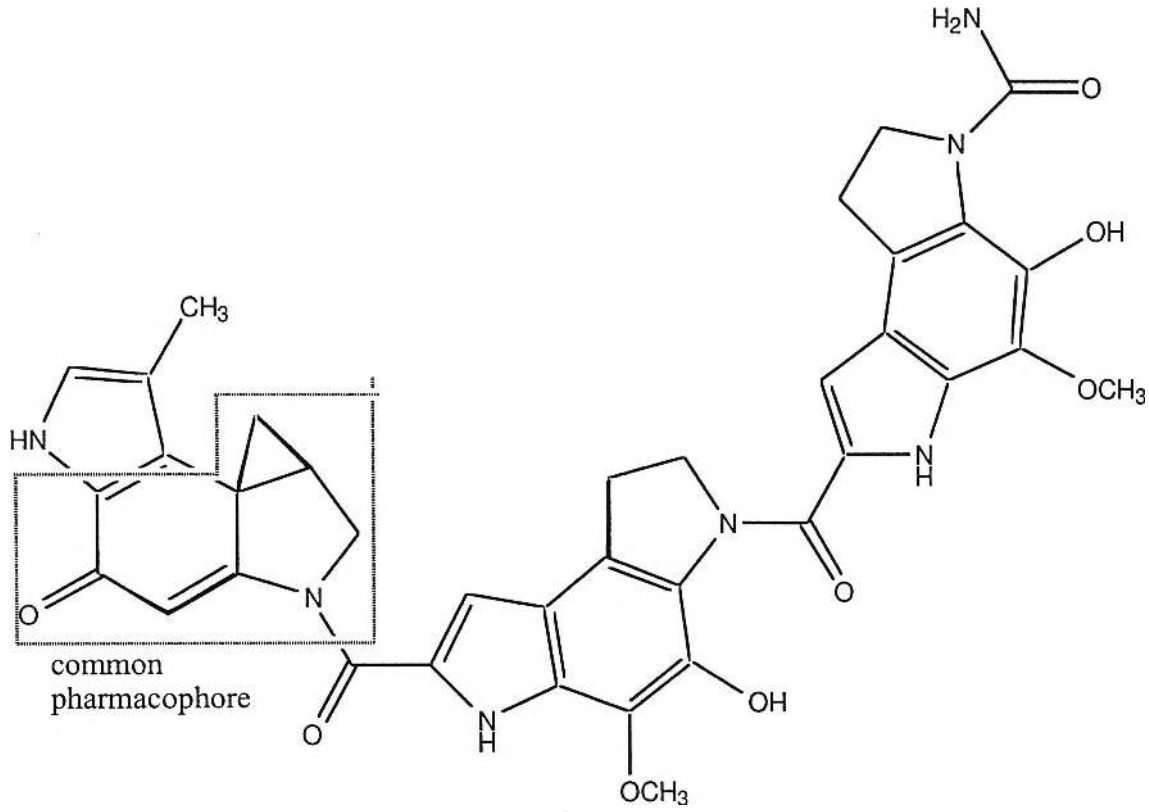


Figure 1.6: Structure of CC-1065 and the Common Pharmacophore

CC-1065 is an extensively characterised antitumour antibiotic and is one of the most cytotoxic antitumour agents known.(176-178) The development of CC-1065 as an antitumour agent was stopped, however, because of the delayed death of mice at subtherapeutic doses. The delayed toxicity of CC-1065 is thought to be due to the extent of noncovalent binding stabilisation which renders the DNA alkylation irreversible. In contrast, the alkylation by the pyrindamycins is reversible and the pyrindamycins do not exhibit the same highly toxic effects.(178-181)

The pyrindamycins act at the cellular DNA level.(178) When the alkylation properties of pyrindamycin A and B and the agent known as duocarmycin A were studied, it was revealed that the sites of alkylation for each of the antitumour agents were indistinguishable. This suggested that a common agent, duocarmycin A (shown in Figure 1.7) was the reacting species.

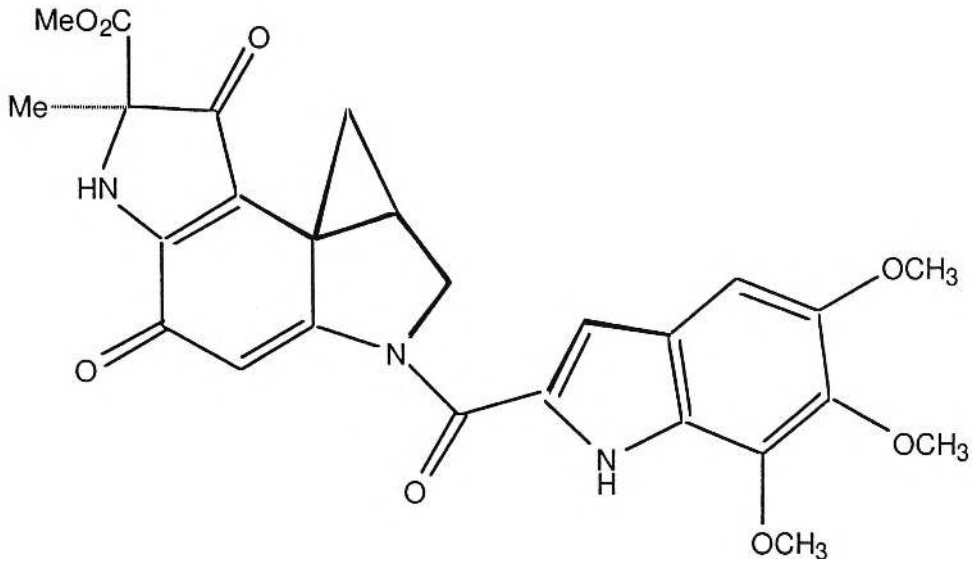


Figure 1.7: Structure of Duocarmycin A

It was supposed that the halogens of pyrindamycin A and B were cleaved in the culture medium or cells, resulting in their conversion to duocarmycin A.(177)(178)(182) Hence, pyrindamycin A and B are considered to be prodrugs of duocarmycin A.(178) Although the sites of alkylation were indistinguishable, the efficiency of the alkylation (judged by the concentration of the agent required for its observation) proved dependent on the structure of the agent and the incubation conditions.(182)(183) For example, the reaction of duocarmycin A at 4°C for 24 hrs, led to more extensive alkylation than with the pyrindamycins (A>B). This compares to the reaction at 37°C for 24 hrs, where the pyrindamycins showed more extensive alkylation than duocarmycin A, (and pyrindamycin B≥A). The dependence on the reaction conditions could be accounted for by the relative availability of the agents (i.e. the stability of pyrindamycin A and B > duocarmycin A) under the reaction conditions and their relative rates of DNA alkylation (duocarmycin A > pyrindamycin A > pyrindamycin B).(177)(182)(184) The depth of minor groove penetration by the agent and the steric accessibility of the alkylation site also contribute to the observed efficiency or extent of DNA alkylation.(179)(185)

1. 5. 1. Sequence Selectivity of Alkylation

Details of the preferred DNA alkylation sites and hence the sequence selectivity of the pyrindamycins have been obtained from the thermally-induced strand cleavage at the sites of covalent modification of double-stranded DNA, after exposure to the agents.

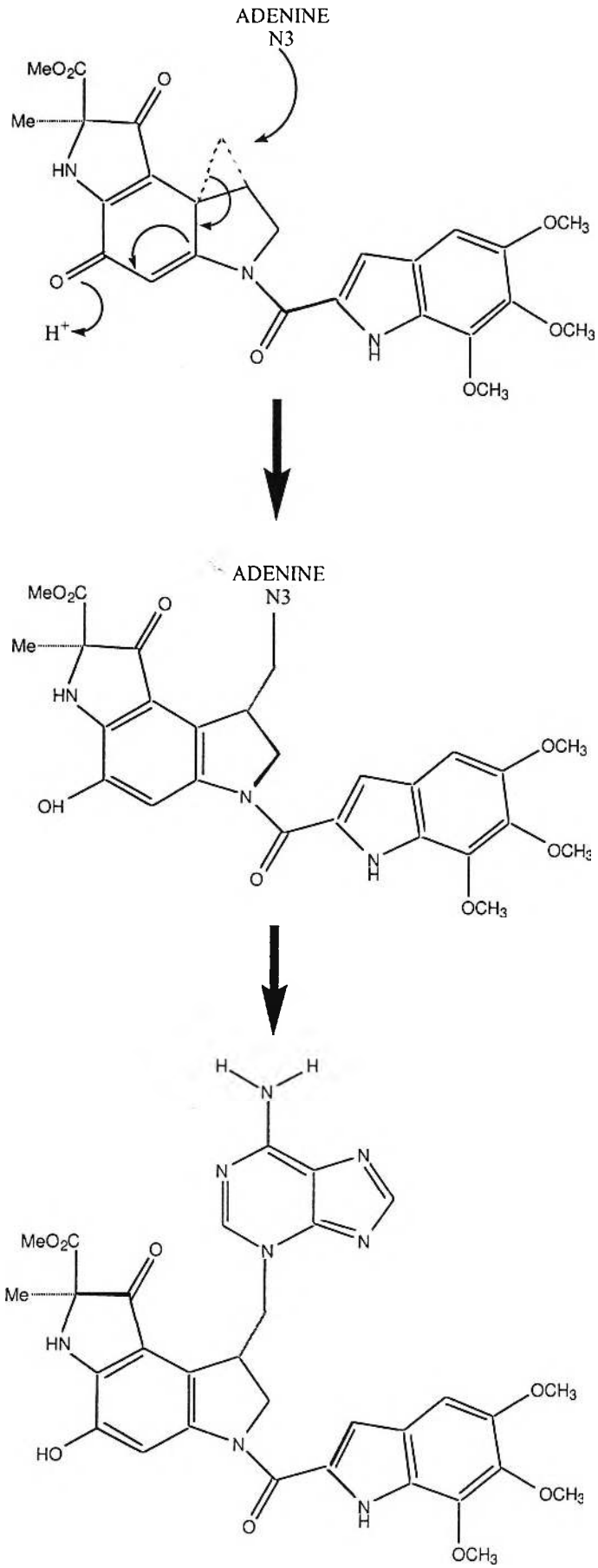
Gel electrophoresis of the resulting DNA, under denaturing conditions, along with Sanger dideoxynucleotide sequence reactions followed by autoradiography, permitted identification of the alkylated sites.(177)(179) The preferred sites of DNA alkylation of the pyrindamycins proved to be an adenine (A) flanked by two 5′- A or T bases. Thus, it has been shown that the pyrindamycins have a strong preference for the sequence of the three bases in the following order :

5′-(AA<u>A</u>)-3′ > 5′-(TT<u>A</u>)-3′ > 5′-(TA<u>A</u>)-3′ > 5′-(AT<u>A</u>)-3′

The first two sequences are high affinity sites and the second two are low affinity sites. In addition, the alkylation exhibited a strong, but not exclusive, sensitivity to the fourth base, preferring an A or T versus G or C as the flanking 5′- base. The lower affinity sequences, more than the high affinity sequences, preferred alkylation sites with a purine (A or G) versus a pyrimidine (T or C) preceding the A alkylation site.(177)(182)

One problem with this sequencing method is that only adducts susceptible to thermal N-glycosidic bond cleavage (adenine N3 and guanine N3 and N7 alkylation) are observed. Any additional alkylation reactions, if present, would not be detected.(179) NMR and mass spectrometry, therefore, have potential advantages over this technique for identifying alkylation sites because it should be possible to observe the presence of any additional adducts, and the process would be much faster. Even with these advantages, the use of these techniques (especially mass spectrometry) in this area has not been very widespread. The work of Boger *et al.*(184) is one of only a handful of papers that have used mass spectrometry, and even then it appears that it was only used in the derivation of molecular weight information concerning the adducts.

The pyrindamycins, once converted within the cell to duocarmycin A, alkylate the N3 position of adenine in the DNA minor groove. This reaction is displayed in Scheme 1.2



Scheme 1.2: The Proposed Mechanism for the Reaction of Pyrindamycins with Adenine Sites within DNA.

Boger *et al.*(185) proposed that adenine alkylation by the pyrindamycins proceeds via the following model: "The binding of the agent spans 3.5 base-pairs in the 3'- to 5'-direction from the adenine N3 alkylation site. The hydrophobic concave face of the agent is deeply imbedded in the minor groove and the polar functionality of the agent lies on the outer face of the complex. This explains the observed absolute requirement for the first three base-pairs, and to a lesser extent the fourth base-pair, of the alkylation site to be A or T."(177-179)(182)(185)(186)

Guanine has been shown to be a second target of duocarmycin A in DNA. Results by Sugiyama *et al.*(98) indicate that the N3 of guanine can nucleophilically attack the cyclopropane subunit of duocarmycin A. This guanine alkylation appears to occur in oligonucleotides that lack a high affinity alkylation site or when adenine alkylation sites within AT-rich regions of labeled restriction fragments are not available.(98)(185)

1. 5. 1. a. The Role of Noncovalent Binding

During the development of the pyrindamycin DNA alkylation selectivity model, the most commonly asked question was, "what role does noncovalent binding play?" There have been many studies that have addressed this question.(179)(181)(187-190) In a study by Boger *et al.* in 1991(187), it was discovered that in the absence of noncovalent binding, the alkylation of DNA by the pyrindamycins was nonselective. Initially it was thought that a DNA autocatalytic phosphate activation of the alkylation reaction (Lewis acid complexation/protonation) and the stereoelectronic effects associated with the cyclopropane alkylation contributed uniquely to the alkylation selectivity. Additional studies by Boger and co-workers in 1993 and 1995 further supported the role of noncovalent binding in alkylation selectivity. Boger *et al.* observed in 1993(181) that the adenine N3 addition reaction does not occur with free adenine. This reinforced the view that it was the noncovalent binding stabilisation derived from hydrophobic binding and van der Waals contacts that led to alkylation selectivity. This was further supported in 1995(188) when studies by Boger *et al.* found that the incorporation and conversion of a natural enantiomer of duocarmycin SA into an agent exhibiting the DNA selectivity of a typical unnatural enantiomer, by reversal of the binding orientation of the agent, is only consistent with a model where the AT-rich noncovalent binding selectivity and

depth of minor groove penetration surrounding the alkylation site are controlling the sites of alkylation.

1. 5. 2. Unnatural Enantiomers

The alkylation properties of some of the unnatural enantiomers of these agents have also been studied.(185)(191-193) The unnatural enantiomers when compared with their natural forms were found to exhibit the same sequence selective alkylation. This results from the diastereomeric nature of the adducts. The unnatural enantiomer binding differs, however, as it starts at the 5′- base preceding the adenine alkylation site and extends across the adenine and the adjacent two bases in the 3′- direction. This reversed orientation binding is required to permit the potential adenine alkylation site access to the least substituted carbon of the activated cyclopropane. (185)(191)(192)

1. 6. Outline of this Work

The literature reviewed in this chapter has shown that there have been relatively few studies of the ESI-MS and ESI-MS/MS of oligonucleotides, especially compared to peptides and proteins. Characterisation of these molecules was difficult prior to the introduction of the ESI and MALDI techniques, however earlier mass spectrometry ionisation methods have been applied extensively to the characterisation of individual bases, nucleosides, and nucleotides, with oligonucleotide studies limited to 13 mers. The gentle ionisation conditions of ESI-MS have also shown it to be feasible for the study of both covalent and noncovalent drug-oligonucleotide interactions. These studies have also, however, been very limited.

The aim of the work presented in this thesis was to characterise small oligonucleotides by ESI-MS and to then investigate the feasibility of this technique for studying interactions of the oligonucleotide with alkylating agents that bind in the minor groove of DNA. The example chosen for this was the antitumour agent pyrindamycin A (PyA). The instrumental and experimental details for the ESI-MS and ESI-MS/MS studies are described in Chapter 2. Chapter 3 describes a study of small oligonucleotides (2-12 mers) by ESI-MS/MS as a precursor to the study of oligonucleotide adducts, since prior to the commencement of this study there had been only a limited number of

ESI-MS/MS studies of oligonucleotides. Chapter 4 details the characterisation of the adducts formed between pyrindamycin A and self complementary oligonucleotides by ESI-MS and ESI-MS/MS. Overall, this work demonstrates the potential of mass spectrometry as an aid in drug design research.

2. Experimental

2.1. Oligonucleotides

All di- and tri-nucleotides were purchased from Sigma (St. Louis, MO) and used without further purification. The deoxyribonucleotides 5′-d(CACGTG)-3′ and 5′-d(AGGCCT)-3′, and the sequences used for the pyrindamycin studies, 5′-d(CGGTAATTACCG)-3′ and 5′-d(CGCGAATTCGCG)-3′, were synthesised "trityl-on" with an Applied Biosystems Model 381A DNA synthesiser, using β-cyanoethylphosphoramidite chemistry. The crude "trityl-on" oligonucleotides were purified on a Waters Delta-Pak C18 reversed-phase HPLC cartridge system with a linear gradient of acetonitrile in 0.1 *M* aqueous ammonium acetate (pH 7.0) at a flow rate of 1 mL min^{-1} over a period of 30 mins for the trityl-on sequence, and 20 mins for the detritylated sequence. The purified oligonucleotides were detritylated with 80% acetic acid and re-chromatographed using the same stationary phase and solvent system. Figure 2.1 displays the HPLC profile for the detritylated oligonucleotide 5′-d(CGGTAATTACCG)-3′. All oligonucleotides purified using this system produced identical HPLC retention times. Confirmation of peak identity was performed using ESI-MS as outlined in Section 2.3 below. Figure 2.2 shows the ESI mass spectrum for the detritylated oligonucleotide 5′-d(CGGTAATTACCG)-3′.

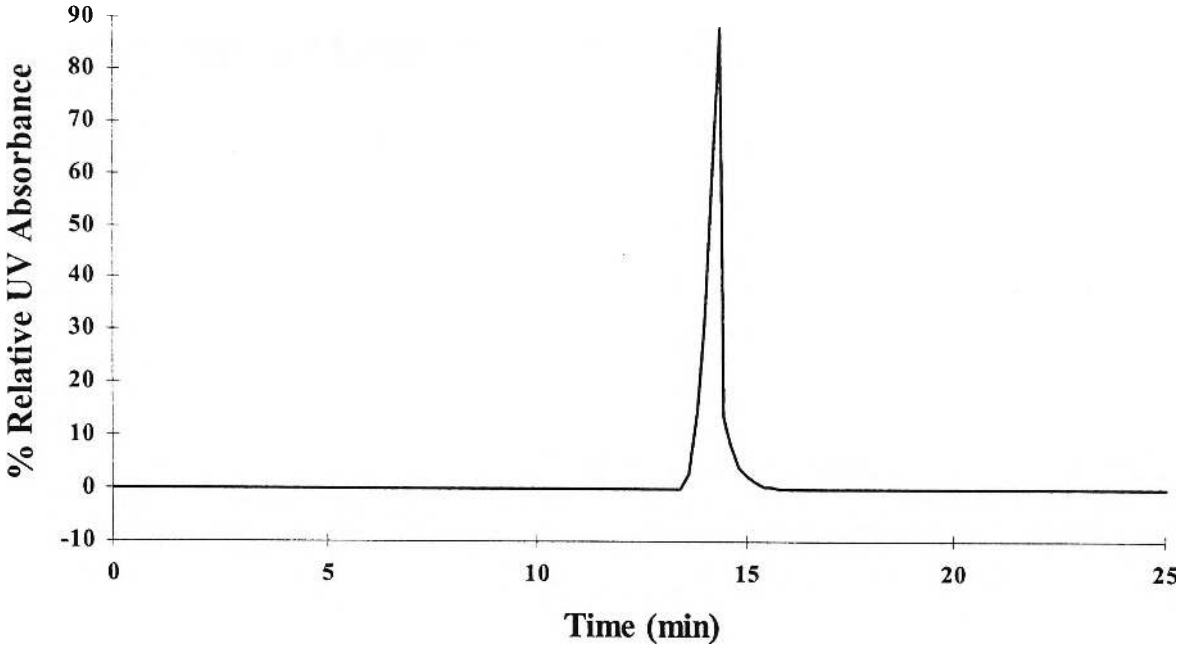


Figure 2.1: HPLC profile of the detritylated 5′-d(CGGTAATTACCG)-3′ oligonucleotide.

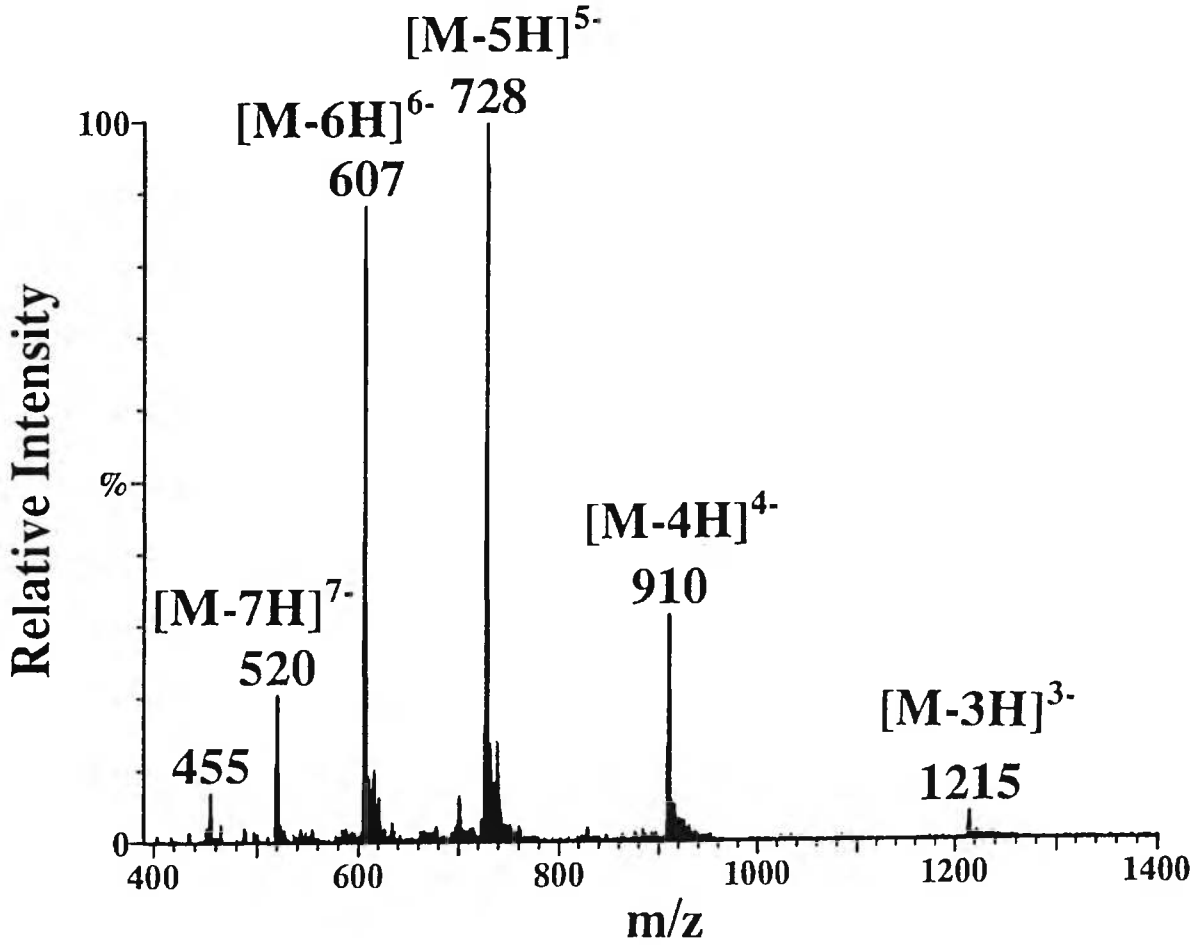


Figure 2.2: ESI mass spectrum of the detritylated 5′-d(CGGTAATTACCG)-3′ oligonucleotide (where M is the oligonucleotide).

2. 2. Pyrindamycin A + Oligonucleotide Reaction

The pyrindamycin A used for this study was obtained from Dr Geoff Wickham of the University of Wollongong.

The pyrindamycin A - oligonucleotide reactions were performed according to the method of Boger and coworkers.(184) A solution of pyrindamycin A (1 mg in 0.5mL DMF) was individually added to both 5′-d(CGGTAATTACCG)-3′ and 5′-d(CGCGAATTCGCG)-3′, in a 10:1 ratio, in 0.1 M sodium phosphate buffer (pH 7.4) and stirred slowly, in the dark and at room temperature for varying reaction times from 2 hours to 14 days. HPLC purification was performed as for the oligonucleotides, only the gradient was run over a period of 40 mins. Confirmation of peak identity was made using ESI-MS as outlined in section 2.3 below.

2. 3. Mass Spectrometry

The majority of ESI mass spectra were acquired on a VG Quattro mass spectrometer (VG Organic, Altrincham, UK) of quadrupole-hexapole-quadrupole configuration equipped with an electrospray ionisation source which allowed mass analysis of singly-charged ions up to 4000 Da. Nucleotide samples were dissolved in 50% aqueous isopropanol (negative ion mode) or water (positive ion mode) at a concentration of 10-200 pmol μL^{-1}. An injection volume of 10 µL was used for each analysis. Samples were introduced into the source by a flow of solvent delivered via an ISCO syringe pump at 5-10µL min^{-1}. A flow of warm, dry nitrogen (65°C, 1 atm) assisted evaporation of the solvent. The electrospray probe tip potential was 3.5 kV, with 0.5 kV on the chicane counter electrode. The mass range was calibrated using sodium iodide in the positive ion mode and a standard sugar mixture was used for negative ions. Skimmer potentials in the range 50-100V were used as indicated. Spectra were acquired at a rate of 100 *m/z* per second and typically 10-15 scans were summed to obtain representative spectra.

2. 4. Tandem Mass Spectrometry (MS/MS)

Low-energy MS/MS spectra were acquired on the VG Quattro mass spectrometer. For the MS/MS studies the collision gas used was argon and the collision energies ranged from 10-70 eV. The resolution in MS1 was set to a minimum and the resolution in MS2 was ~ 1.5 Da (peak width at half-height). The photomultiplier was 650 V.

High energy MS/MS spectra were acquired on a VG Autospec magnetic sector-orthogonal acceleration (Mag-oa-TOF) mass spectrometer (VG Organic, Wythenshawe, UK). Samples were approximately 200-400 pmol/μL in 50% aqueous acetonitrile or 50% aqueous isopropanol. The samples were continuously infused via a Harvard Syringe pump at a flow rate of 10 μL min^{-1} or in the case of the pyrindamycin A reaction products injected using a rheodyne 7125 injector. The collision gas was either methane (5′-ApCpC-3′) (5'-d(CGGTAATTACCG)-3') and ((PyA-Cl) + base A) or xenon (5′-d(AGGCCT)-3′). The laboratory collision voltage was 400 eV for singly-charged ions and 200 eV for multiply-charged ions.

3. ESI-MS/MS of Small Oligonucleotides

The initial work in this study involved characterisation of a large set of dideoxyribonucleotide monophosphates (i.e. d(AC), d(CA), TT, d(AT), TdA, TdG, TdC, d(AG), d(AA), d(GG)) at a range of laboratory collision energies and skimmer potentials. As mentioned in chapter one, studies utilising FAB-MS/MS have been performed on the complete set of dinucleotides.(148) It was of interest, therefore to determine if the same fragmentation pathways were observed in ESI-MS/MS spectra of the same compounds, since the initial internal energy of the ions was expected to be lower in the case of ions generated by ESI compared to FAB.

3.1. Protonated Dinucleotides

Figure 3.1 shows the MS/MS spectra of the $[M+H]^+$ ion of d(AC) obtained at a laboratory collision energy of 40 eV and skimmer potentials of 30 V (Figure 3.1a) and 70 V (Figure 3.1b) respectively. The skimmer potential determines the degree of acceleration of the ions through the intermediate pressure region in the source such that higher skimmer potentials generally increase the excitation of the ions and more fragmentation results.(194) In this example, more abundant fragment ions are observed when the higher skimmer potential was used, although the nature of the fragments is unchanged. The most abundant ion in these spectra is at m/z 112 owing to loss of the 3′ base (cytosine) with charge retention and hydrogen transfer i.e. so-called BH_2^+ ions(43). The peak owing to the BH_2^+ ion for adenine (m/z 136) is less intense. In Figure 3.1b there are "pairs" of the BH_2^+ ions, which correspond to losses of neutral cytosine and adenine, at m/z 430 and m/z 406 respectively. The ion at m/z 308 presumably arises from cleavage of the 3′C-O bond in the sugar from which cytosine is lost (i.e. $[w_1+2H]^+$ according to scheme 1.1). The remaining ions in the spectrum are due to the $[PO_3H_2]^+$ (m/z 81) or fragments of the phosphate and sugar residues (m/z 179, 197, 216).

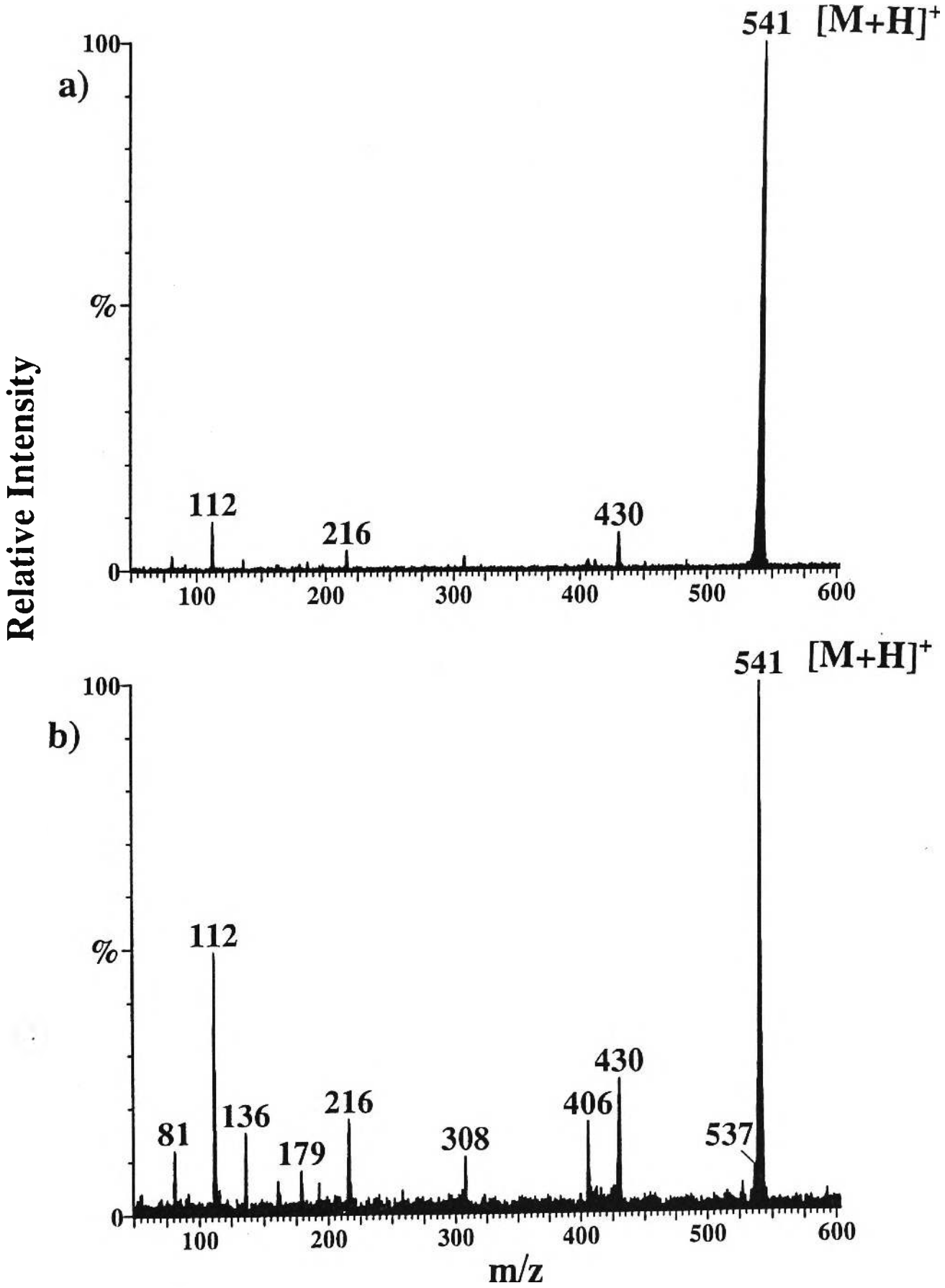


Figure 3.1: ESI-MS/MS spectra of the $[M+H]^+$ ions of d(AC), with E_{lab}=40 eV and skimmer potential of **a)** 30 V, and **b)** 70 V

Further investigation by ESI-MS/MS of d(CA) and d(AG) showed results consistent with those obtained for d(AC). Figure 3.2 a and b show the ESI-MS/MS mass spectrum of the $[M+H]^+$ ion of d(CA) using a laboratory collision energy of 50eV with a skimmer potential of 40V and 80V respectively. In these spectra, the most abundant ion at m/z 332 was the $[w_1+2H]^+$ ion, closely followed by m/z 430, which is owing to the loss of the 5′ base from the molecular ion. Other ions observed in the spectra, m/z 136 and m/z 112, corresponded to the BH_2^+ ions of the bases adenine and cytosine respectively.

Figure 3.3 a and b shows the ESI-MS/MS spectra for the $[M+H]^+$ ion of d(AG) at a laboratory collision energy of 50eV when the skimmer potential is at 40V and 80V respectively. Both of these spectra are dominated by m/z 152 which is the BH_2^+ ion of the 3′ base, guanine. The ions observed at m/z 446 and 430 are the result of the loss of each of the bases adenine and guanine respectively from the protonated molecular ion. Ions due to the adenine (m/z 136), $[PO_3H_2]^+$ (m/z 81) and fragmentation of the phosphate and sugar residues (m/z 216) were also observed.

Dominance of the BH_2^+ ion of the 3′ base was found to be a general trend in the MS/MS spectra of $[M+H]^+$ ions of all the dinucleotides examined, except when the 3′ base is thymine, in which case the BH_2^+ ion of the 5′ base was generally more intense and in most cases no peak was observed for the thymine. Figure 3.4 highlights these observations using TT (Figure 3.4a), and d(AT) (Figure 3.4b). Figure 3.4c shows that when thymine is the 5′ base there is no deviation from the general trend. These observations are in accord with the earlier FAB-MS/MS study(148) in which it was suggested that the lack of a BH_2^+ ion from 3′ thymine can be understood in terms of charge distribution in a model structure and the large difference in proton affinity of thymine compared to the other bases.

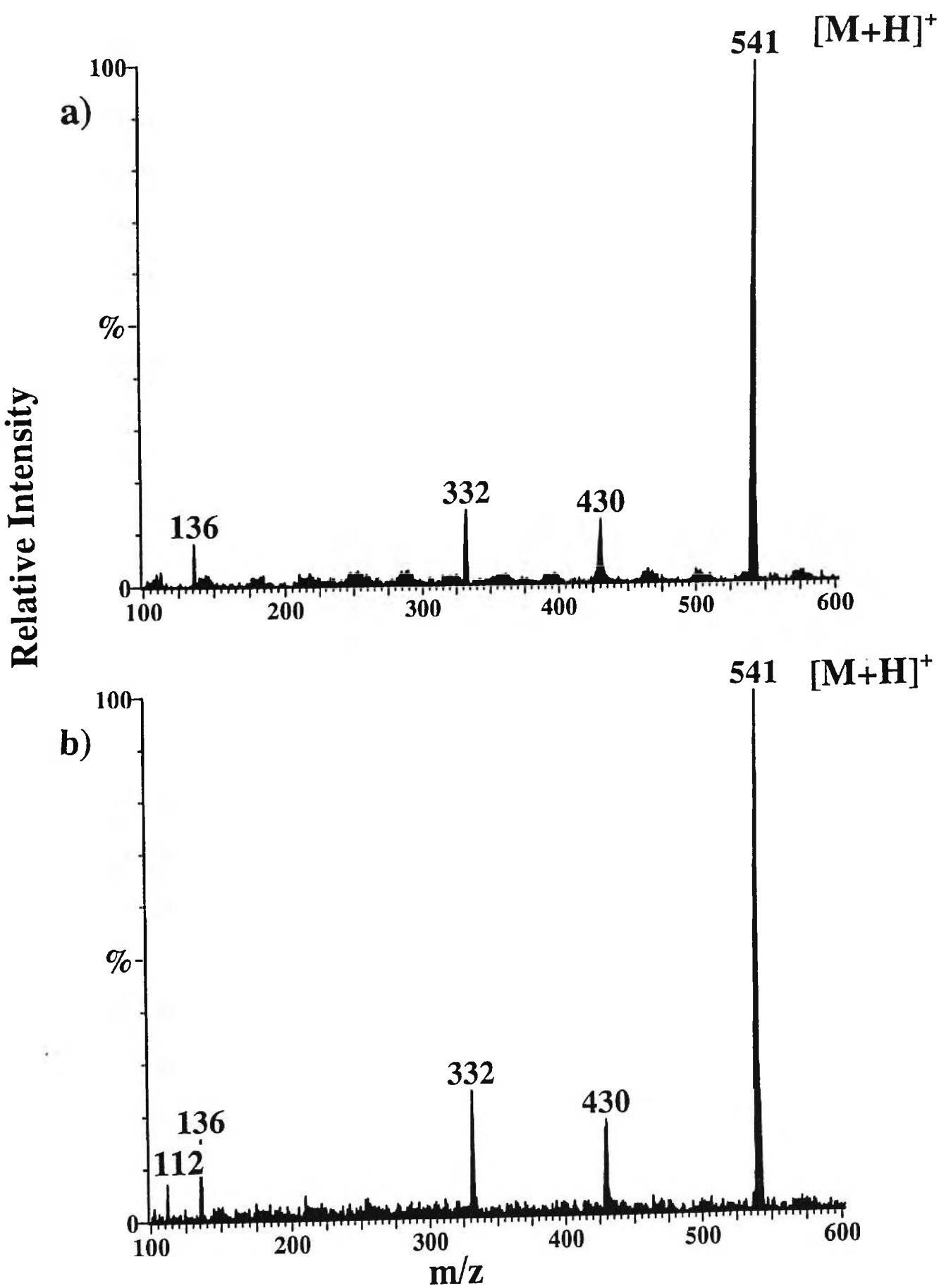


Figure 3.2: ESI-MS/MS spectra of the $[M+H]^+$ ions of d(CA) with E_{lab}=50 eV and a skimmer potential of **a)** 40 V, and **b)** 80 V

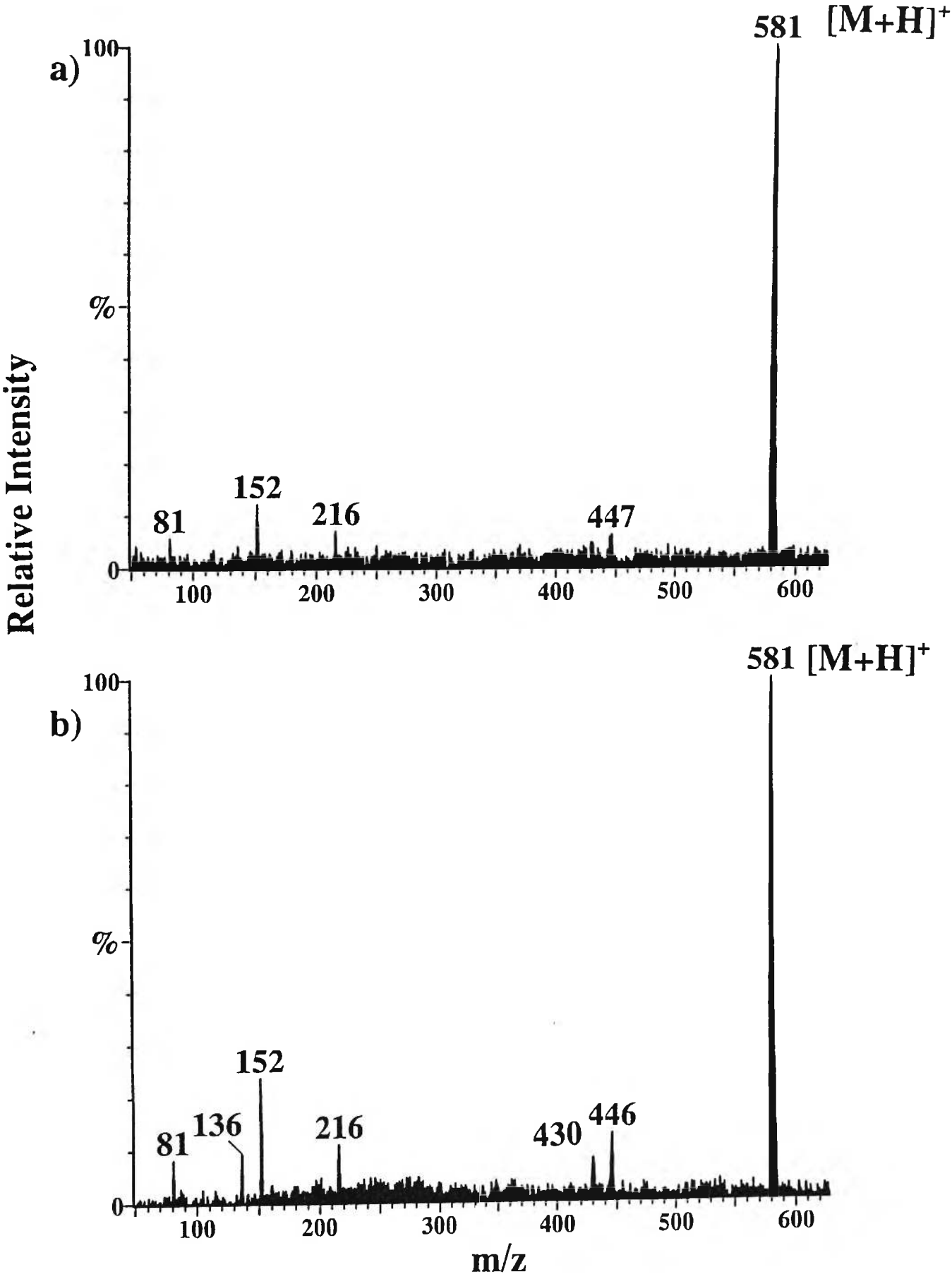


Figure 3.3: ESI-MS/MS spectra of the $[M+H]^+$ ions of d(AG) with E_{lab}=50 eV and a skimmer potential of **a)** 40 V, and **b)** 80 V

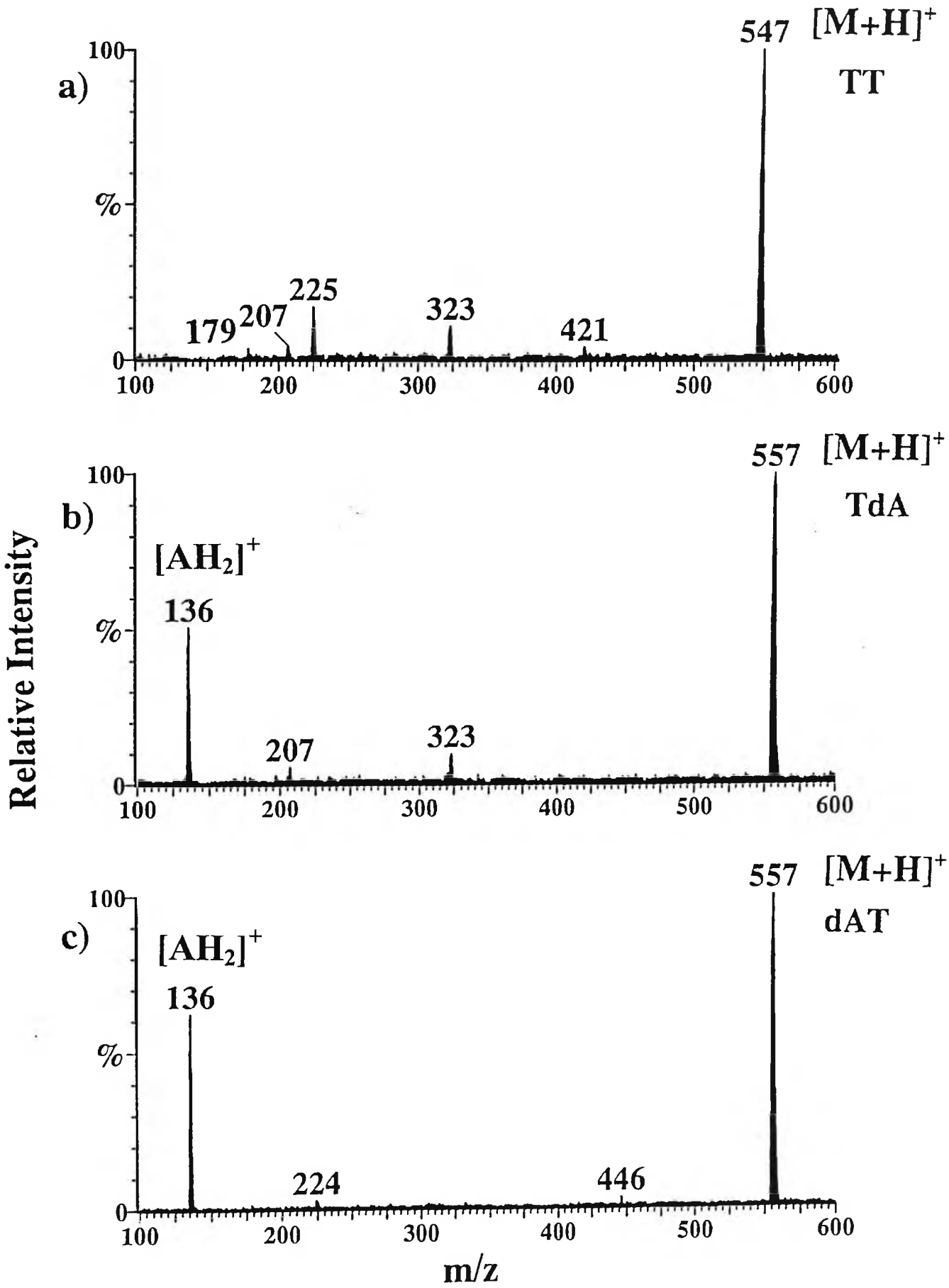
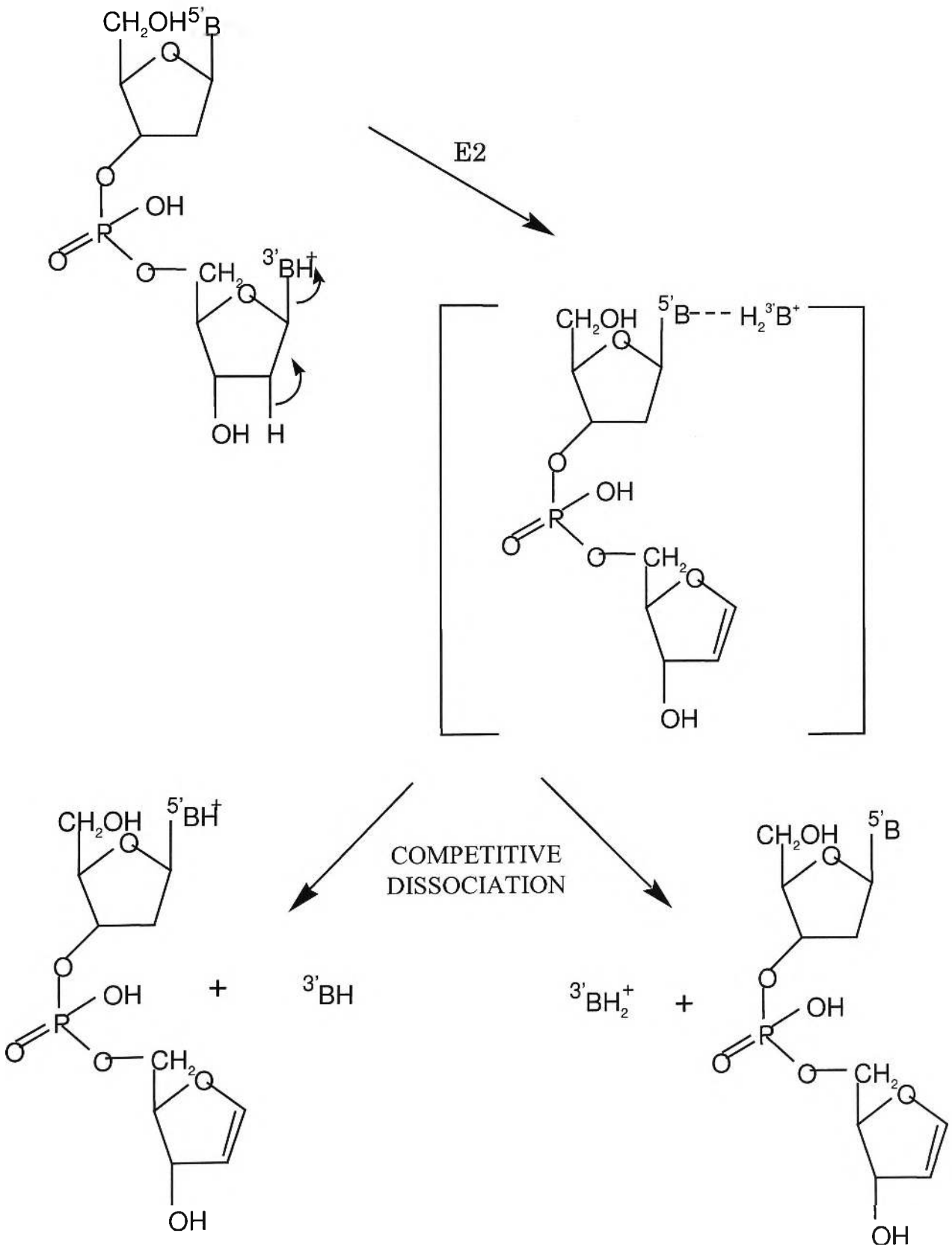


Figure 3.4: ESI-MS/MS spectra of the $[M+H]^+$ ions of a) TT b) dAT c) TdA at E_{lab}=50 eV and skimmer potentials of 45, 60 and 60 V respectively

The MS/MS results obtained in this study for the protonated molecular ion produced similar fragmentation products to those obtained in experiments performed by Rodgers *et al.* (194a) on a FTICR mass spectrometer. In both cases, the major fragment ion observed was the BH_2^+ ion of the 3′ base. Additional fragments were dinucleotide species dependant but generally the same for the different mass spectrometers. Differences between the instruments were only observed when the dinucleotide contained T.(194a) The proposed reaction mechanism for the formation of the BH_2^+ fragment ion is shown in Scheme 3.1. (194a)

Scheme 3.1: Reaction Mechanism for the Major Fragmentation Pathway of Protonated Dinucleotides



3.2. Deprotonated Dinucleotides

Figure 3.5 shows the MS/MS spectra of the $[M-H]^-$ ion of d(AC) at different skimmer potentials. In this case, only one fragment, the B^- ion of adenine at m/z 134, is evident in the spectrum obtained at the lower skimmer potential (30 V). Additional fragment ions observed at the higher skimmer potential (70 V) are the B^- ion of cytosine (m/z 110), the cleavage of the ribose phosphate 3'C-O in the sugar from which cytosine is lost, (w_1^-, at m/z 306) and the ion resulting from loss of neutral adenine (m/z 404). There are also peaks corresponding to the PO_3^- phosphate group (m/z 79) and the phosphate plus the sugar residue (m/z 195).

Figure 3.6 displays the ESI-MS/MS spectra for d(CA) using a laboratory collision energy of 50eV and skimmer potentials of 20V and 80V. At the low skimmer potential, no fragmentation is observed. In contrast, the spectrum obtained at the high skimmer potential exhibits peaks at m/z 428 which is due to the loss of cytosine from the deprotonated molecular ion, the w_1^- ion at m/z 330, a_1^- at m/z 210, and m/z 195, the phosphate plus the sugar residue. The ions at m/z 134 and m/z 110 correspond to the B^- ions of adenine and cytosine respectively, with cytosine being the more intense of the two.

Figure 3.7 shows the two ESI-MS/MS spectra obtained at a laboratory collision energy of 50eV for d(AG) with skimmer potentials of 40V and 80V. The only fragment observed for the low skimmer potential is m/z 134, due to the 5′ base adenine. In the spectrum for the higher skimmer potential, the most intense peak (m/z 134) is the B^- ion of the 5′ base adenine. The remaining ions in the spectrum are m/z 444, owing to the loss of adenine from the molecular ion; the w_1^- ion at m/z 346; m/z 195 arising from the phosphate plus the sugar residue; and the m/z 79 ion owing to the PO_3^- group. This example also demonstrates that at least qualitatively, the skimmer potential has a greater influence on the MS/MS spectra of the deprotonated dinucleotides than on the protonated species. This can be seen when comparing Figures 3.3a and 3.7a for dAG, in which several peaks in addition to the BH_2^+ ions are observed for the protonated species, whereas the deprotonated species showed only the B^- ion.

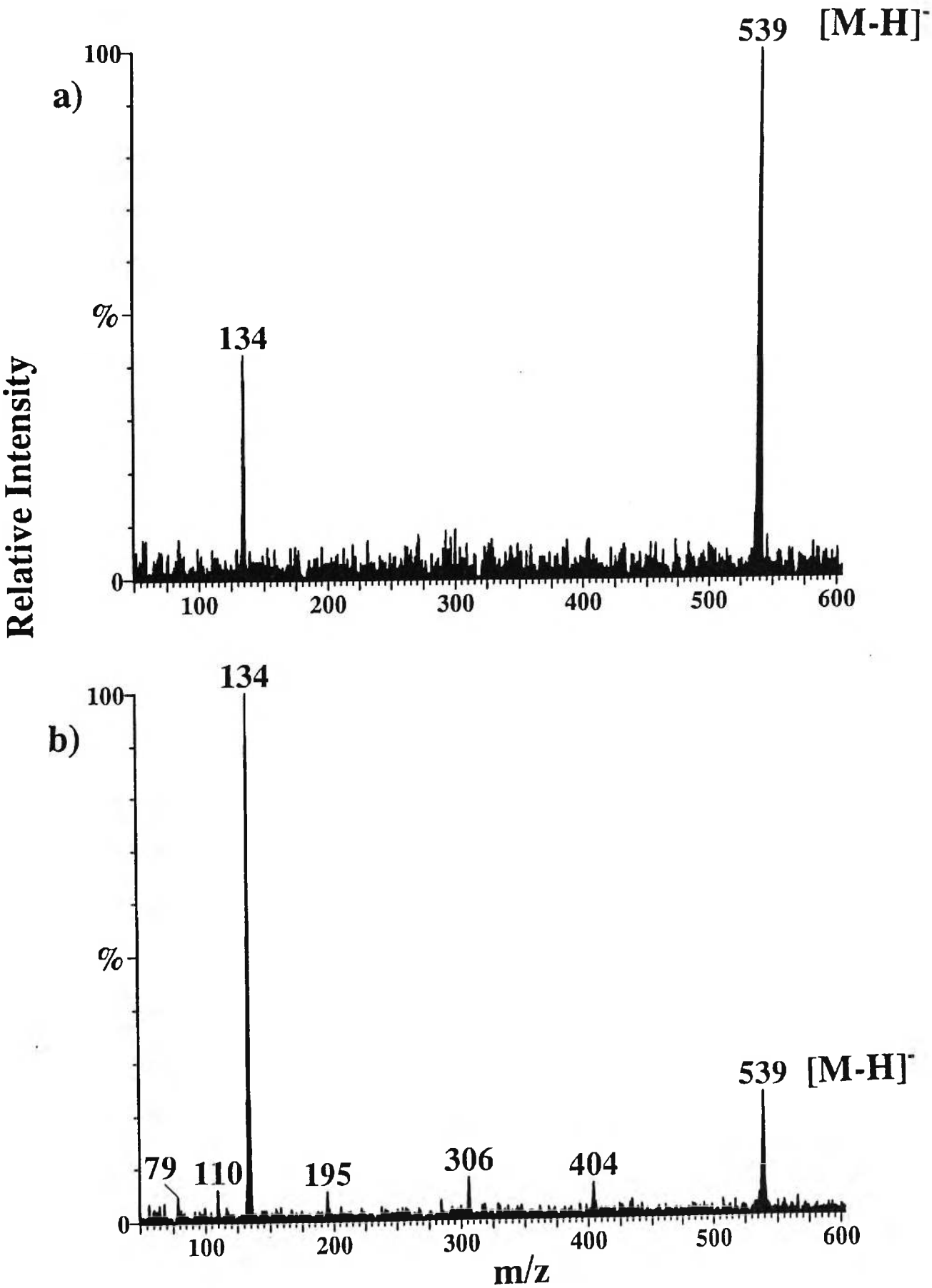


Figure 3.5: ESI-MS/MS spectra of the $[M-H]^-$ ions of d(AC) E_{lab}=40 eV skimmer potential a) 30 V b) 70 V

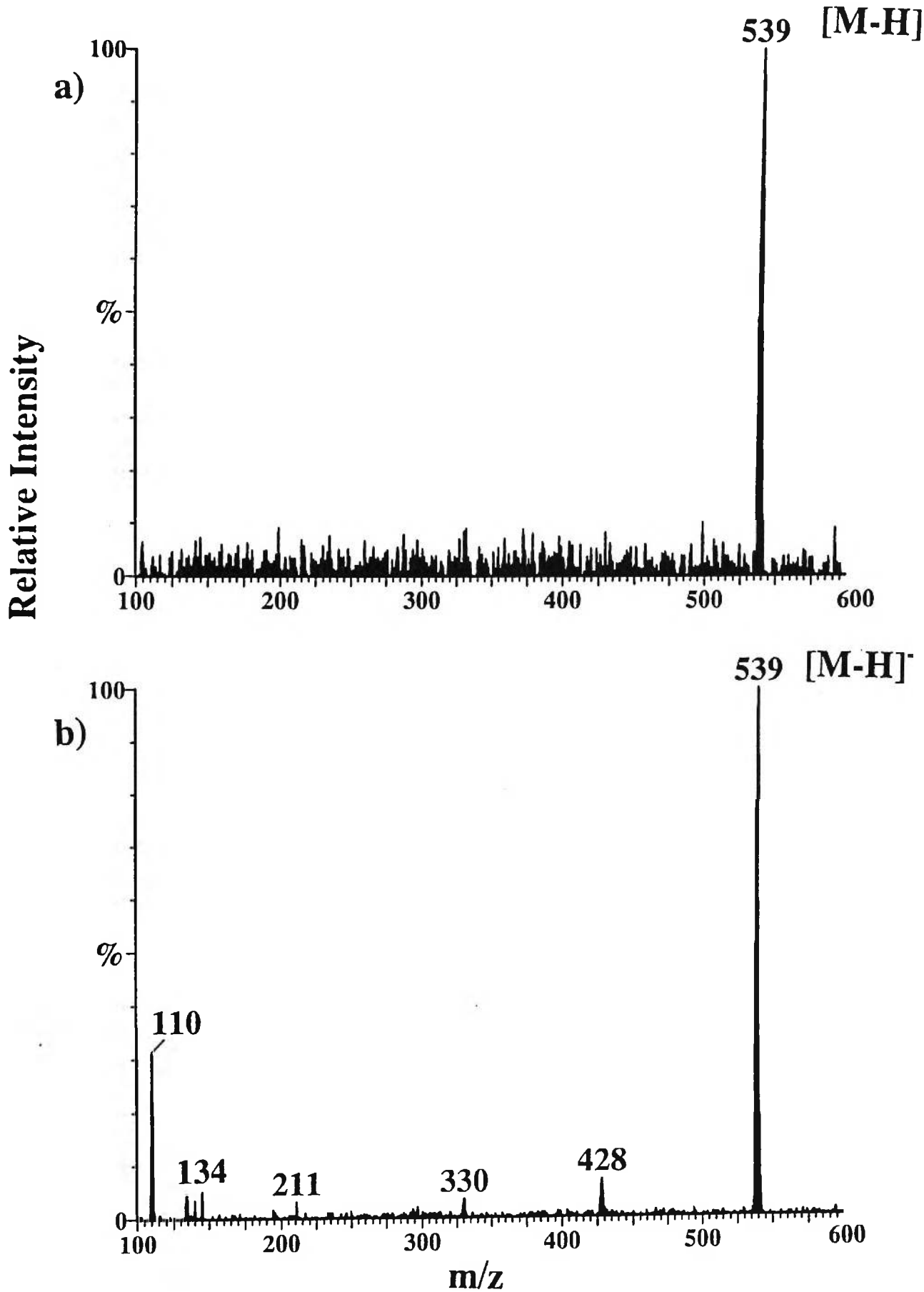


Figure 3.6: ESI-MS/MS spectra of the $[M-H]^-$ ions of d(CA) with E_{lab}=50 eV and a skimmer potential of **a)** 20 V, and **b)** 80 V

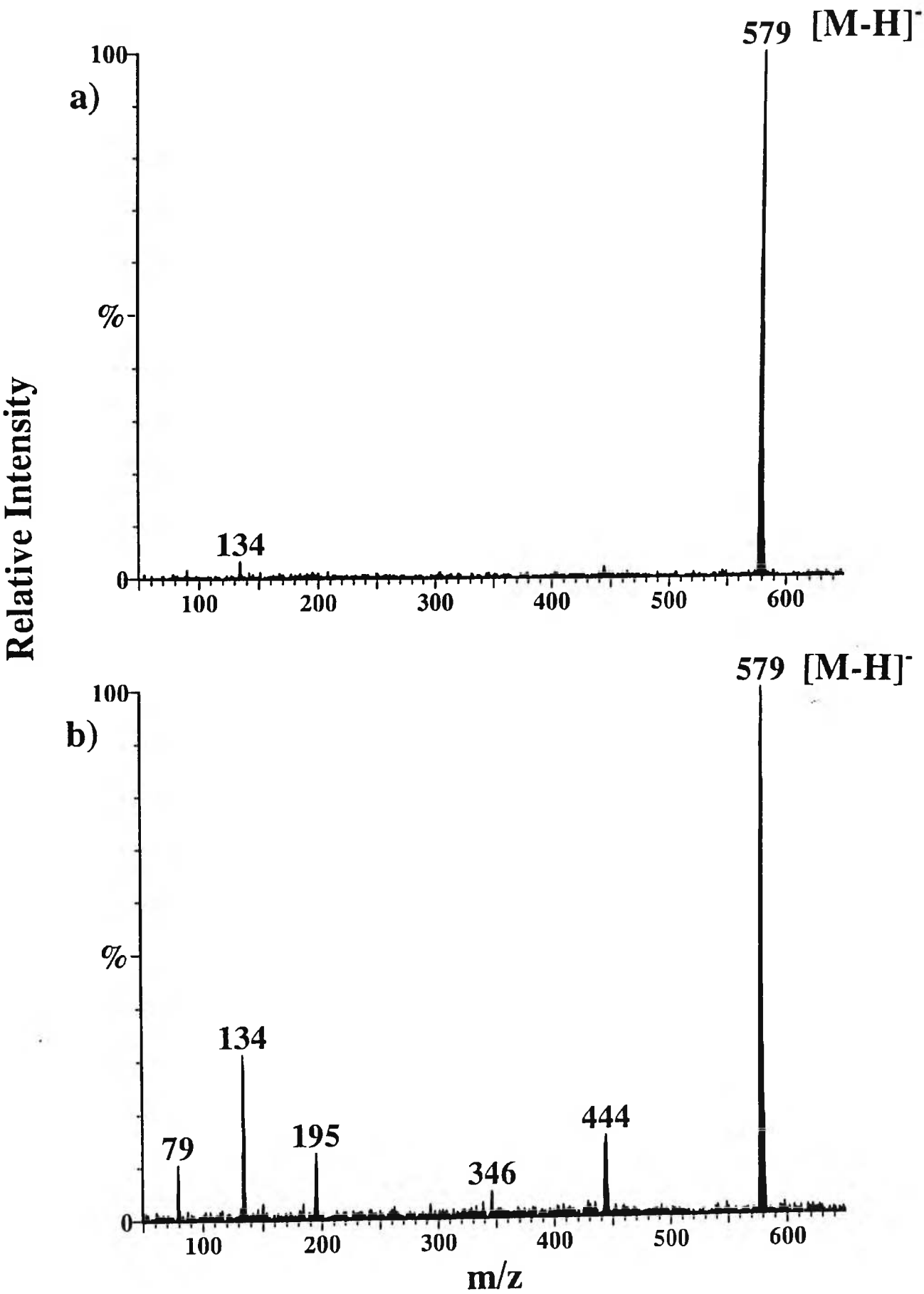
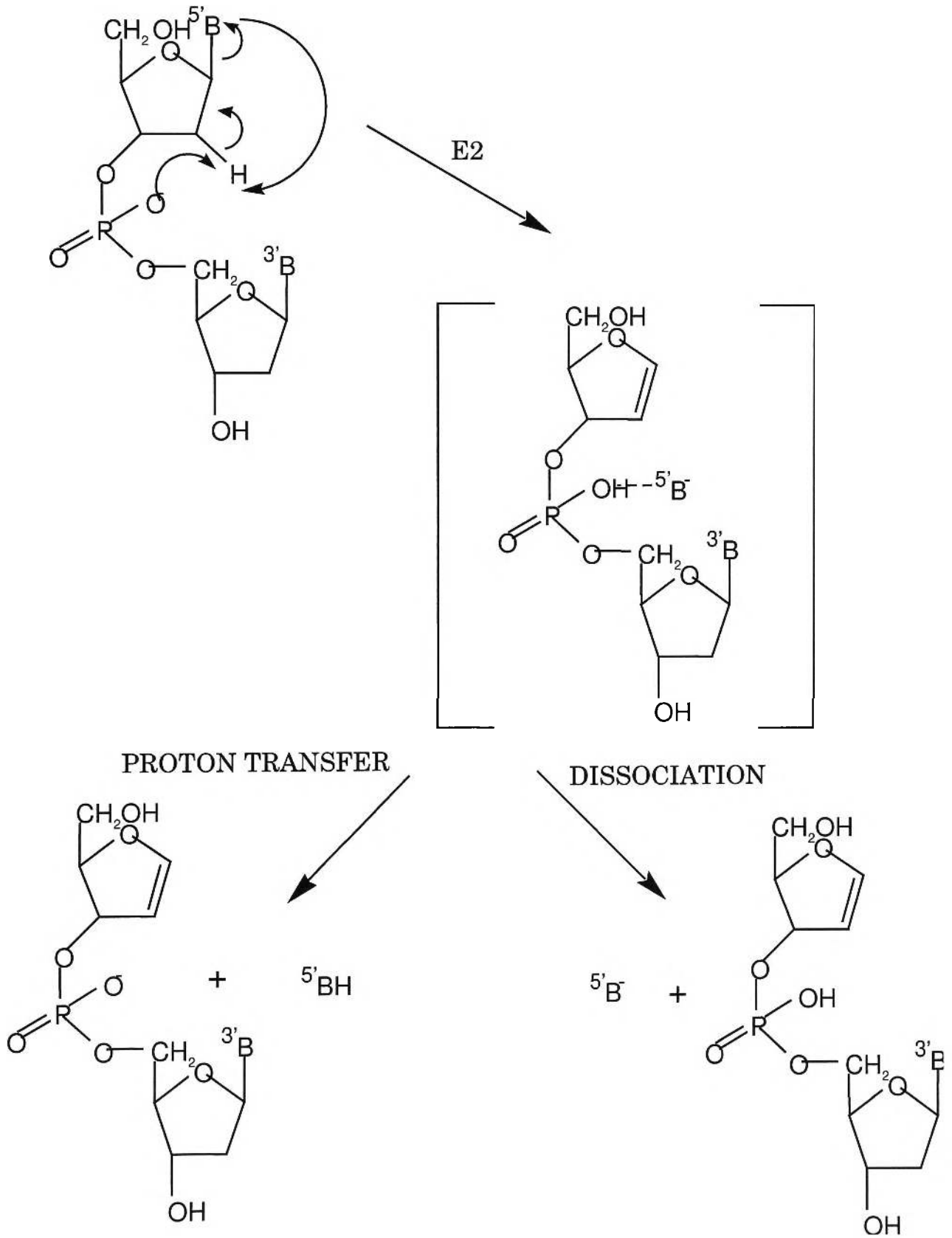


Figure 3.7: ESI-MS/MS spectra of the $[M-H]^-$ ions of d(AG) with E_{lab}=50 eV and a skimmer potential of **a)** 40 V, and **b)** 80 V

The MS/MS results obtained for the deprotonated dinucleotides in this study were found to resemble those obtained by Rodgers *et al.* (194b) on a FTICR mass spectrometer. The major product ions observed were independent of the dinucleotide species and the mass spectrometer type. The major fragments observed were the 5′ base B^- ion and the fragment resulting from the loss of the neutral 5′ base from the molecular ion.(194b) The reaction mechanism for the production of these two fragment ions is given in Scheme 3.2. (194b)

Scheme 3.2: Reaction Mechanism for the Major Fragmentation Pathway of Deprotonated Dinucleotides



In contrast to the spectra of the $[M+H]^+$ ions, the dominant fragment ions in the spectra of the $[M-H]^-$ ions arise from loss of the 5′ base. This was found to be a general trend for all the dinucleotides examined, including those containing thymine. Thus the MS/MS spectra of $[M-H]^-$ ions of dinucleotides appear to be more diagnostic of base position than those of the $[M+H]^+$ ions. This is also consistent with MS/MS studies of $[M-H]^-$ ions following fast atom bombardment (FAB).(148) The spectra obtained at the higher skimmer potentials are similar to the FAB MS/MS spectra of the same dinucleotides. This supports the view that ions generated by FAB have higher initial internal energies than following ESI.(195) As previously reported,(194) the MS/MS spectra of deprotonated dinucleotides should have applications in determining the sequence of modified dinucleotides that may be obtained following digestion of large segments of DNA, in which the action of the nucleases is blocked by the modification. These results demonstrate that MS/MS spectra of dinucleotides following ESI may be equally as useful in this regard as the FAB-MS/MS spectra.

3.3. Cationised oligonucleotides

The MS/MS spectra of cationised adducts of dinucleotides were also examined in detail in an attempt to see if these adducts yielded more information. When ESI-MS/MS was performed on the $[M+Na]^+$ adduct of TTT (Figure 3.8a) there was no significant increase in fragmentation observed compared with the ESI-MS/MS of the $[M+H]^+$ molecular ion (Figure 3.8c). The spectra of the $[M+K]^+$ ion (Figure 3.8b) yielded no fragment ions even under relatively harsh collision conditions.

The ESI-MS/MS spectra of the $[M+Li]^+$ adduct of the dinucleotides showed a greater number of fragments compared to the corresponding $[M+H]^+$ and $[M-H]^-$ ions. Figure 3.9a shows the MS/MS spectra of the $[M+Li]^+$ ion of d(AC), and the corresponding ribonucleotide ApC is shown in Figure 3.9b. For d(AC), the sequence ion $[w_1+H+Li]^+$(m/z 314) is the major fragment, rather than the BH_2^+ ions. There are also ions such as $[y_1+H+Li]^+$(m/z 234), $[d_1-2H+Li]^+$, and fragments resulting from additional cleavages of the sugar residue (m/z 204), none of which are present in the spectrum of the $[M+H]^+$ ions. This is not surprising, given that the Li attachment has previously been shown to enhance the fragmentation of saccharides.(196)(197)

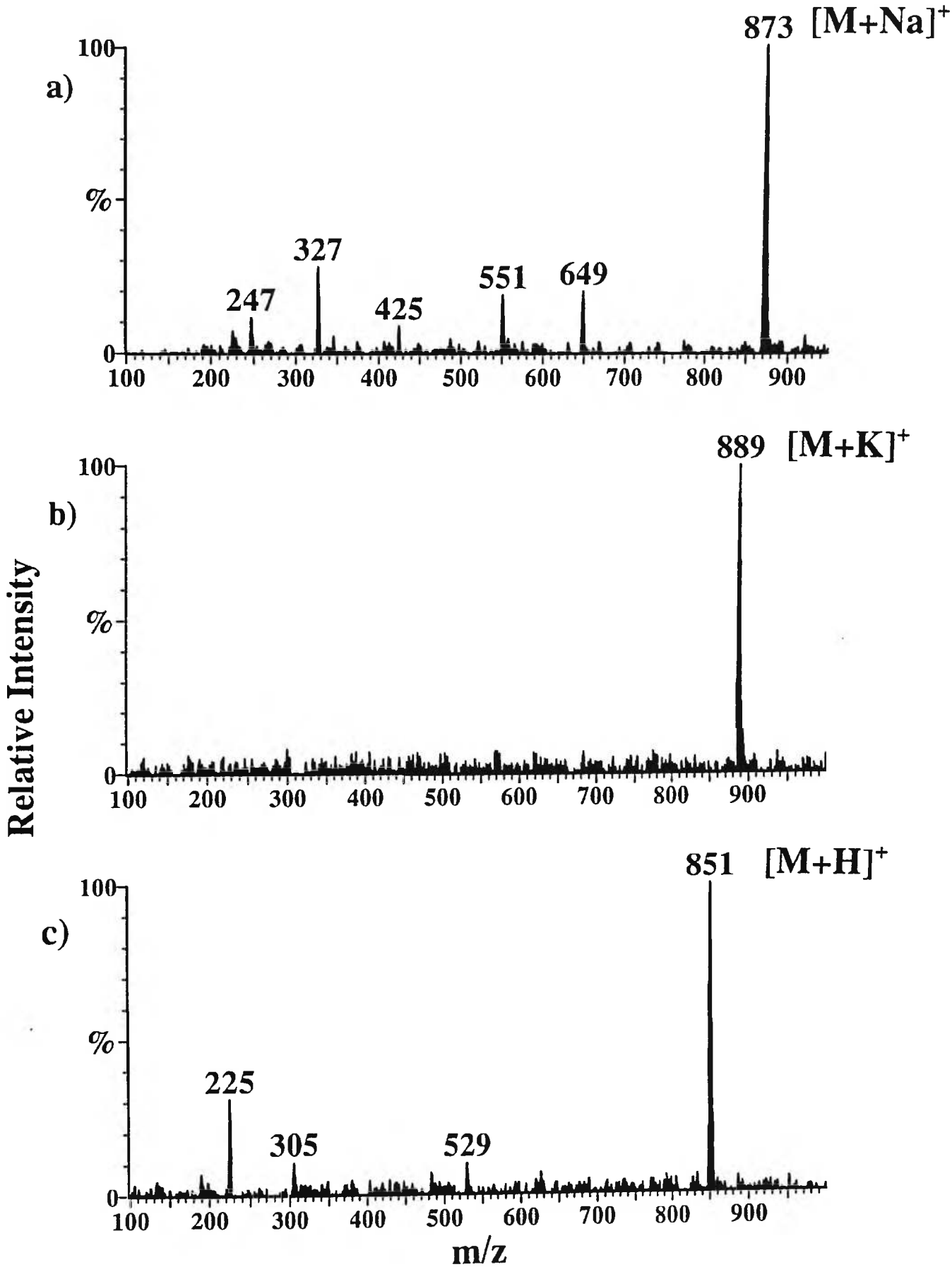


Figure 3.8: ESI-MS/MS spectra of the TTT ions **a)** $[M+Na]^+$ with E_{lab}=50 eV, **b)** $[M+K]^+$ with E_{lab}=100 eV, and **c)** $[M+H]^+$ with E_{lab}=75 eV, with skimmer potential 65 V

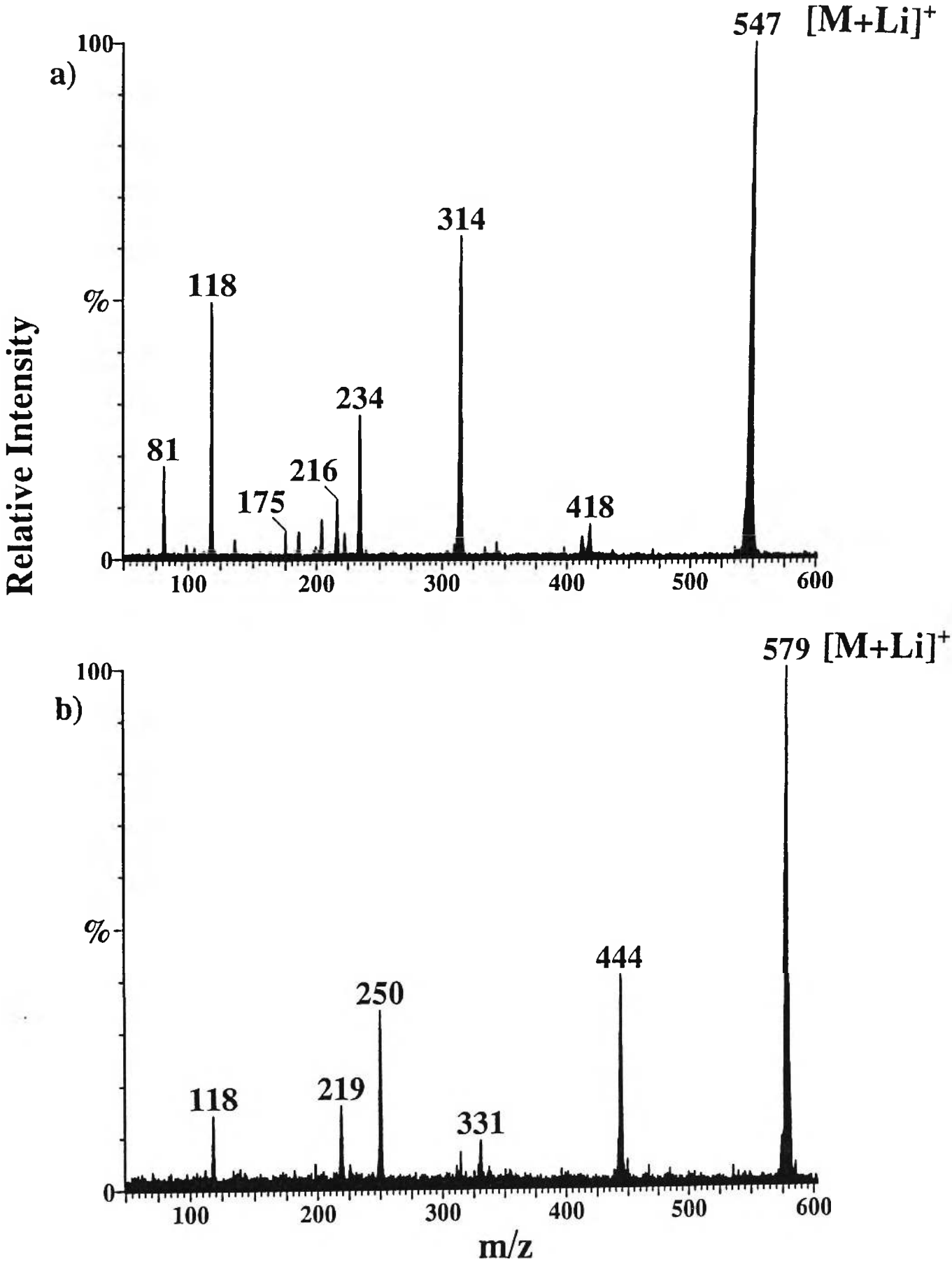


Figure 3.9: ESI-MS/MS spectra of the $[M+Li]^+$ ions of **a)** d(AC) and **b)** ApC, with E_{lab}=50 eV and skimmer potential of 50 V

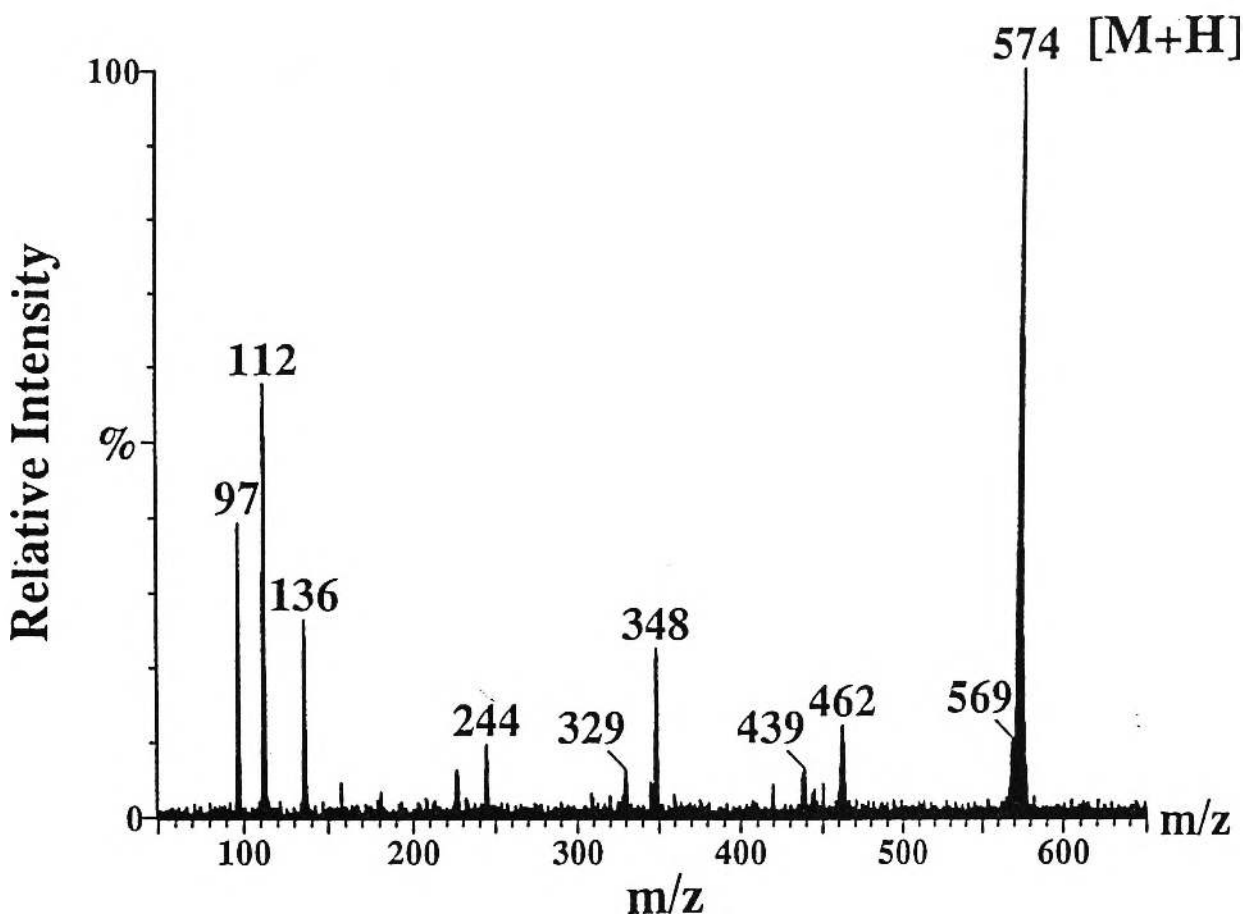


Figure 3.10: ESI-MS/MS spectra of the $[M+H]^+$ ion of ApC with E_{lab}=50 eV and a skimmer potential of 50 V

It is interesting to note that there are also several cases in which Li appears to have been lost, with the neutral fragment resulting in protonated fragment ions, for example the BH_2^+ ion of adenine and the ions at m/z 197 and m/z 216, which are fragments of the phosphate and ribose. The MS/MS spectrum of the $[M+Li]^+$ ion of the ribodinucleotide monophosphate ApC (Figure 3.9b) shows intense peaks due to the loss of adenine (m/z 444), the $BHLi^+$ ion of cytosine (m/z 118), the $[w_1+2H+Li]^+$ ion (m/z 331), and the $[y_1+H+Li]^+$ ion (m/z 250). These two sequence ions were not present in the MS/MS spectrum of the $[M+H]^+$ ion of ApC (Figure 3.10) obtained under the same conditions.

3. 4. Larger Oligonucleotides

The MS/MS spectra of the $[M-H]^-$ and $[M-2H]^{2-}$ ions of the ribonucleotide ApCpC were obtained on the triple quadrupole and are shown in Figure 3.11. Assignments of the major fragment ions are given in Table 3.1. The spectrum of the doubly-charged ion yielded a smaller number of fragments than the corresponding singly-charged ion.

Figure 3.12 shows the MS/MS spectrum of the $[M-H]^-$ ion obtained on the magnetic sector-time-of-flight (mag-TOF) instrument for comparison with the spectrum in Figure 3.11b. There are several additional fragment ions, and the signal-to-noise ratio is superior in the spectrum obtained on the magnetic sector - TOF instrument, reflecting both the higher collision energy available and the better sensitivity of the latter instrument. In all cases, both sequence and base ions are observed. Using Scheme 1.1, in which the oligonucleotide is shown as neutral, these are denoted as deprotonated species.

Table 3.1. Assignments of Major Fragment Ions in MS/MS spectra of 5′-ApCpC-3′

FRAGMENT IONS m/z			ASSIGNMENT
$[M-H]^-$ E_{lab}= 400 eV	$[M-H]^-$ E_{lab} = 50 eV	$[M-2H]^{2-}$ E_{lab} = 50 eV	(refer to scheme 1.1)
79	79	79	PO_3^-
97	97	97	$H_2PO_4^-$
110	110	110	$B_{2/3}(C)^-$
134	134	134	$B_1(A)^-$
177	-	-	sugar + PO_3
195	-	-	sugar + PO_4H_2
211	211	211	$[d_1\text{-}B_1\text{ -H}]^-$ or $[w_1\text{-}B_3\text{ -H}]^-$
305	-	-	$[x_1\text{- H}]^-$
322	322	322	w_1
346	347	-	d_1
420	420	-	$[z_2\text{ -}B_2\text{-H}]^-$
438	-	438	$[y_2\text{-}B_2\text{-H}]^-$
442	-	-	$[a_2\text{-}B_2\text{-3H}]^-$
530	-	-	z_2^-
547/8	547	-	y_2^-
554	-	-	$[a_2\text{-H}]^-$
610	609	-	$[x_2\text{ -H}]^-$
634	-	-	$[c_2\text{ -H}]^-$
	708	-	-
766	-	-	loss of $B_{2/3}$ (C)
877			$[M-H]^-$

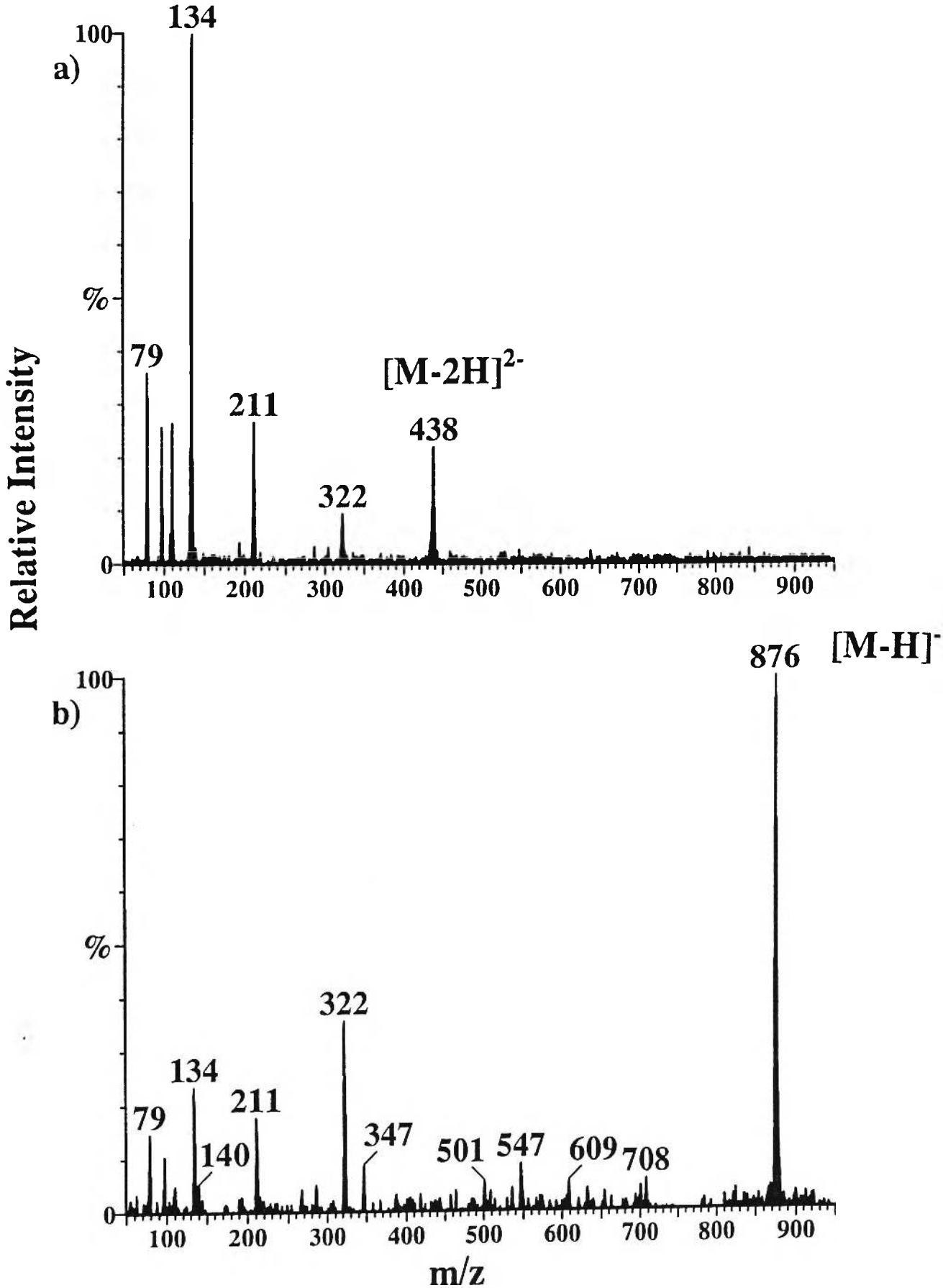


Figure 3.11: ESI-MS/MS of 5′-ApCpC-3′ obtained on the triple quadrupole for the **a)** $[M-2H]^{2-}$ ion with E_{lab}=50 eV and skimmer potential of 40 V, and the **b)** $[M-H]^-$ ion with E_{lab}=50 eV and skimmer potential of 65 V

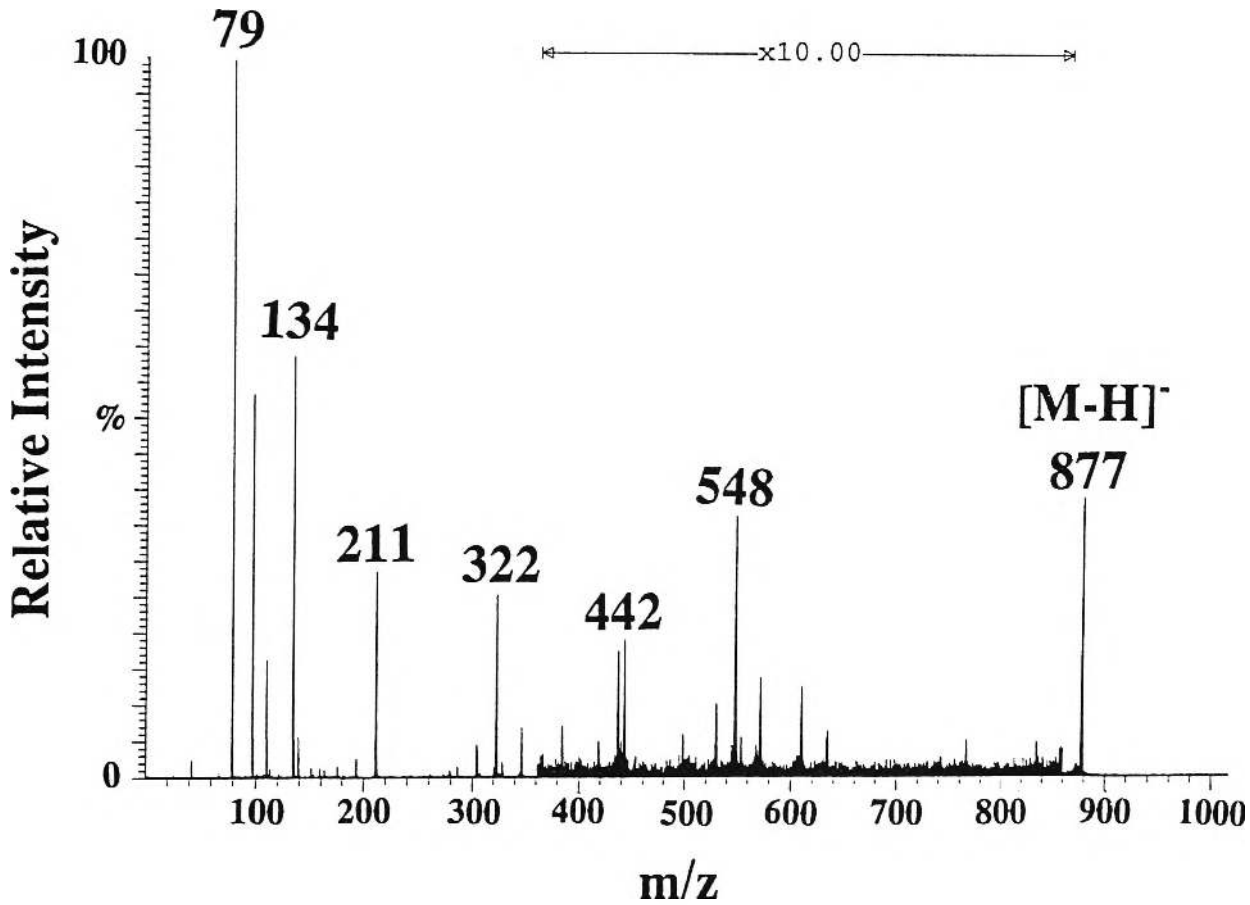


Figure 3.12: ESI-MS/MS spectrum of the $[M-H]^-$ ion of 5′-ApCpC-3′ obtained on the magnetic sector-time-of-flight instrument with the collision cell voltage=400 eV

A limited number of larger oligonucleotides were also examined on both instruments. Figure 3.13 shows the MS/MS spectrum of the $[M-2H]^{2-}$ of 5'-CACGTG-3' obtained on the triple quadrupole instrument with a laboratory collision energy of 40 eV and a relatively high pressure (8×10^{-4} mbar) of argon collision gas. At lower pressures, only the ions due to the base and the phosphate group are observed. Assignments of the major fragments in the spectrum labelled according to the nomenclature shown in Scheme 1.1 are given in Table 3.2.

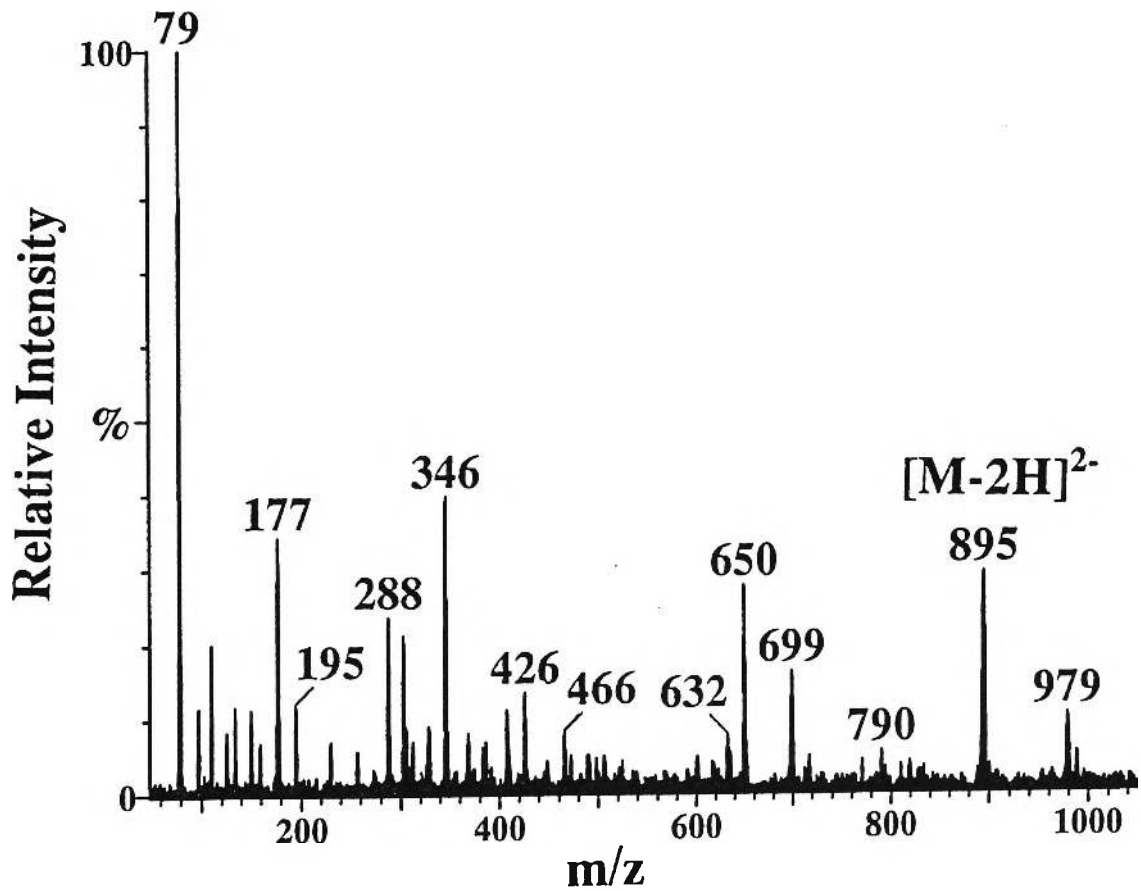


Figure 3.13: ESI-MS/MS spectrum of the $[M-2H]^{2-}$ ion of 5′-d(CACGTG)-3′ obtained on the triple quadrupole E_{lab}=40 eV skimmer potential 55 V

Table 3.2. Major Fragment Ions in MS/MS spectrum of 5′ d(CACGTG)-3′

Observed m/z	Assignment	Observed m/z	Assignment
988	$[a_4\text{-}B_4(G)\ \text{-}3H]^-$	386	$[a_2\text{-}B_2(A)\text{-}3H]^-$
979	w_3^-	346	w_1^-
895	$[M\text{-}2H]^{2-}$	329	nucleotide** G
833	$[M\text{-}B_5(T)]^{2-}$	312	nucleotide** A
819	$[M\text{-}B_{4/6}(G)]^{2-}$	303	nucleotide** T
810	$[M\text{-}B_{4/6}(G)\text{-less } H_2O\text{-}H]^{2-}$	288	nucleotide** C
790	$[w_5\text{-}H]^{2-}$	257	-
771	$[d_5]^{2-}$	230	G + sugar (C_5H_7O)
699	$[a_3\text{-}B_3(C)\ \text{-}3H]^-$	195	[sugar ($C_5H_7O_2$) + PO_4H_2]$^-$
650	w_2^-	177	[sugar ($C_5H_7O_2$) + PO_3]$^-$
632	$[x_2\text{-}H]^-$	159	[sugar ($C_5H_7O_2$)+PO_3-H_20]$^-$
616	$[d_2\text{-}3H]^-$	150	$[G]^-$
601	$[c_2\text{-}2H]^-$	134	$[A]^-$
506	$[d_2\text{-}B_2(C)\text{-}3H]^-$	125	$[T]^-$
490	$[w_3\text{-}H]^{2-}$	110	$[C]^-$
466	$[c_2\text{-}B_2(A)\text{-}3H]^-$	97	$H_2PO_4^-$
426		79	PO_3^-
408	$[a_4\text{-}4H]^{2-}$		

Based solely on the masses, it appears that both singly- and doubly-charged fragments are observed. The base peak in the spectrum is the ion due to the deprotonated phosphate group PO_3^- (m/z 79), however there are also intense peaks characteristic of the sequence. For example, there is almost a complete set of "w" ions (w_1, w_2, w_3 and w_5). McLuckey *et al.* have reported this as a major fragmentation pathway in the MS/MS spectra of multiply-charged oligonucleotide anions with a preference for base loss in the order A > T >> C > G.(153) The same strong preference for loss of adenine is not observed here (in which case the w_2 ion at m/z 650 would be expected to be the major fragment). Initially it was not clear whether this was because of the different collision conditions in the triple quadrupole compared to the ion trap, or whether the difference arises from the lower charge state of the oligonucleotides examined.

**nucleotide refers to the base + the sugar + PO_3H

Barry *et al.* have observed loss of all non-terminal bases for the triply-charged ions of modified oligonucleotides with similar sequence (CACGXG) in MS/MS spectra obtained on a triple quadrupole,(198) it is likely that the different collision conditions is the important factor. Weimann *et al.*(199) have also shown that the charge state is not the dominant factor for determining the nature of the fragment ions in MS/MS spectra. There are many other so-called sequence ions present in the spectrum. A number of the ions which appear to result formally from transfer of one or more hydrogens to the neutral fragment are consistent with those observed by Barry *et al.*, who also reported $[c_n\text{-}2H]^-$ ions.(198) The hydrogen transfer appears to be internally consistent, as there are both w_3^- and $[w_3\text{-}H]^{2-}$ ions present in the spectrum, the x_n ions all have masses corresponding to $[x_n\text{-}H]^-$, and the $[d_n\text{-}3H]^-$ series is also present. There have been insufficient studies of oligonucleotide fragmentation to allow hydrogen transfer to be predicted in the same way as can be done for peptides. This is one aspect of oligonucleotide fragmentation that needs to be examined in more detail in future studies.

A further difficulty with interpretation of the MS/MS spectra of multiply-charged ions is the determination of the charge state of the fragment ions if all the complementary ions are not observed. This problem has been approached by various groups in different ways. If a high resolution MS/MS spectrum is available such as with Fourier transform mass spectrometry (FT-MS), then charge states can be determined directly.(155) Barry *et al.* have used complementary ions containing sodium to determine the charge states in the low resolution MS/MS spectra of oligonucleotides obtained on a triple quadrupole.(198) In these experiments, a singly-charged parent ion was indicated by a mass shift of 22 Da (22/1), doubly-charged by 11 Da (22/2), etc. The magnetic sector-time-of-flight combination has the potential to resolve some of the possibilities based on peak widths.

Figure 3.14 shows the MS/MS spectrum of the $[M\text{-}2H]^{2-}$ ion of 5′-AGGCCT-3′ obtained on the mag-TOF instrument, and Table 3.3 gives the major fragment ions in the spectrum.

Table 3.3. Major Fragment Ions in the MS/MS spectrum of 5′ d(AAGGCT)-3′

Observed m/z	Assignment	Observed m/z	Assignment
895	$[M-2H]^{2-}$	305	nucleotide** T
778	$[w_5-H]^{2-}$	288	nucleotide** C
739	y_5^{2-}	257	$[y_1 + OH-H]^-$
641	$[c_2-2H]^{2-}$	239	$[y_1-2H]^-$
610	w_2^-	195	$[sugar + PO_4H_2]^-$
592	$[a_4-2H]^{2-}$	177	$[sugar + PO_3]^-$
508	$[d_2-B_2(G)- H]^-$	150	$[G]^-$
408	-	134	$[A]^-$
368	-	125	$[T]^-$
346	$[dGp-H]^-$	110	$[C]^-$
329	nucleotide** G	96	$H_2PO_4^-$
321	w_1^-	79	PO_3^-

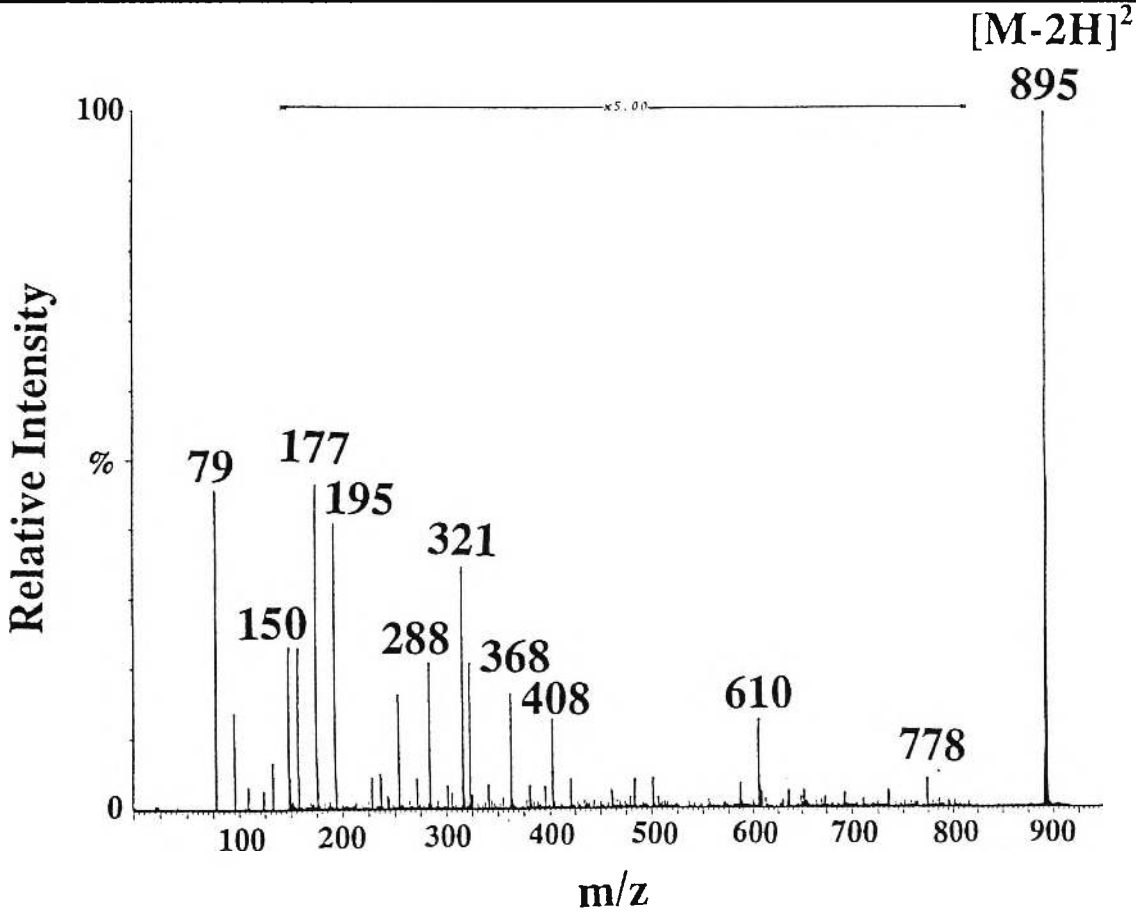


Figure 3.14: ESI-MS/MS spectrum of the $[M-2H]^{2-}$ ion of 5′-d(AGGCCT)-3′ obtained on the magnetic sector-time-of-flight instrument collision cell voltage=200 eV

**nucleotide refers to the base + the sugar + PO_3H

A number of sequence ions are observed in the spectrum which demonstrates that useful MS/MS spectra can be generated under the collision conditions available on that instrument. Hence it should be possible to extend this to a more detailed study of fragmentation of doubly-charged oligonucleotide anions using the ^{13}C-approach for charge state determination.

Figure 3.15 shows the ESI-MS/MS spectrum for the $[M-4H]^{4-}$ ion of the oligonucleotide 5′-d(CGGTAATTACCG)-3′ obtained on the mag-TOF instrument. Table 3.4 displays the assignments of the fragment ions observed in that spectrum.

Table 3.4: Fragment ion assignments for the $[M-4H]^{4-}$ ion of 5′-d(CGGTAATTACCG)-3′

Observed m/z	Assignment	Observed m/z	Assignment
910	$[M-4H]^{4-}$	383	$[a_4-H_2O-2H]^{3-}$
886	$[c_9-B_9(A)]^{3-}$	368	$[w_4-B_9(A)]^{3-}$
882	$[b_3-2H]^-$ or $[y_6]^{2-}$	346	$[w_1]^-$
876	$[z_6]^{2-}$ or $[x_9-B_4(T)]^{3-}$	328	nucleotide** G
872	$[c_6-B_6(A)]^{2-}$	312	nucleotide** A
861	$[a_9-B_9(A)]^{3-}$	303	nucleotide** T
857	$[y_9-B_4(T)]^{3-}$	288	nucleotide** C
794	$[d_8-B_8(T)]^{3-}$ or $[z_8]^{3-}$	230	G + sugar ring (C_5H_7O)
785	$[c_8-B_8(T)]^{3-}$ or $[c_5]^{2-}$	216	A + sugar ring (C_5H_7O)
775	$[x_8-B_5(A)]^{3-}$	201	$[b_2-B2(G)]^{2-}$
719	$[w_7]^{3-}$ or $[a_3-B_3(G)]^-$	195	$[sugar + PO_4H_2]^-$
715	$[c_5-B_5(A)]^{2-}$	177	$[sugar + PO_3]^-$
674	$[a_5-B_5(A)]^{2-}$	159	$[sugar + PO_3 - H_2O]^-$
635	$[w_2]^-$ or $[d_2]^-$	150	$[G]^-$
514	$[w_5]^{3-}$	134	$[A]^-$
506	$[x_5]^{3-}$	125	$[T]^-$
490	$[x_2-B_{11}(C)-H_2O-H]^-$	110	$[C]^-$
481	$[d_3]^{2-}$	97	$H_2PO_4^-$
408	$[d_3-B_3(G)]^{2-}$ or $[w_3-B_{10}(C)]^{2-}$	79	PO_3^-
386	$[y_4]^{3-}$		

A number of ions corresponding to sequence ions were observed. There were no ions observed at m/z values higher than that for the $[M-4H]^{4-}$ molecular ion. The most common series was that of the w ions for which approximately half of the ions were observed. Difficulty was encountered while trying to assign the peaks because the ions

could have been charged singly, doubly or triply. The assignments made here were based on the most logical ions corresponding to those masses, however, ions such as m/z 719 could be assigned to either w_7^{3-} or $(a_3\text{-B3(G)})^-$ for example. These results do show that ESI-MS/MS on the mag-TOF is feasible for the study of quadruply charged parent ions. In addition, the strong preference for loss of adenine is not observed as was also the case for the $[M-2H]^{2-}$ ions of the two 6-mers.

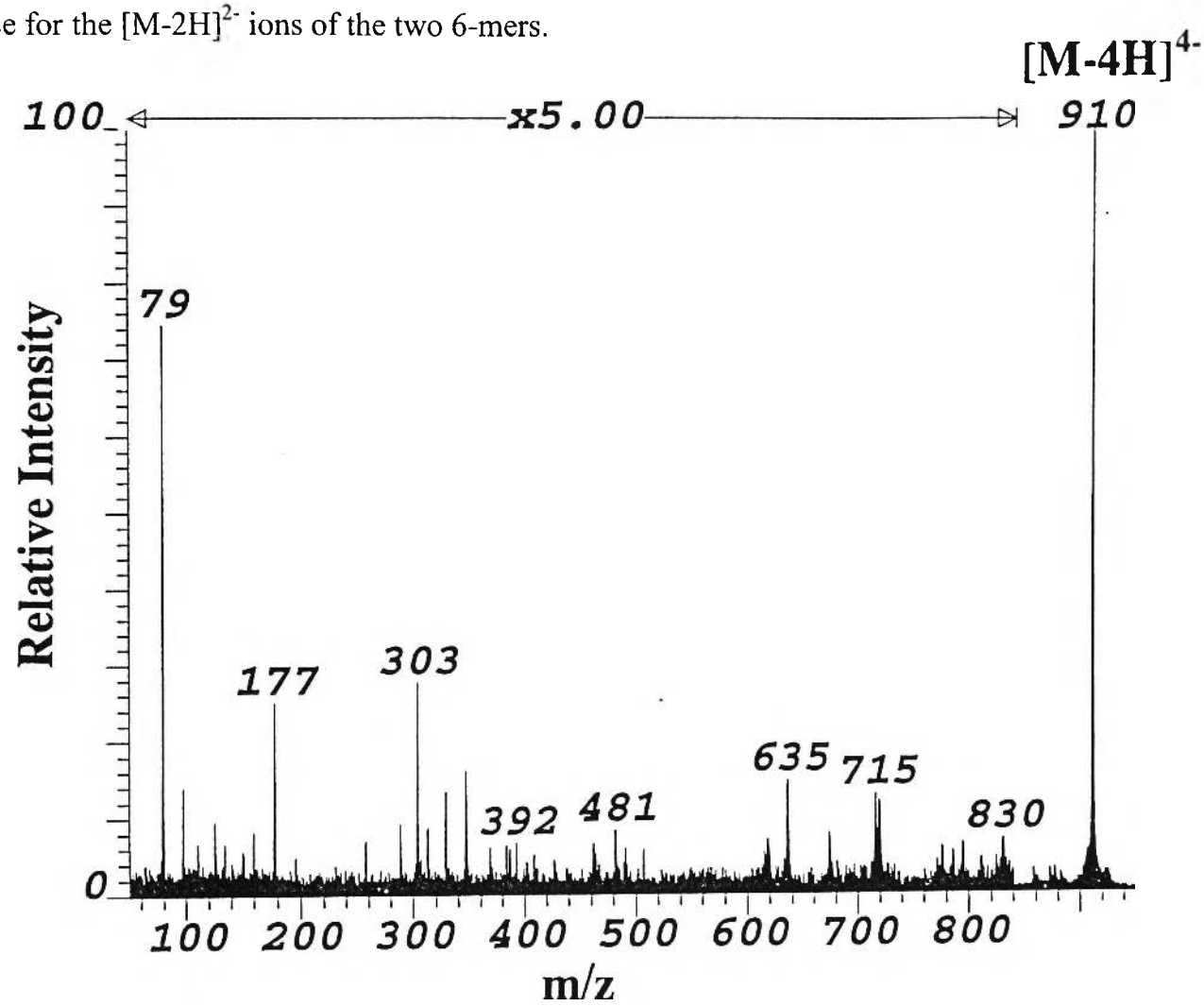


Figure 3.15: ESI-MS/MS spectrum of the $[M-4H]^{4-}$ ion of 5′-d(CGGTAATTACCG)-3′ obtained on the magnetic sector-time-of-flight instrument collision cell voltage=200 eV

3. 5. Summary

In summary, this study has shown that at least for dinucleotides, more extensive fragmentation (including those cleavages which result in sequence ions in large oligonucleotides) is observed using higher skimmer potentials, as well as when examining the MS/MS spectra of $[M+Li]^+$ adducts. The use of Li needs to be explored for larger oligonucleotides. In contrast to previous MS/MS spectra of oligonucleotide anions obtained on an ion trap, a strong preference for loss of adenine is not observed in the MS/MS spectra of hexamers obtained on either a triple quadrupole (E_{lab} = 40 eV) or a magnetic sector time-of-flight instrument (E_{lab}=400 eV). Similarly, the $[M-4H]^{4-}$ ion of the 12 mer did not display a dominant loss of adenine, suggesting that differences in collision energy between the ion trap and scanning instruments accounts for these differences rather than the charge state of the precursor ion. Along with previous studies of tandem mass spectra of oligonucleotide anions, the data presented here shows the potential of MS/MS following electrospray ionisation for determining oligonucleotide sequence, however this work has also highlighted the need for more detailed investigations of the fragmentation of these anions (especially in relation to hydrogen transfer between neutral and charged fragments and fragment ion pairs) before these spectra are as readily interpretable as MS/MS spectra of peptides.

4. Investigation of Pyrindamycin A Binding to DNA.

As noted in the introduction to this thesis (Section 1.5) pyrindamycin A (PyA) is an antitumour agent known to alkylate adenine in DNA. This chapter describes the purification and the analysis of adducts of PyA and the oligonucleotides 5'-CGG TAA TTA CCG-3' and 5'-CGC GAA TTC GCG-3'. These sequences have a number of potential binding sites for pyrindamycin A. 5'-CGG TAA TTA CCG-3' contains two potential binding sites, a high affinity site at A9 and a low affinity site at A6. The 5'-CGC GAA TTC GCG-3 sequence does not contain any of the highly preferred binding sites, however binding to either of the two adenines may still occur. The reaction conditions for PyA and the self complementary oligonucleotides are described in Chapter two (Section 2.2).

4.1. ESI-MS and HPLC of Pyrindamycin A

In order to check the purity of PyA itself, and as a background to the studies on PyA-oligonucleotide mixtures, the free PyA was analysed by ESI-MS in the negative ion mode. Figure 4.1 shows the ESI mass spectrum for PyA. There are two peaks at m/z 506 and m/z 542 which correspond to $[M\text{-}HCl\text{-}H]^-$ and $[M\text{-}H]^-$ respectively, where M represents PyA. There was also a peak at m/z 544 as expected owing to the $[M\text{-}H]^-$ species containing one ^{37}Cl isotope

Free PyA was also analysed by reverse phase HPLC under the same conditions used for purification of the reaction mixtures (see Section 2.1) in order to determine the retention time of PyA. The resulting HPLC profile, shown in Figure 4.2, indicates PyA elutes at 42 mins under these conditions.

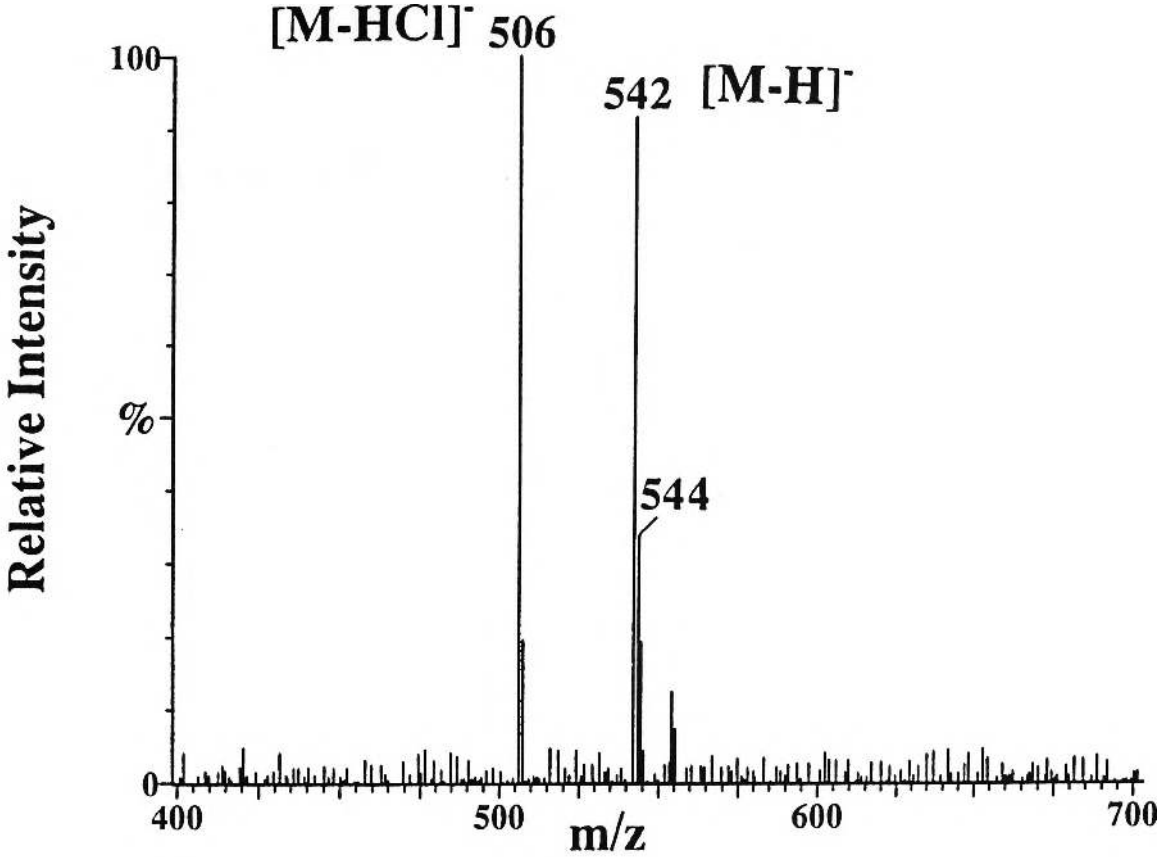


Figure 4.1: ESI mass spectrum of Pyrindamycin A

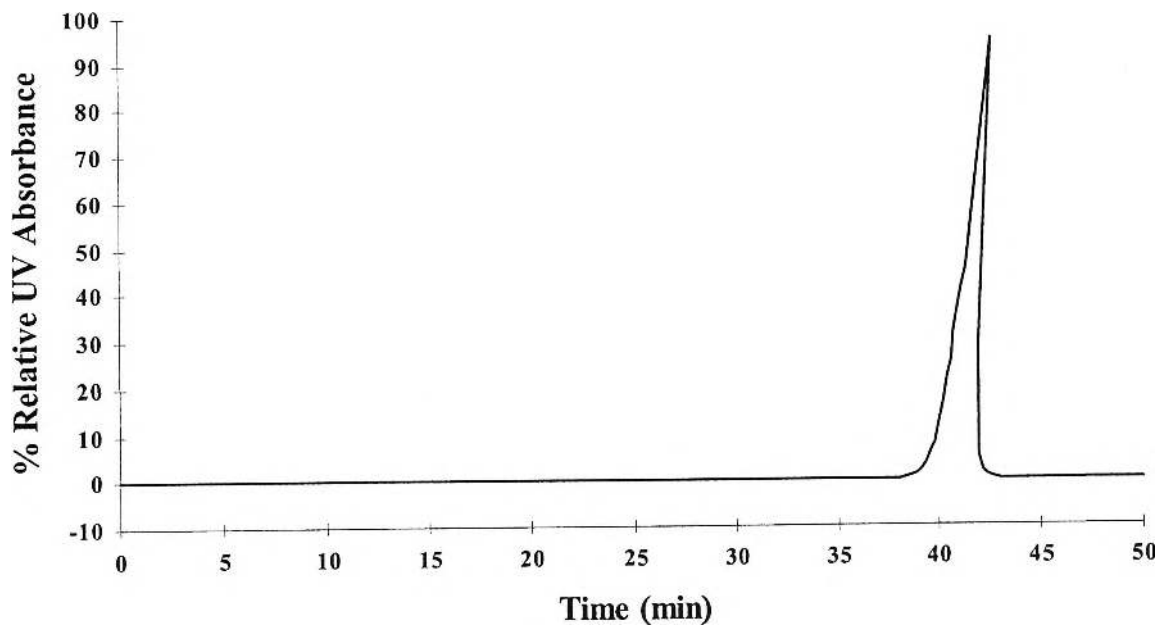


Figure 4.2: HPLC profile of Pyrindamycin A

4. 2. Analysis of the Reaction Between PyA and 5'-CGG TAA TTA CCG-3'

The binding of pyrindamycin A (PyA) to the oligonucleotide 5'-CGG TAA TTA CCG-3' was studied under a wide range of reaction conditions. A HPLC profile of the PyA, 5'-CGG TAA TTA CCG-3' reaction mixture following incubation for 6 days is given in Figure 4.3. The major peaks were collected and analysed by ESI-MS in the negative ion mode. Figure 4.4a shows the ESI mass spectrum for the HPLC peak eluting at 26 mins. This was shown to be the free oligonucleotide (referred to as M) with m/z 606 being $[M-6H]^{6-}$, m/z 728 $[M-5H]^{5-}$ and m/z 910 the $[M-4H]^{4-}$ ion. Figure 4.4b shows the ESI-MS spectrum acquired for the HPLC peak eluting at 28 mins. This spectrum displayed peaks at m/z 701 and m/z 877 corresponding to the $[M-A-5H]^{5-}$ and $[M-A-4H]^{4-}$ molecular ions respectively, for the oligonucleotide loss of adenine (A) species. Also seen in this mass spectrum is a peak at m/z 641. This represents the $[(PyA-HCl) + A]^-$ adduct. That both of these species are observed from a single HPLC peak suggests that these arise from decomposition of the $[M+(PyA-HCl)]^-$ adduct (discussed in more detail in section 4.2.1). Figure 4.4c displays the mass spectrum for the third HPLC peak which eluted at 36 mins. The main peak in this spectrum is m/z 641 which corresponds to the $[(PyA-HCl)+A]^-$ adduct.

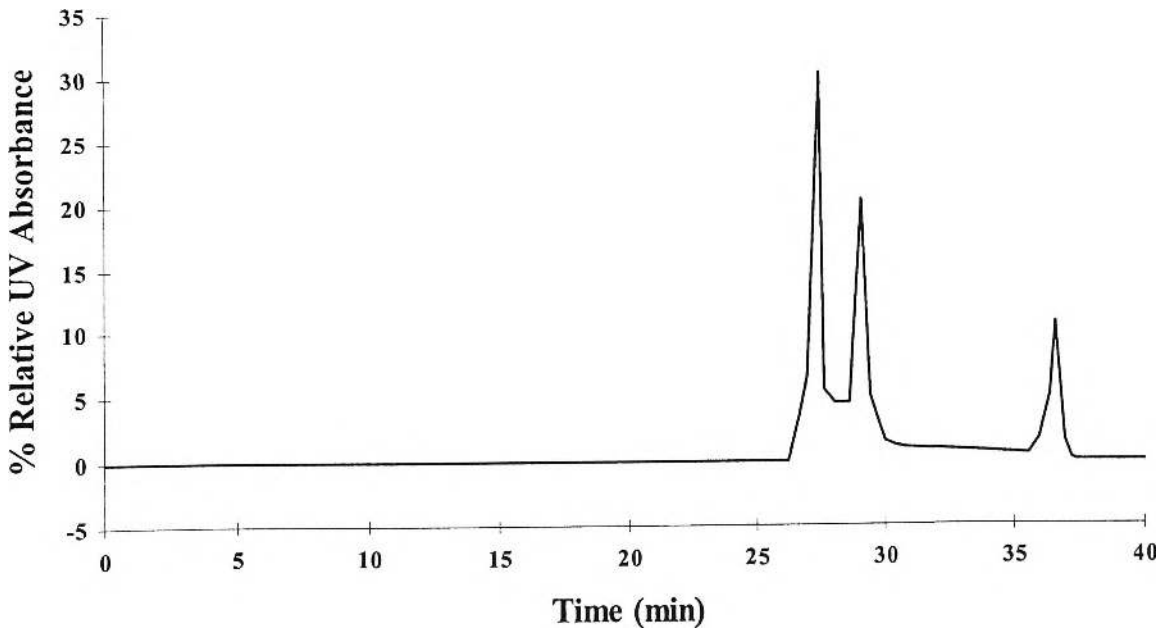


Figure 4.3: Typical HPLC profile for the PyA oligonucleotide reaction

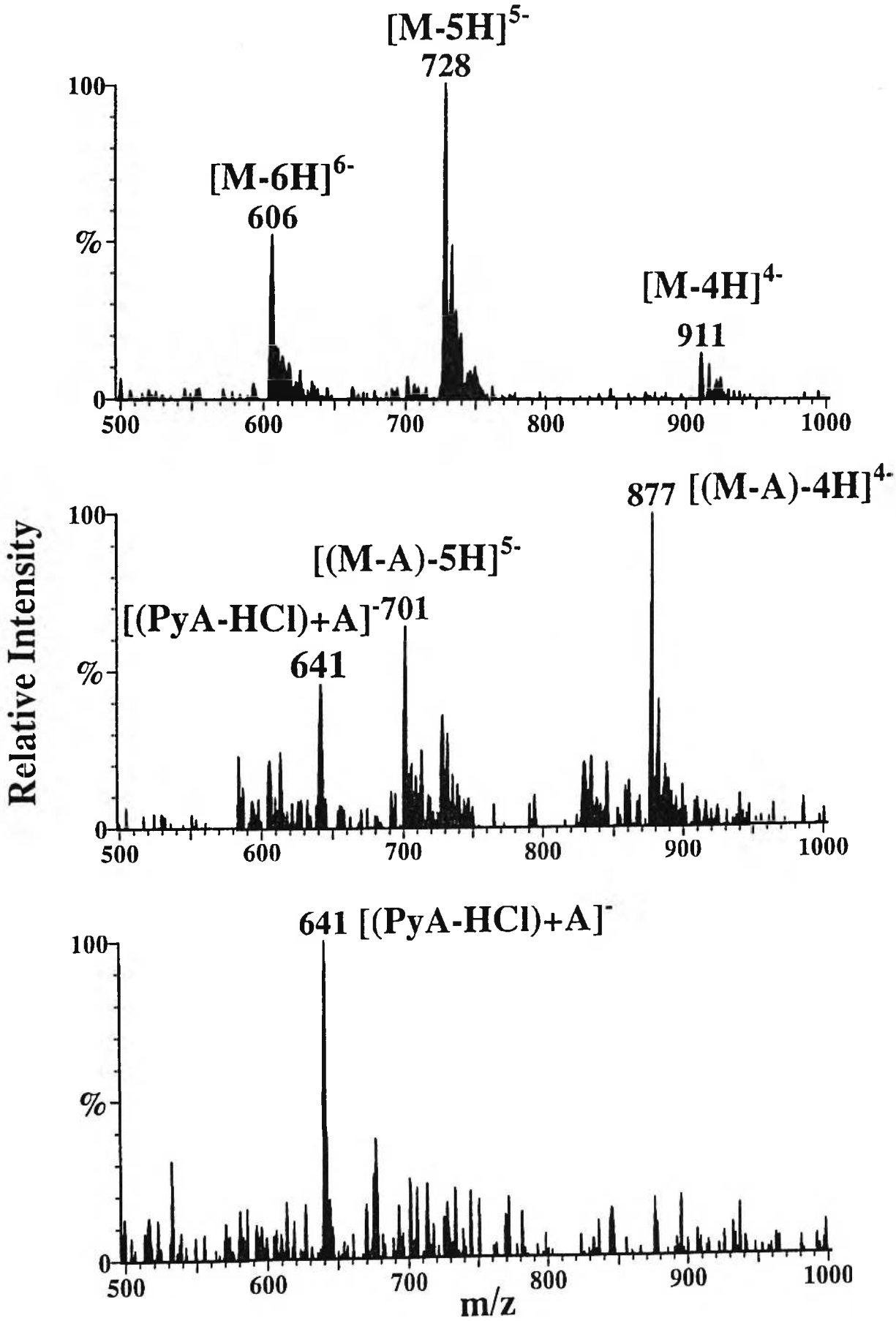


Figure 4.4: ESI mass spectrum for the HPLC fractions obtained at retention times of **a)** 27 mins, **b)** 29 mins, and **c)** 37 mins.

During all of the reactions of PyA with this oligonucleotide, the HPLC profile did not display a peak corresponding to the free ligand. This was thought to be caused by some of the ligand precipitating out of solution during the stirring of the reaction, following loss of the dimethylformamide (DMF) which is necessary to dissolve PyA.

PyA absorbs well in the UV range because of its two benzene and three amino rings. Even though free PyA is not observed in the HPLC and ESI mass spectrum, occasionally hydrolysed PyA ((PyA-HCl) + H_2O) was present. The mass spectrum in Figure 4.5 shows the $[M-H]^-$ ion of hydrolysed PyA at m/z 524 and its dimer at m/z 1050.

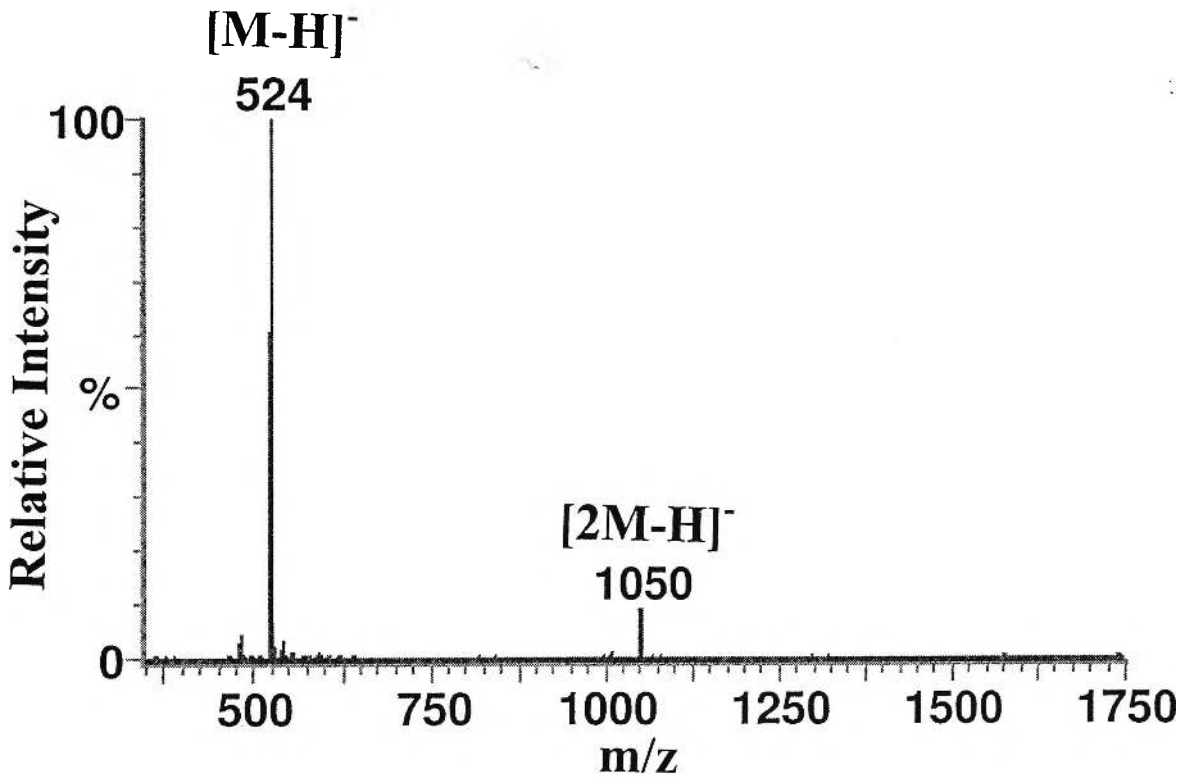


Figure 4.5: ESI mass spectrum of hydrolysed Pyrindamycin A (M=PyA-Cl+H_2O)

4.2.1. Confirmation of Peak Identity

The identity of the $[M-HCl+A-H]^-$ (m/z 641) adduct was confirmed by MS/MS. This spectrum is displayed in Figure 4.6. The main fragments observed were the peaks at m/z 134 and m/z 506 which are known from MS studies to correspond to the base A^- and $[PyA-HCl-H]^-$ ions respectively. At present the identity of the (oligonucleotide-A) species represented by the $[M-5H]^{5-}$ molecular ion (m/z 701) is only based on the molecular mass since this peak was not sufficiently abundant for MS/MS analysis.

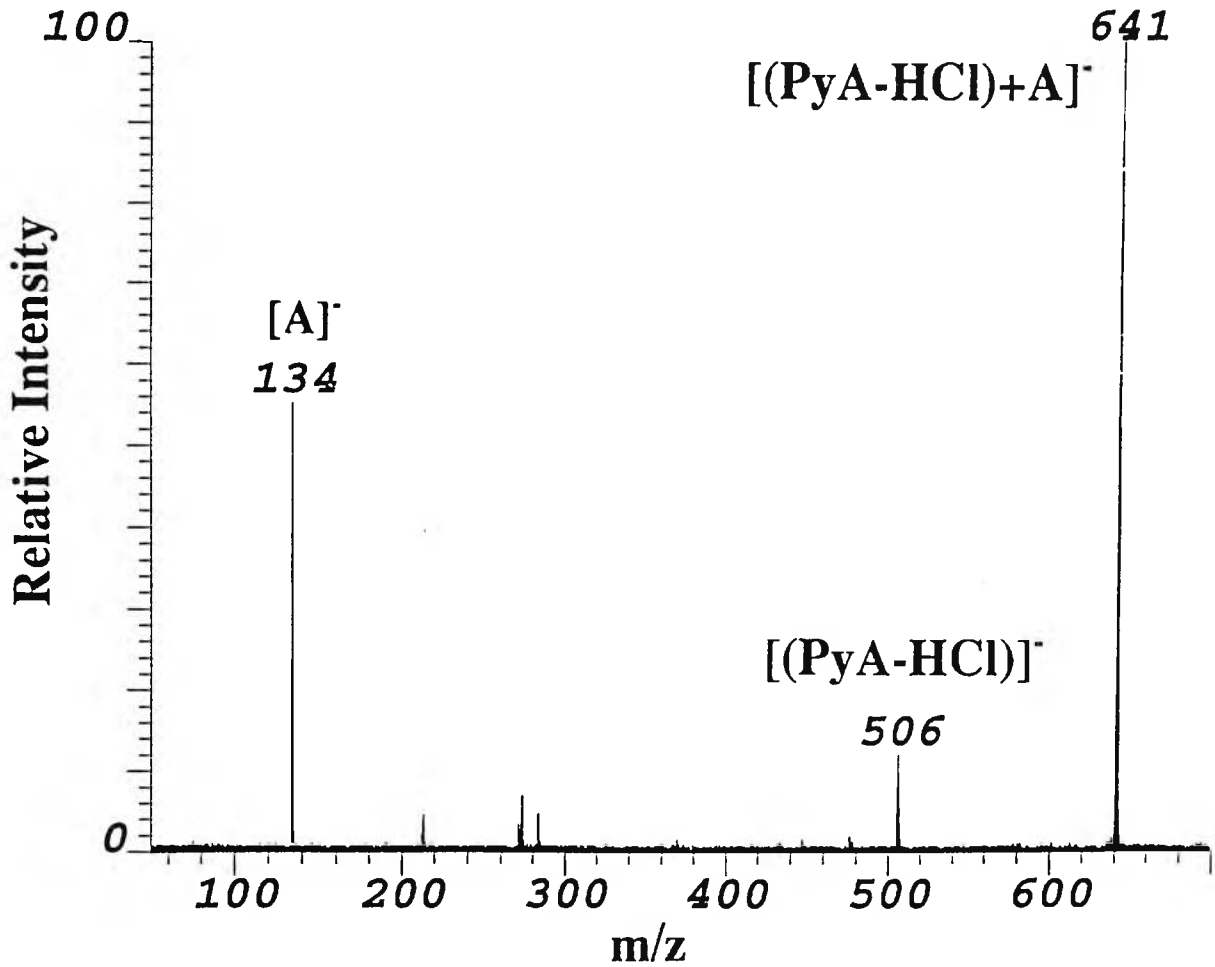


Figure 4.6: MS/MS of [(PyA-HCl)+A]⁻

4. 2. 2. Evidence for Decomposition of the Oligonucleotide-PyA Adduct During ESI-MS

The ESI mass spectrum of the HPLC peak at 28 minutes (Figure 4.4b) did not show evidence of an intact PyA-oligonucleotide adduct. This raised the question of whether such an adduct was sufficiently stable in solution to be detected. Alternatively, if the adduct is present in solution, was it decomposing during sample introduction or ionisation? The latter explanation was thought to be more likely for several reasons.

Firstly, after reacting for approximately 3 days, the third peak eluting at 36 mins is observed and corresponds to the [(PyA-HCl)+A]⁻ adduct alone, suggesting that when this species is present in the original reaction mixture it is well resolved from other species by the HPLC.

Secondly, if decomposition occurred in solution the (oligonucleotide-A) product would contain an additional H_2O which has not been observed. Such products are observed

with reactions between the alkylating agent hedamycin, which alkylates the N7 position of guanine.(200)

The dependence of the (PyA-HCl + oligonucleotide) adduct formation on the reaction conditions was studied using variations to the standard reaction method, in terms of the reaction time, the pH, and the buffer used (which may have affected the final pH of the reaction mixture and hence the stability of the adducts). Table 4.1 provides a summary of the reactions studied. In this table the peak occurring at 28 mins is referred to as the (PyA-HCl + oligonucleotide) adduct, despite the fact that in some cases only the decomposition products are observed.

The table indicates the reaction conditions under which the (PyA + oligonucleotide) adduct, the ((PyA-HCl) + A) adduct, and the (oligonucleotide-A) species were observed. From the data given in the table it is then possible to determine the optimum reaction conditions for the formation for each of the adduct species. The ((PyA-HCl) + A) adduct and the (oligonucleotide-A) species were present under most of the reaction conditions studied. The (PyA + oligonucleotide) adduct was not often seen, presumably because of its dissociation into the other two species.

Table 4.1: Summary of the reactions between pyrindamycin A and the oligonucleotides

BUFFER SOLUTION	pH	TIME	SPECIES OBSERVED		
			PyA+oligo	PyA+A	oligo-A
5'-CGG TAA TTA CCG-3'					
DMF		1,6,8 days *	-	-	-
0.1M Na_2HPO_4		2,3,4 days *	-	-	-
0.1M Na_2HPO_4	~9	½,1,1½,2day *	-	-	-
0.1M Na_2HPO_4	9.01	½ day +			
		1 day	-	X	X
		1½ days	-	X	X
		3 days #			
0.1M NH_4OAc	6.95	½,1,1½,3day *	-	-	-
0.1M NH_4HCO_3	8.06	½ day	-	X	X
		1 day	-	X	X
		1½ days •			
		3 days #			
0.1M Na_2HPO_4	7.52	2 hours	-	-	-
		4 hours	-	-	X
		6 hours	-	X	X
		½ day	-	X	X
		1 day #			
		1½ days		X	X
		2 days	-	X	X
		6 days	X	X	X
0.1M Na_2HPO_4	7.82	3 hours	-	-	-
		5 hours	-	X	X
		7 hours	-	X	X
		½ day	-	X	X
		1 day	-	X	X
		1½ days	-	X	X
		2 days	-	X	X
		6 days	X	X	X
0.1M Na_2HPO_4	7.79	3 days	X	X	X
		7 days	X	X	X
		9 days	X	X	X
		11 days	X	X	X
0.1M Na_2HPO_4	7.96	10 days	v.sml pk	X	X
5'-CGC GAA TTC GCG-3'					
0.1M Na_2HPO_4 Dickerson sequence	8.1	3 days	X	X	X
		7 days	X	-	-
		10 days	X	X	-

X species present as determined by ESI-MS. For more explanation see text

* Only free oligonucleotide observed

+ HPLC had some problems but appears to be the same as for day 1.

• No ESI-MS confirmation but HPLC appears to be the same as for day 1.

No ESI-MS confirmation but HPLC appears to be the same as for day 1½.

Three different buffers, 0.1 M Na_2HPO_4 with pH 9.01, 0.1 M NH_4OAc (pH 6.95), and 0.1 M NH_4HCO_3 (pH 8.06), were also trialed. Reactions of PyA reported in previous studies used 0.1 M Na_2PO_4. The two ammonium buffers were assessed as they were thought to be better for subsequent mass spectrometry analysis, because they do not contain sodium. From Table 4.1 and the HPLC profiles in Figure 4.7, 4.8, and 4.9, it was shown that the best results were obtained for Na_2HPO_4 and NH_4HCO_3. This might be a consequence of the lower buffering capacity of NH_4OAc such that variations in the final pH of the reaction mixture affect the final stability of the adducts. Therefore, to overcome the potential problem of sodium contamination with the Na_2HPO_4 buffer, HPLC purification and on-line desalting on the mass spectrometer were used.

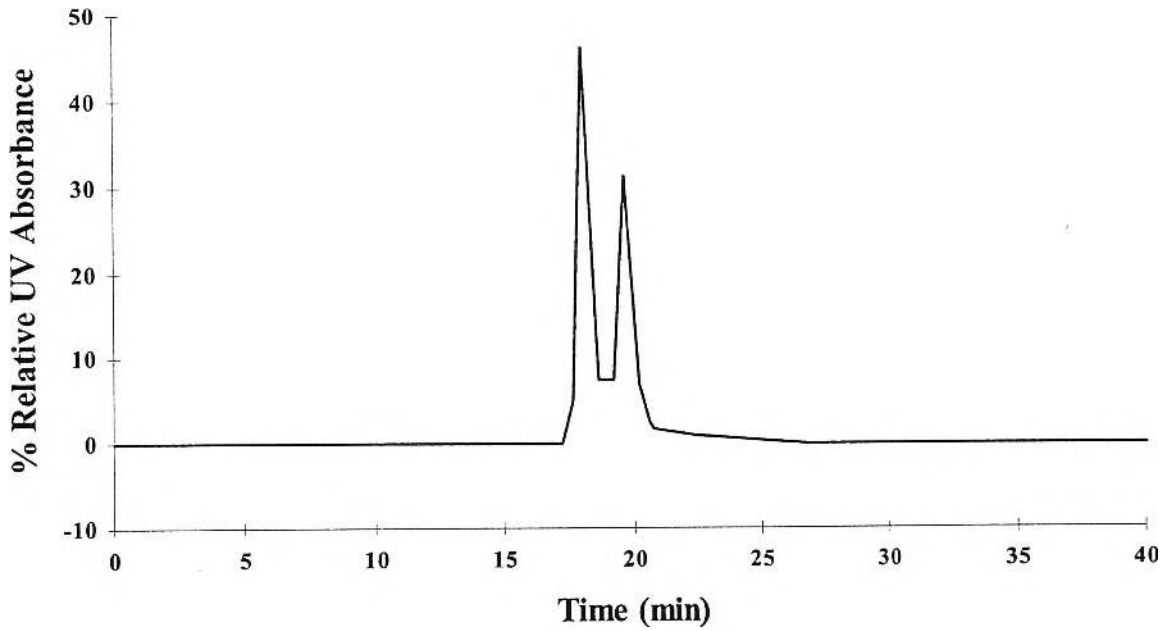


Figure 4.7: HPLC profile obtained using Na_2HPO_4 to buffer the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3', pH 9.01

Note that these and subsequent HPLCS in Fig. 4.7, 4.8 and 4.9 were run using different buffers to that of Fig. 4.3.

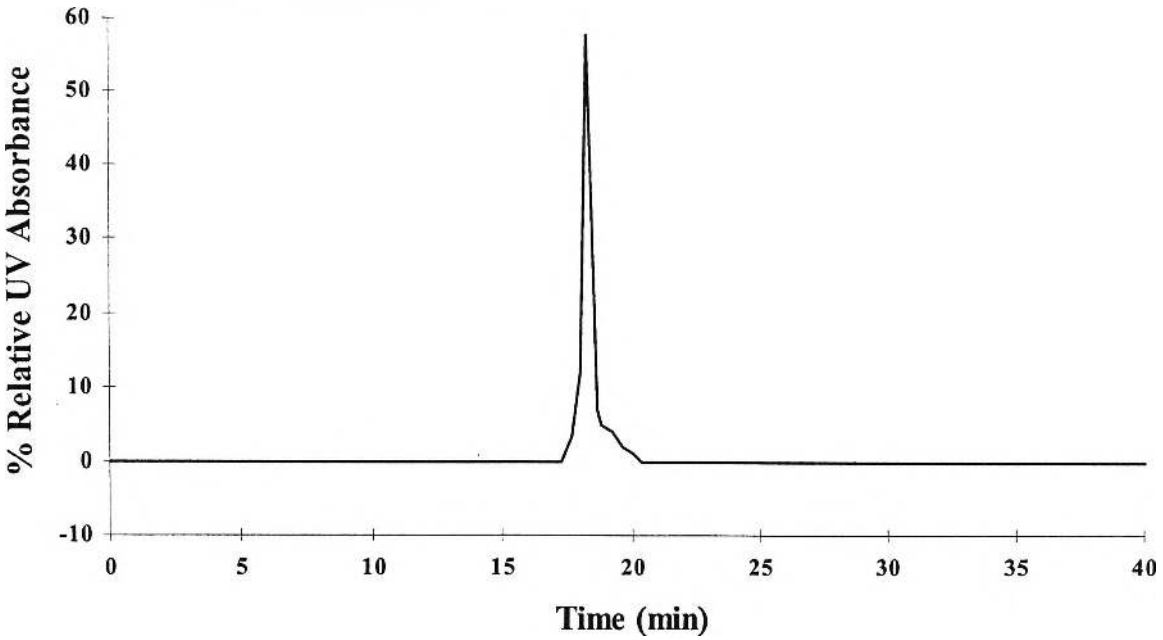


Figure 4.8: HPLC profile obtained using NH_4OAc to buffer the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3', pH 6.95

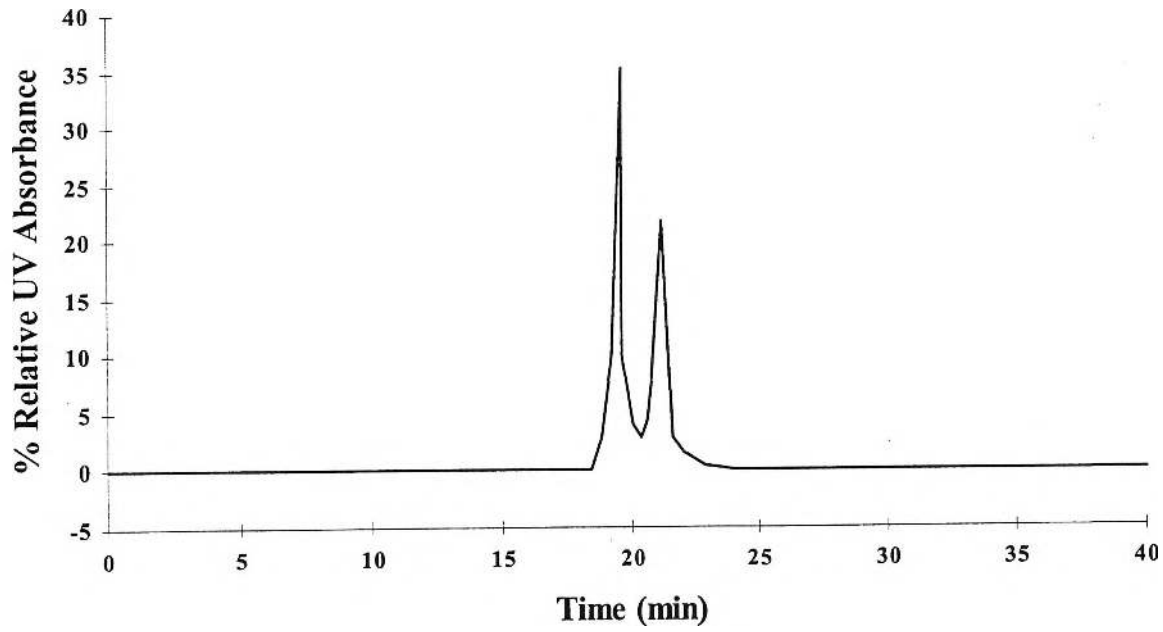


Figure 4.9: HPLC profile obtained using NH_4HCO_3 to buffer the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3', pH 8.06

By varying the reaction time and keeping the buffer (Na_2HPO_4) and the pH (7.80) constant, the number of species observed in the HPLC profile was increased. Figure 4.10 shows the amount of each of the two reaction products, relative to the unreacted oligonucleotide over a period of time, from the HPLC profile. Note that this is only an arbitrary measure since the UV extinction coefficients for the oligonucleotide and adduct will be different.

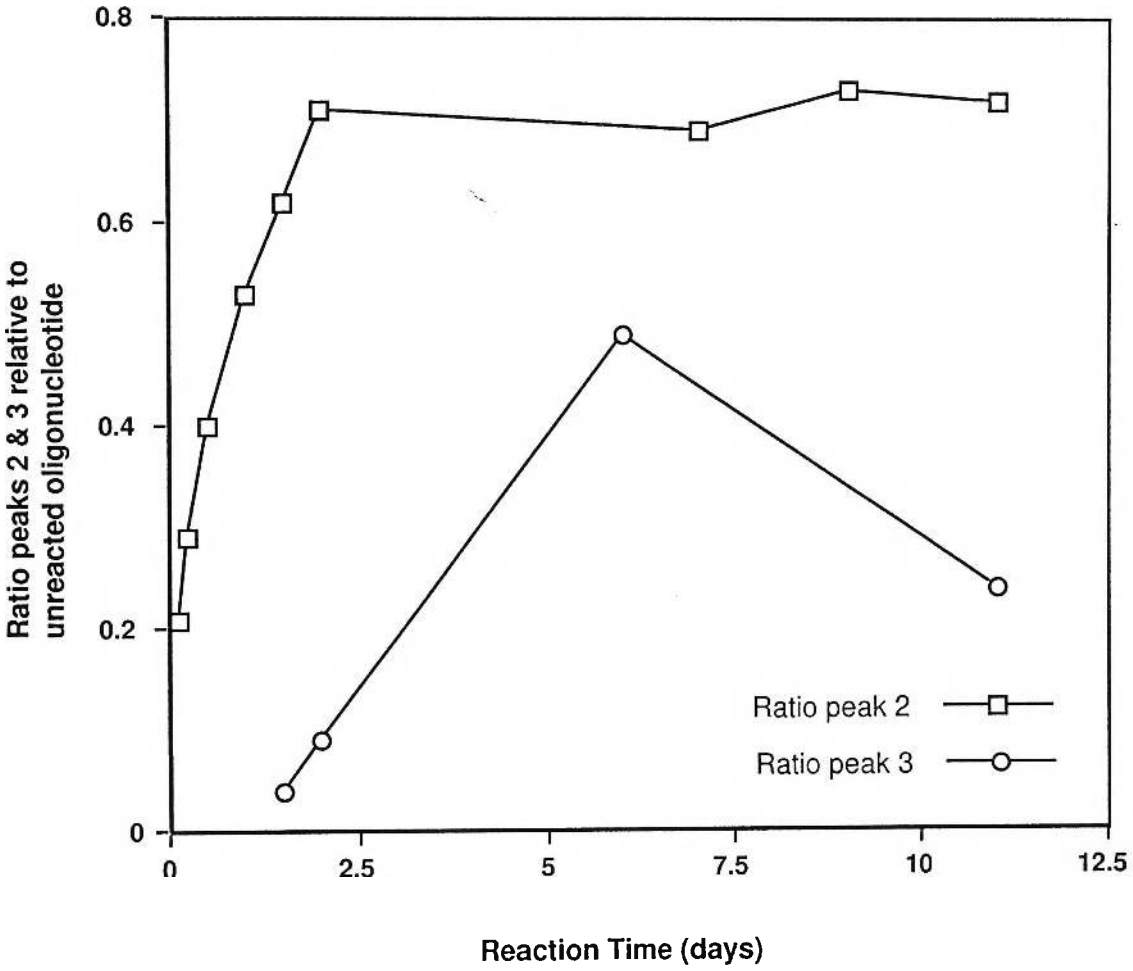


Figure 4.10: Ratio of the relative peak heights of (oligonucleotide+(PyA-Cl)) (peak 2) and ((PyA-Cl)+A) (peak 3) to unreacted oligonucleotide with time

From the data in this graph it was concluded that the (oligonucleotide + PyA) adduct (the 2nd peak in the HPLC profile) reaches its maximum intensity after 2 days and stays at relatively the same value after that. The third peak owing to primarily the ((PyA-Cl) + base A) appears after 2 days but was not present in sufficient concentrations

until day 6 to be analysed by ESI-MS. This peak was never observed to be greater than the second HPLC peak.

Table 4.2 shows the adducts observed for each reaction buffered by Na_2HPO_4. From the data shown in this table it can be observed that when only two peaks are present (one being the free oligonucleotide) only ions corresponding to the (oligonucleotide-base A) and ((PyA-Cl)+A) species are observed in the ESI mass spectrum for the second peak. As time progresses, on average after about 6 days, a third peak is observed. This peak contained ((PyA-Cl)+A) (641 m/z) and the second peak contained some (oligonucleotide + (PyA-Cl)) and (oligonucleotide-A) (701 m/z). The optimum reaction time for observing the (oligonucleotide + (PyA-Cl)) adduct is after 9 days. However, to observe the other two species 1½ -2 days is sufficient.

Table 4.2: Presence of Adducts using the Na_2HPO_4 buffer

pH	time	Presence of Adduct		
		PyA + oligo	PyA –Cl + A	oligonucleotide - A
9.01	1 day	-	19 mins	unresolved from PyA+A
	1½ days	-	18 mins	unresolved from PyA+A
7.52	4 hours	-	-	27 mins
	6 hours	-	25 mins	unresolved from PyA+A
	½ day	-	26 mins	unresolved from PyA+A
	1 day	-	28 mins	unresolved from PyA+A
	1½ days	-	27 mins	unresolved from PyA+A
	6 days	in 29 min peak	37 mins	29 mins
7.82	5 hours	-	26.5 mins	unresolved from PyA+A
	7 hours	-	26 mins	unresolved from PyA+A
	½ day	-	28 mins	unresolved from PyA+A
	1 day	-	25 mins	unresolved from PyA+A
	1½ days	-	27 mins	unresolved fromPyA+A
	2 days	-	29 mins	unresolved from PyA+A
	6 days	in 28 min peak	36 mins	28 mins
7.79	3 days	17 mins	all species	unresolved
	7 days	12 mins	all species	unresolved
	9 days	17.5 mins	all species	unresolved
	11 days	20 mins	in 20 min peak	32 mins
7.96	10 days	17.5 mins	18.5 mins vsml	17.5 mins v.sml
		21.5 mins	21.5 mins	18.5 mins
8.1 Dickerson sequence used	3 days	21 mins	25 mins	in 21 mins peak v.sml
		23 mins	28 mins	in 25 mins peak v.sml
		also25&28mins		
	7 days	36 mins	-	-
	(run for 1hr)	55 mins	-	-
	10 days	24 mins		
		in 35 mins peak	35 mins	

Figure 4.11, 4.12, and 4.13 show the effect of pH on the HPLC profile when the reaction time was kept constant at 1½ days. The reactions were performed at pH 7.52, 7.82, and 9.01 respectively. Of the three different reaction pH's studied, pH 9.01 was observed to produce the greatest ratio of free oligonucleotide to (oligonucleotide + (PyA - Cl)). This may result from the higher pH increasing the rate of the reaction, due to the phenolic hydroxyl being first converted into a C=O group in order for the alkylation of adenine to take place. This conversion is favoured at high pH. ESI-MS of these HPLC peaks does not show the (oligonucleotide + (PyA - Cl)) adduct presumably because it is decomposed to ((PyA-HCl) + A) and (oligonucleotide - A) during the ionisation process.

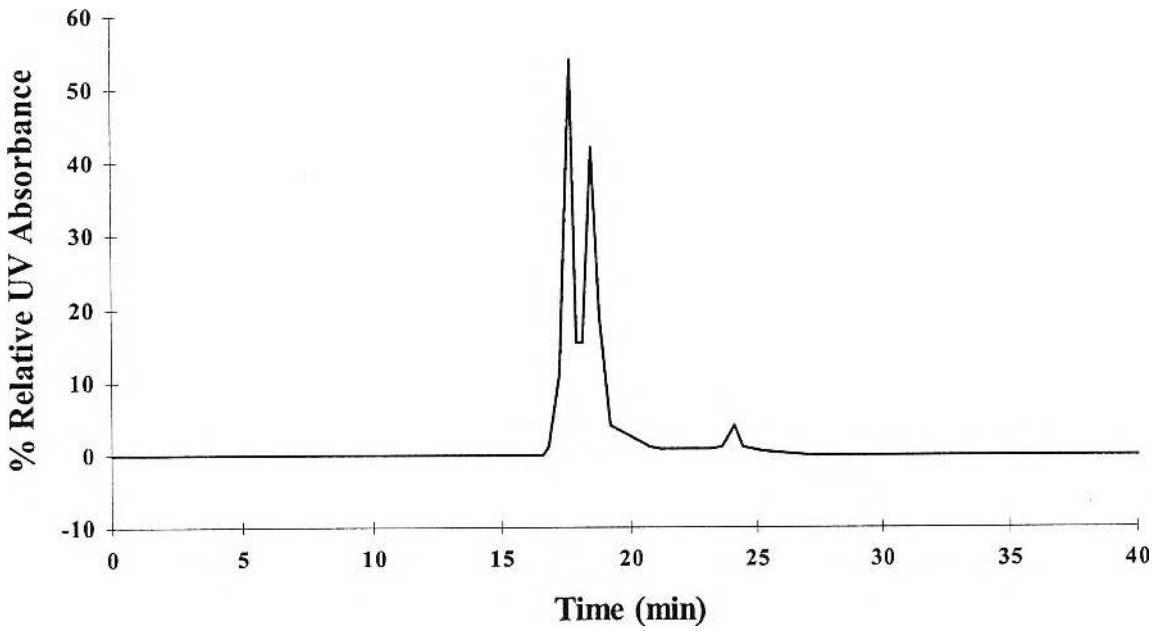


Figure 4.11: HPLC profile for the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3' performed at pH 7.52

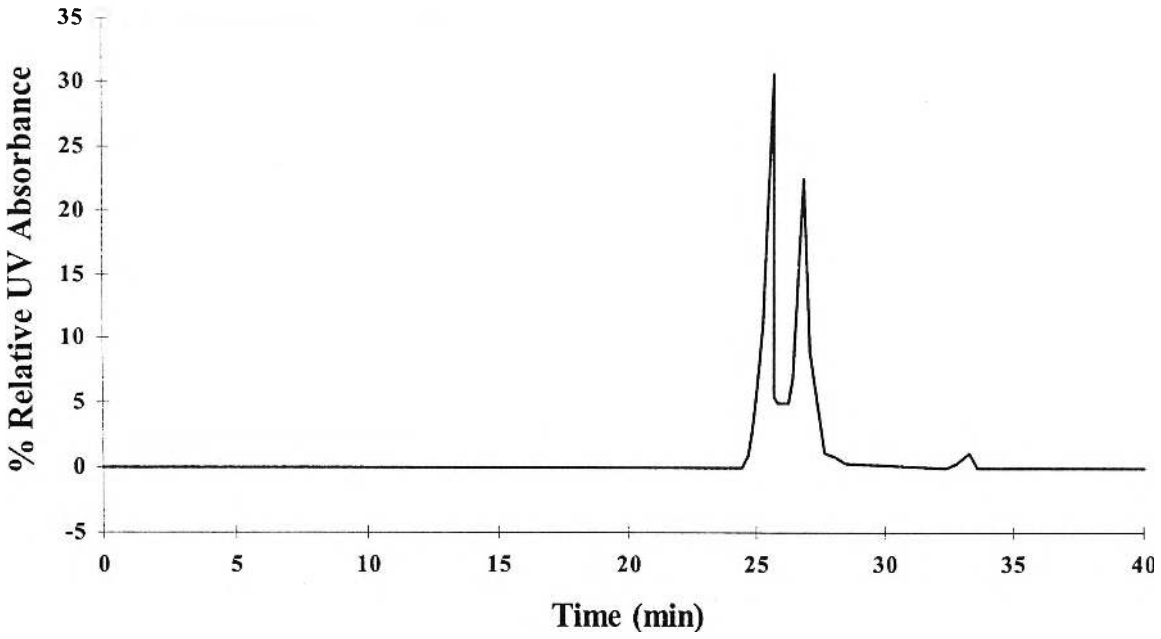


Figure 4.12: HPLC profile for the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3'performed at pH 7.82

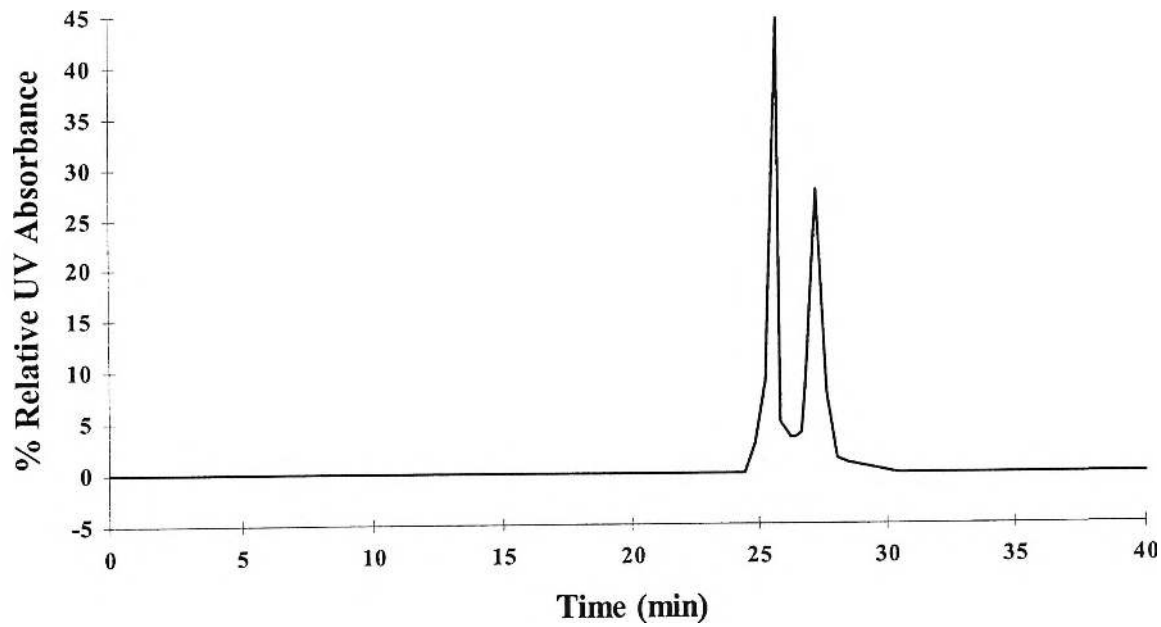


Figure 4.13: HPLC profile for the reaction between PyA and the oligonucleotide 5'-CGG TAA TTA CCG-3' performed at pH 9.01

4. 2. 3. Optimisation of HPLC Purification

In order to determine if the PyA + oligonucleotide adduct was being formed but not resolved from the free oligonucleotide, the gradient used for the HPLC purification was varied. The usual gradient was a linear run from 0 to 100% of 60% aqueous acetonitrile in 0.1 M NH_4OAc over 40 mins. This gave rise to the HPLC profile shown in Figure 4.14. When the gradient was run slowly to approximately 45% of the total time and then fast for the remainder of time, the HPLC profile shown in Figure 4.15 resulted. From these two profiles it can be seen that a slightly better separation is obtained for the nonlinear gradient. This difference was not, however, sufficient to warrant its use for all of the subsequent reactions.

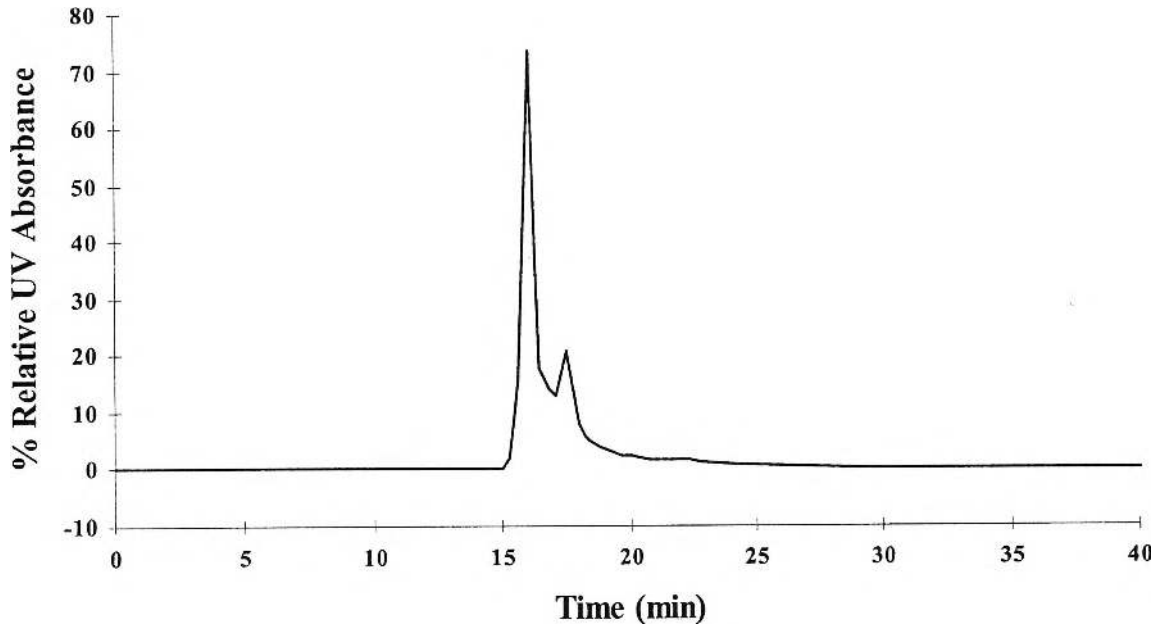


Figure 4.14: HPLC profile using the linear gradient

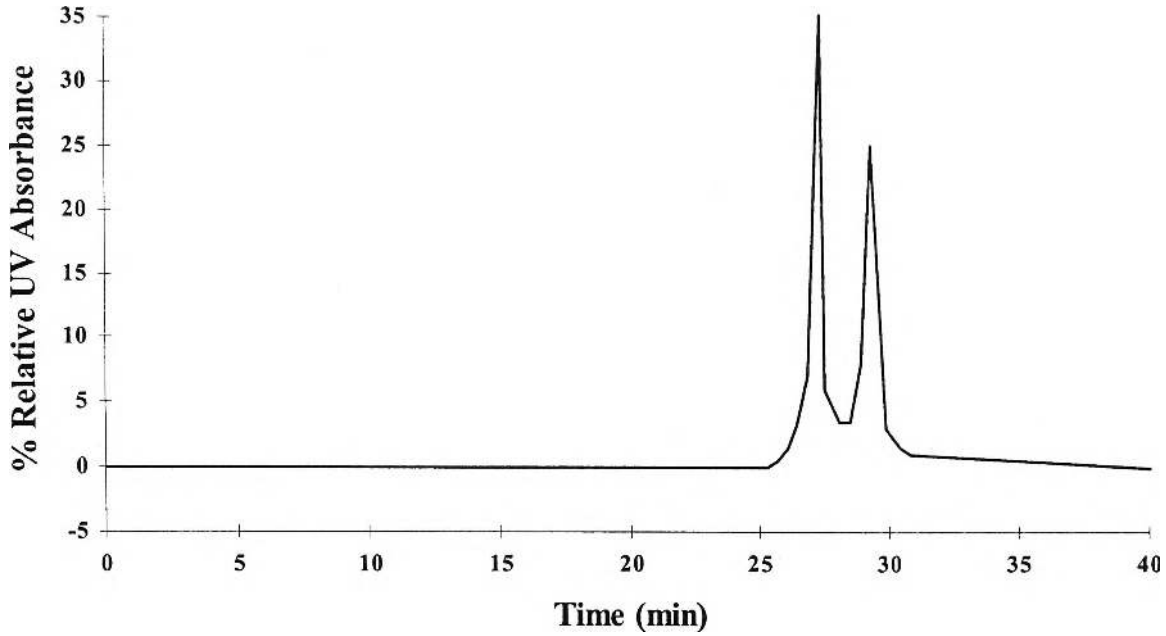


Figure 4.15: HPLC profile using the non-linear gradient

4. 2. 4. Summary

In summary, from the ESI mass spectra and the HPLC profiles it can be concluded that the best conditions for the observation by ESI-MS of the (oligonucleotide + (PyA-Cl)) adduct were 11 days at pH 7.8. The best conditions for observation of the ((PyA-HCl)+A) adduct were 10 days with pH 7.96, and for the (oligonucleotide - A) species were 3 days and pH 7.79. As only one HPLC fraction contained species relating to the alkylated oligonucleotide, alkylation presumably only occurred at one site indicating that the alkylation is very specific for this sequence. The most likely position for this alkylation was at A9 as this is a high affinity alkyaltion site (refer Section 1.5.1), however, further MS/MS experiments to confirm this were limited by the lability of the adduct.

4.3. Analysis of the Reaction Between PyA and 5'CGCGAATTCGCG-3'

The pyrindamycin A reaction with the oligonucleotide sequence known as the Dickerson sequence, 5'-CGC GAA TTC GCG-3', was also examined. Figure 4.16 shows the HPLC profile for this reaction after 9 days. From the ESI mass spectra for this reaction and the data given in Table 4.1, the (oligonucleotide + (PyA - Cl)) adduct is more readily apparent by ESI-MS than the previous sequence. From the HPLC profile in Figure 4.16 four main peaks were observed. ESI-MS confirmed that the peak eluting at 16 mins is free oligonucleotide. The peaks eluting at 18 and 24 mins both contain the decomposition products of (oligonucleotide + (PyA - Cl)), (oligonucleotide - A) and ((PyA-HCl) + A), and some of the intact adduct. The presence of the two peaks containing the (oligonucleotide + (PyA - Cl)) adduct are evidence for alkylation at more than one site in the DNA sequence. Thus the adducts have different retention times owing to different steric influences but the molecular mass is the same. This might be expected because the Dickerson sequence does not contain adenine in any of the preferred binding sites, so alkylation is less specific. The final peak eluting at 35 mins is due to the hydrolysed form of PyA. The ESI mass spectra shows a peak at m/z 525 for the molecular ion of ((PyA-Cl)+H_2O) and a dimer is also observed at m/z 1050 (as was the case in Figure 4.5). A peak was also observed at a retention time of 29mins, however it was not present in a high enough concentration for mass spectral analysis.

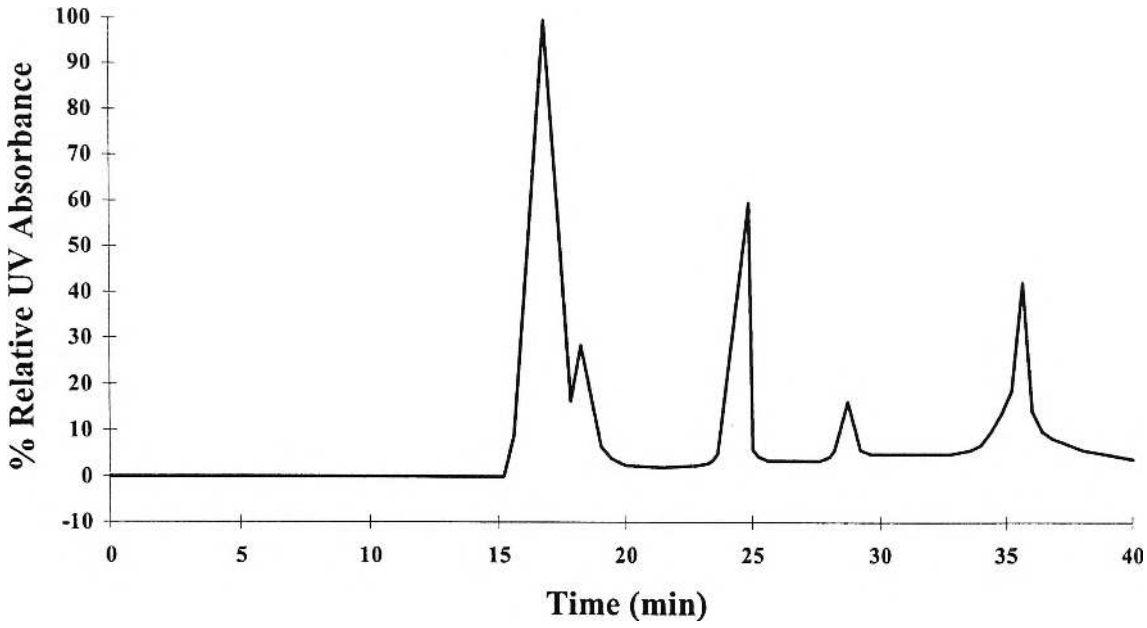


Figure 4.16: HPLC profile for the reaction of PyA with the 5′-CGC GAA TTC GCG-3′ oligonucleotide

5. Conclusion

This study has shown that for dinucleotides, more extensive fragmentation (including those cleavages which result in sequence ions in large oligonucleotides) is observed using higher skimmer potentials, or when examining MS/MS spectra of $[M+Li]^+$ adducts. The $[M+Na]^+$ and $[M+K]^+$ adducts were also investigated by ESI-MS/MS. These did not exhibit significantly increased fragmentation compared with $[M+H]^+$ adducts. No fragmentation of the $[M+K]^+$ ions were observed, and MS/MS of the $[M+Na]^+$ ion only produced a slight increase in fragmentation compared to the $[M+H]^+$ ion when very harsh collision conditions were used.

The $[M+H]^+$ spectra of dinucleotides were generally dominated by the BH_2^+ ion of the 3′ terminal base except when that base was thymine. In this case, no BH_2^+ ion was observed, presumably because thymine has a very low proton affinity. The ESI-MS/MS spectra of $[M-H]^-$ ions of the dinucleotides were dominated by the B^- ion of the 5′ base. No exceptions to this trend were observed. This preferred loss of the 5′ base to the 3′ base in negative ions becomes less significant for larger oligonucleotides so it cannot be used for these molecules to assign the 5′ base.

In contrast to previous MS/MS spectra of oligonucleotide anions obtained on an ion trap, a strong preference for the loss of adenine is not observed in the MS/MS spectra of hexamers, or for the 12 mer, obtained on either a triple quadrupole (E_{lab}=40 eV) or a magnetic sector time-of-flight instrument (E_{lab}=400 eV). The MS/MS spectra for the 12 mer provides a great deal of sequence information owing to cleavage of the oligonucleotide backbone. The w series is predominant for this sequence with approximately half of this series being observed. The whole sequence may not be able to be determined from the fragmentation, however confirmation of most of the sequence can be achieved. This is true also for the two hexamers for which the w series predominates but again only half to two thirds of the ions in the series were observed.

Along with previous studies of tandem mass spectra of oligonucleotide anions, the data presented here shows the potential of MS/MS following electrospray ionisation for

determining oligonucleotide sequence, however this work has also highlighted the need for more detailed investigations of the fragmentation of these anions (especially in relation to hydrogen transfer between neutral and charged fragments and fragment ion pairs) before these spectra are as readily interpretable as MS/MS spectra of peptides.

The reaction of the alkylating agent pyrindamycin A (PyA) with the oligonucleotides 5′-CGGTAATTACCG-3′ and 5′-CGCGAATTCGCG-3′ was studied by reverse phase HPLC followed by electrospray ionisation mass spectrometry (ESI-MS). The intact $[M+(PyA-HCl)]^-$ adduct (where M is the oligonucleotide) was not often observed by ESI-MS but was observed as two decomposition products, the $[(PyA-HCl)+A]^-$ adduct and the $[oligonucleotide-AH]^-$ species. This was assumed to be because this adduct is particularly labile and thereby decomposes during ionisation. From the ESI mass spectral data and the HPLC profiles, it can be concluded that the best conditions for the observation of the $[M+(PyA-HCl)]^-$ adduct was after 11 days at pH 8.0. As only one HPLC fraction contained the species relating to the alkylated 5′-CGGTAATTACCG-3′ oligonucleotide, alkylation presumably only occurred at one site, most likely ATTA, indicating that the alkylation is very specific for this sequence. ESI-MS/MS confirmed that m/z 641 was the $[(PyA-HCl)+A]^-$ adduct, as fragments were observed for the adenine m/z 134 and the $[PyA-HCl]^-$ m/z 506. The other decomposition product the $[M-AH]^-$ species has only at this stage been postulated on the basis of molecular weight.

The HPLC profile for the 5′-CGCGAATTCGCG-3′ reaction with pyrindamycin A was shown to be nonspecific, as two HPLC peaks were observed for the adduct. This might be expected as this sequence does not contain any preferential binding sites.

The data presented here for the interaction of the antitumour agent pyrindamycin A with the oligonucleotides 5′-CGGTAATTACCG-3′ and 5′-CGCGAATTCGCG-3′ shows that ESI-MS is feasible for characterising the resulting adducts. Initial studies that were performed utilising ESI-MS/MS to obtain structural information of the adducts appears to be very promising, however the stability of the resulting adducts can be a limiting factor in some cases.

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