CHEMICAL DERIVATIZATION FOR LIQUID CHROMATOGRAPHY AND
MASS SPECTROMETRY OF NUCLEOSIDES AND THEIR ANALOGS

by

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A Thesis
Submitted to the School of Graduate Studies in
Partial Fulfillment of the Requirement for the
Degree
Master of Science

McMaster University
April, 1981
CHEMICAL DERIVATIZATION, LIQUID CHROMATOGRAPHY
AND MASS SPECTROMETRY OF NUCLEOSIDES
To Charlotte
Master of Science (1981) McMaster University
(Chemistry) Hamilton, Ontario

TITLE: Chemical Derivatization for Liquid Chromatography and Mass Spectrometry of Nucleosides and their Analogs.

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NUMBER OF PAGES: x, 78
ABSTRACT

Currently, nucleosides and their analogs are routinely analysed by reverse phase (RP) or ion exchange (IEX) high performance liquid chromatography (HPLC) with UV detection. These methods require the use of buffer solutions which are incompatible with the newly introduced method of combined liquid chromatography-mass spectrometry (LC-MS). The LC-MS interface is not capable of eliminating the inviolate salts in the buffers. Accumulation of these salts would cause arcing in the ion source. In addition, the nucleosides are polar and their analysis by this method, without chemical modification, would lead to the mass spectra of pyrolysis products. In this thesis a method involving chemical derivatization and combined LC-MS of the nucleosides and their analogs is discussed.

N,N'-methylene-bridged dinucleosides were derivatized with tert-butyl-dimethylsilyl (TBDMS) reagent, purified and analysed by liquid chromatography and combined LC-MS. Mixed derivatization of the nucleosides to produce the N-dimethylaminomethylene-O-tertbutyldimethylsilyl (DMAM-TBDMS) derivatives and optimization of conditions are examined. The liquid chromatographic and combined LC-MS analyses of these derivatives are discussed. The electron-impact mass spectral fragmentation and rearrangement mechanisms of the TBDMS derivatives of the dinucleosides and the DMAM-TBDMS derivatives of the nucleosides are discussed.
ACKNOWLEDGEMENTS

I would like to express my sincere thanks and gratitude to Dr. M.A. Quilliam for the supervision and advice he provided in the course of this research. I would also like to thank him for enabling me to attend the 1979 and 1980 Anlab Expositions.

I would like to thank Dr. Francois Messier, Mr. Joe Boison and other colleagues for the many valuable discussions and encouragement, and Mr. F.A. Ramelan for the help he gave me in mass spectrometry.

My sincere thanks go to McMaster University for the financial assistance, in the form of research scholarship and teaching assistantship, it provided me.

Finally, I would like to thank Ms. Linda Palmer for typing this thesis.
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ABBREVIATIONS

A
Adenosine

C
Cytidine

dA
Deoxyadenosine

dC
Deoxycytidine

dG
Deoxyguanosine

DMAM
Dimethylaminomethylene

DMAM-TBDMS
Dimethylaminomethylene-O-tertbutyldimethylsilyl

DMF-DMA
Dimethylformamide-dimethylacetal

DNA
Deoxyribonucleic acid

dU
Deoxyuridine

FID
Flame ionization detector

G
Guanosine

GC
Gas Chromatography

GC-MS
Gas Chromatography-Mass spectrometry

HPLC
High Performance Liquid Chromatography

LC
Liquid Chromatography

LC-MS
Liquid Chromatography-Mass spectrometry

MS
Mass spectrometry

TBDMS
Tertbutyldimethylsilyl

TBDMSCl
Tertbutyldimethylchlorosilane

TFA
Trifluoroacetyl

TLC
Thin-layer chromatography

TMS
Trimethylsilyl

U
Uridine
CHAPTER ONE

1. INTRODUCTION

1.1. OBJECTIVE

The objective of the research project is to develop an alternative analytical method for nucleosides and their analogs utilizing the techniques of chemical derivatization, liquid chromatography (LC) and mass spectrometry (MS).

1.2. BACKGROUND

1.2.1. Nucleosides

Nucleosides play a very important role in biological systems. As nucleotides they form the basic units of nucleic acids, the genetic apparatus of all living organisms. They are also present in energy transfer systems in living cells.

There are two types of nucleosides found in nucleic acids: Deoxyribonucleic acids (DNA) contain the deoxyribonucleosides while the ribonucleic acids (RNA) contain the ribonucleosides. One other difference between DNA and RNA is that the nucleoside thymidine (T) in DNA is replaced by uridine (U) in RNA. The structures of the nucleosides are given in Scheme 1.

Also present in nature are modified nucleosides, many of which occur as antibiotics (1) and in transfer RNA's. Numerous nucleoside analogs have also been synthesized for in vivo and in vitro studies (2-4). One example of these analogs includes the homologous series containing the N, N'-methylene-bridged dinucleosides. The structures of these dinucleosides are given in Scheme 1.
Scheme I: Structures of Nucleosides Studied

\( R_1 = R_2 = H, \text{DEOXYURIDINE, dU (I)} \)
\( R_1 = H, R_2 = \text{OH, URIDINE, U (II)} \)
\( R_1 = \text{Me}, R_2 = H, \text{THYMIDINE, T (III)} \)
\( R_1 = \text{Me}, R_2 = \text{OH, 5-METHYLURIDINE, } m^5\text{U (IV)} \)

\( R_2 = \text{OH, CYTIDINE, C (V)} \)
\( R_2 = H, \text{DEOXYCYTIDINE, dC (VI)} \)

\( R_2 = \text{OH, ADENOSINE, A (VII)} \)
\( R_2 = H, \text{DEOXYADENOSINE, dA (VIII)} \)

\( R_2 = \text{OH, GUANOSINE, G (IX)} \)
\( R_2 = H, \text{DEOXYGUANOSINE, dG (X)} \)
$R_1=R_2=H$, $n=1$, $dU(CH_2)dU$ (XI)

$R_1=R_2=Me$, $n=1$, $T(CH_2)T$ (XIII)

$R_1=R_2=Me$, $n=6$, $T(CH_2)_6T$ (XV).

Scheme 1b: Structures of Nucleoside Analogs Studied.
1.2.2. Analytical Methods for Nucleosides

1.2.2.1. General Techniques

The nucleic acid components (bases, nucleosides, and nucleotides) and their analogs have been routinely analyzed using paper chromatography, electrophoresis (5-8), thin-layer chromatography (TLC) (9-11), and classical column chromatography including ion exchange and ion exclusion (12-15). Detection of the compounds may be based on UV absorption of the purine and pyrimidine bases, on colorimetric methods and on labelling with radionuclides.

Despite the availability of these established classical methods, modern instrumental methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC) are highly desirable since they can provide fast, high resolution separations and qualitative as well as quantitative information. They can also provide high sensitivity for trace analysis and a degree of automation.

1.2.2.2. Gas Chromatography

GC, being a gas phase analytical method, requires that compounds in a sample be volatile and thermally stable. Its application to nucleosides and nucleotides is, therefore, limited by the multifunctional nature of these compounds and their consequent polarity, low volatility and thermal lability. Conversion to chemical derivatives is essential for the successful GC analysis of these compounds. A number of these have been examined, the best studied being acetyl (16), alkyl (16,18,19), trifluoroacetyl (17), and trimethylsilyl (TMS) (20-24) derivatives. The most successful derivatization method has been trimethylsilylation. Most of the TMS derivatives are suitable for quantitative GC and are also the preferred derivatives for mass spectrometry.
Unfortunately, TMS derivatives are very easily hydrolysed and therefore are not easily stored and cannot be subjected to liquid chromatography. Derivatives based on other silyl groups have also been examined. The more sterically crowded tert-butyldimethylsilyl (TBDMS) derivatives have been shown to be more stable to hydrolysis and to have very favourable electron-impact mass spectral behavior (25-27), including abundant \((M-57)^+\) ions corresponding to elimination of the tert-butyl radical and a structurally informative fragmentation - directing capability. Two disadvantages of silyl derivatives in general (TMS and TBDMS) are that the more polar cytosine and guanine nucleosides are not easily chromatographed by GC, and that there is incomplete formation of N-silyl derivatives leading to two products for nucleosides containing the primary amino function.

1.2.2.3. High Performance Liquid Chromatography

In recent years HPLC has become a powerful tool for nucleic acid analysis. It has been used either in the reverse phase mode (28-32) or the ion exchange mode (33, 34).

The technique of RP-HPLC involves a non-polar stationary phase such as a hydrocarbon, chemically-bonded to silica support and a polar mobile phase (e.g., water, methanol, acetonitrile, or mixtures thereof). Buffers are usually added to the mobile phase to suppress the ionization of nucleosides. The method is very useful for the separation of compounds that belong to an homologous series.

HPLC is found to be very suitable for quantitative analysis of the nucleosides. The compounds may be analysed without any chemical modification and they are easily detected through their strong absorption in the near-UV region.
1.2.2.4. Mass Spectrometry

Mass spectrometry, like chromatography, has proved to be a very important method in nucleic acid chemistry. The application of the method in this field has rapidly expanded since 1962 when Biemann and McCloskey (35) first obtained the mass spectra of free bases and nucleosides. McCloskey (36) has reviewed the application of mass spectrometry in the field of nucleic acid chemistry.

There are some major difficulties in the application of mass spectrometry to underivatized nucleosides in general. Their low volatility requires that the sample be introduced via the solid probe inlet system. The problems of low volatility and thermal decomposition are most severe in compounds such as the guanine nucleosides. Extensive pyrolysis occurs in these compounds resulting in irreproducible and sometimes confusing mass spectra.

Newer methods of ionization, such as chemical ionization (37-40), field desorption (41,42), field ionization (43), laser desorption (44), fission-fragment induced desorption (45,46), and secondary ion MS (47), can extend mass spectrometry to the more labile nucleosides. These "soft" ionization techniques offer less pyrolysis, more abundant molecular ions (or quasi-molecular ions) and less extensive fragmentation. Unfortunately, the simplified spectra do not usually give as much structural information as do electron-impact spectra.

Chemical derivatization prior to mass spectrometry offers a solution to many of the above problems. Chemical derivatives can enhance volatility and thermal stability and allow the use of GC as an alternative means of sample introduction. Derivatization procedures that have been investigated include alkylation (19), acetylation (48), trifluoroacetylation (17), isopropylation and boronation (49) for cis-diols of ribosides, and silylation (20-28). As in GC, trimethyl-
silylation has been the most valuable method. Several of these derivatives have been examined for combined gas chromatography-mass spectrometry (GC-MS) (17, 19, 28).

### 1.2.2.5. Combined Liquid Chromatography-Mass Spectrometry

The relatively new technique of liquid chromatography-mass spectrometry (LC-MS) offers to be a powerful alternative method to GC-MS. Two types of this technique have been investigated, the first of which is a belt transport system. In this type of LC-MS technique, a moving belt system transports the LC effluent through a series of vacuum locks where the solvent is evaporated and the sample is introduced into the ion source for analysis by electron-impact ionization or chemical ionization. The second type involves direct introduction system, usually utilizing chemical ionization with the mobile phase as the reagent gas.

Although the LC-MS technique is still in its infancy, it has been used for the analysis of some polar organic compounds including some underivatized nucleosides (50). As was pointed out earlier in the chapter, many of the HPLC analyses of these polar compounds have been performed on reverse phase and/or ion exchange columns using polar solvents and buffer solutions. Such mobile phases are not compatible with the LC-MS belt transport system which is capable of handling only volatile solvents. Non-volatile buffer salts would of course accumulate in the LC-MS interface leading to arcing of the ion source high voltage. It would, therefore, be advantageous to be able to use non-polar solvents as the chromatographic eluent for separation of the compounds before they reach the interface for transport to the mass spectrometer's ion source. Chemical derivatization should offer a solution to this problem, since it will make the compounds non-polar and therefore allow the use of non-polar solvents for their chromatographic separation.
1.3. RESEARCH PROPOSAL

Nucleic acids and their constituents form an important area of biochemical analysis and for that matter there has been a constant search for better methods for their analysis. Although GC-MS has proved to be very useful in this area, some of the derivatives are not amenable to the technique while others provide unsatisfactory mass spectra.

The objective of this project is to investigate derivatization as a means of improving the compatibility of HPLC and mass spectrometry for combined HPLC-MS analysis of nucleosides. Chemical derivatization of nucleosides to low polarity derivatives should allow the use of non-polar, volatile organic chromatographic mobile phases that could be easily removed in the LC-MS interface. Also it should increase the volatility and thermal stability of the nucleosides for their eventual volatilization into the mass spectrometer.

The project will involve an examination of various derivatization methods, the optimization of reagent systems and conditions for simple, rapid, and quantitative preparations, the evaluation of chromatographic behavior, the confirmation of structure with mass spectrometry and the interpretation of the electron-impact induced fragmentations and rearrangements. The derivatization methods to be investigated will include silylation, alkylation, and acylation. The TBDMS derivatives are anticipated to have the following advantages: (a) they are easily prepared and are suitable for a variety of functional groups; (b) they have better mass spectral properties than other derivatives; and (c) they possess some of the valuable features of both classical and non-classical derivatives: hydrolytic and thermal stability, volatility, and good behavior in liquid chromatography.
However, earlier investigations have shown that primary amino functions are not quantitatively silylated, resulting in the formation of a mixture of N, O-persilyl and O-persilyl derivatives. To overcome this problem, the approach illustrated in Scheme 2 is proposed. This involves a mixed derivatization method. The primary amino group will be reacted with dimethylformamide/dimethylacetel (DMF) followed by O-silylation with Corey's reagent (52) to form the N-dimethylaminomethylene-O-tert-butyldimethylsilyl (DMAM-TBDMS) derivatives.

The proposed method will compete with existing methods for the analysis of nucleosides and, if it is to be accepted, it must satisfy certain criteria: (a) the reaction time must be short to allow the use of the method for routine analysis of large numbers of samples in a given day; (b) the work-up must be simple; (c) the derivatization should be quantitative, resulting in a single derivative for each nucleoside; (d) the derivatives must show good liquid chromatographic behavior and be well separated; (e) their polarities must be low enough to allow the use of volatile solvents in their HPLC analysis; (f) the derivatives must be volatile and thermally stable for their volatilization into the mass spectrometer; and (g) they must have favorable electron-impact mass spectral behavior, including abundant molecular ion or a fragment indicative of the molecular ion.

The method will be evaluated on the basis of the above-mentioned criteria.
CHAPTER TWO

2. EXPERIMENTAL

2.1. REAGENTS

Dimethylformamide-dimethylacetal (DMF-DMA), trifluoroacetic anhydride (TFAA), and silylation grade pyridine, acetonitrile, and dimethylformamide (DMF) were purchased from Pierce Chemical Co., Rockford, Ill. Reagent grade DMF (Caledon, Georgetown, Ontario) was distilled under vacuum (bp 40°, 100 torr) and stored over molecular sieve 5A. Tert-butyl(dimethyl)chlorosilane (TBDMSCl) and imidazole were purchased from Sigma, St. Louis, Mo. Analytical grade ammonium dihydrogen phosphate and ammonium acetate were purchased from British Drug House, Poole, England.

Nucleosides were purchased from Sigma. The N,N'-methylene-bridged dinucleosides were obtained from Dr. K.K. Ogilvie (McGill University, Montreal, P.Q.)

HPLC grade methanol, acetonitrile, chloroform, methylene chloride, hexane, ethyl acetate, and isopropanol were purchased from Caledon, Georgetown, Ontario. Distilled water was treated in a Millipore Milli-Q water purification system (one ion exchange, two carbon and a 0.2 μm filters).

2.2. PREPARATION OF THE TBDMS REAGENT

The TBDMS reagent was prepared in dry septum-capped 3-mL screw-cap vials. The reagent contained 1M TBDMSCl and 2M imidazole in dry DMF. It was kept under nitrogen atmosphere to prevent exposure to atmospheric moisture.
2.3. PREPARATION OF DERIVATIVES

2.3.1. General

Derivatizations were performed in dry, teflon-lined septum-capped vials. The vials could be inserted into controlled temperature aluminum blocks for reactions that required temperatures other than ambient. One hundred µg to 1 mg of substrate was weighed into the vials and reagents were added so that the resultant substrate concentration was 0.2 to 1 mg/mL with a reagent to substrate molar ratio of about 40 to 100.

2.3.2. TBDMS Derivatives of Dinucleosides

The TBDMS silylation reactions were carried out at 80°C for 1 hr., or allowed to stand at room temperature, with stirring, overnight to ensure complete reaction of all hydroxyl groups on the sugar moiety.

Sephadex LH-20 (Pharmacia Fine Chemicals, Upsalla, Sweden) column clean-up of the silyl derivatives was performed by the method of Kelly and Taylor (57). Mini columns (6.5 x 0.5 cm) were prepared with a slurry of Sephadex LH-20 in hexane/ethyl acetate (3:1, v/v). Up to 300 µL of the reaction mixture was applied to the column and eluted with 3:1 hexane/ethyl acetate. The first 1 mL of the eluent was collected and subsequently evaporated to dryness under a nitrogen stream. The sample was dissolved in acetonitrile or methylene chloride for further analysis.

2.3.3. DMAM-TBDMS Derivatives of Nucleosides

The N-dimethylaminomethylene (DMAM) derivatives of adenosine, guanosine, and cytidine were prepared at ambient temperature by allowing substrate and reagent in dry DMF to stand for 2.5 hours. Methylation of secondary amino functions in the nucleoside bases does not take place under these conditions. Excess reagent and solvent were then evaporated under a stream of nitrogen.
TBDMS silylation and final clean-up proceeded as in the previous section.

2.3.4. Trifluoroacetyl Derivatives of Nucleosides

The trifluoroacetyl derivatives of the nucleosides were prepared by the method of Koenig and coworkers (17). Approximately 100 mg of nucleoside was weighed into a dry teflon-lined septum screw-capped 3-mL vial, followed by the addition of 100 mL of methylene chloride and 7 mL of trifluoroacetic anhydride. The mixture was heated at 100°C for 30 minutes except for deoxyguanosine which was heated at 150°C for 5 minutes. The reaction mixtures were evaporated to dryness under a nitrogen stream. Acetonitrile or methylene chloride was added to give 1 mg/mL solutions for analysis.

2.4. GAS CHROMATOGRAPHY

Gas chromatography was performed on a Varian model 3700 gas chromatograph equipped with an off-column flash injector at 250°C and a flame ionization detector (FID) at 300°C. The column oven temperature was programmed from 150° to 280°C at 10°/min. The column (glass, 0.5 m x 2 mm I.D.) was packed with 1% SP 2100 (methyl silicone) on 100/120 mesh Supelcoport (Supelco, Bellefonte, Pa.). The carrier gas was nitrogen at a flow rate of 20 mL/min.

2.5. THIN-LAYER CHROMATOGRAPHY

The derivatization reactions were monitored by thin-layer chromatography performed on a 2 x 10 cm plastic plates coated with silica gel 1B2-F (J.T. Baker Chem. Co., Phillipsburg, N.J.). The reaction mixtures were applied directly with capillary tube and dried with a nitrogen stream. The development solvent for the DMAM nucleoside derivatives was chloroform/methanol (10:3, v/v) (58), and methylene chloride/methanol (98:2, v/v) for the DMAM-TBDMS derivatives.
Preparative separations by TLC were performed with 7 x 15 cm glass plates coated with a 250 μm layer of silica gel GF (Applied Science, State College, Penn). The plates were activated at 100°C for 1 hour prior to use. Anhydrous ether containing 1% ethanol was used as the developing solvent for the TBDMS derivatives of the nucleosides. The bands were visualized with UV lamp, marked and scrapped into sintered glass funnels. The product was extracted from the gel with ether and the ether subsequently evaporated to dryness under a nitrogen stream. The compounds were dissolved in acetonitrile if LC analysis followed.

2.6. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC analyses were performed on Spectra Physics model 8000 liquid chromatograph equipped with a model 770 variable wavelength UV absorption detector. Reverse phase chromatography was performed using a Spectra Physics RP-8 (chemically-bonded octysilane) column (4.6 mm I.D. x 250 mm), and normal phase partition chromatography, with a Whatman Partisil PXS-PAC (chemically bonded mixed amino-and cyano-propylsilane) column (4.6 mm I.D. x 250 mm). Effluents were sometimes collected for probe mass spectrometry; in the case of the underivatized compounds, they were isolated and derivatized prior to mass spectrometry.

2.7. MASS SPECTROMETRY

Electron impact mass spectra of the nucleoside derivatives were obtained with the VG 7070 mass spectrometer (VG Micromass, Altrincham, UK.) Samples were introduced via a direct insertion probe system, through a GC column via a jet separator, or via an LC-MS interface. The ion source temperature was 200°C.
The spectra were obtained at an accelerating voltage of 4 KV and electron energy of 70eV with emission of 100 to 200 μA. In the case of the dinucleoside derivatives, the accelerating voltage was 2 KV. The spectra were acquired and processed with the VG 2035 data system.

2.8. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Combined liquid chromatography-mass spectrometry was performed with an LC-MS interface from VG Micromass attached to the VG 7070 mass spectrometer. The effluent from the liquid chromatograph was passed through a splitting tee (the splitting ratio of which was adjustable by a needle valve), through the UV absorption detector, and to the interface via a 50 cm length of micobore (0.25 mm I.D.) stainless steel tubing. The tip of this tubing was sharpened to a point and positioned about 2 mm above the LC-MS Kapton belt. The VG LC-MS interface is shown in figure 1.

The dinucleoside derivatives were analysed with the RP-8 column using 100% acetonitrile at 0.9 mL/min. The DMAM-TBDM nucleoside derivatives were analysed with the PAC column using 1% methanol in methylene chloride at 0.8 mL/min. The interface belt speed was set at 1 cm/sec with the solvent and sample evaporators at 6.50 and 8.0 settings, respectively. The ion source housing pressure readings varied between 2 X 10^-6 and 7 X 10^-6 torr. The interface housing pressure was set at -0.6 bar by bleeding in helium via the auxiliary pumping line. This prevented sputtering of the mobile phase as it entered the interface housing.
Fig. 1: VG Micromass Liquid Chromatography-Mass Spectrometry Interface.
CHAPTER THREE

3. DEVELOPMENT OF ANALYTICAL METHODS

3.1a N,N'-METHYLENE-BRIDGED DINUCLEOSIDES

The N,N'-methylene-bridged dinucleosides were chosen as model compounds for derivatization and LC-MS for several reasons. Firstly, these compounds require derivatization for electron-impact mass spectrometry otherwise they provide only the mass spectra of pyrolysis products. Secondly, only hydroxyl groups are available for derivatization, thus making the derivatization reaction quite straightforward. Thirdly, the derivatives will have high molecular weights, which should pose a challenge to the new LC-MS interface. Finally, the methods developed for these compounds could be useful in any future analytical work associated with their use in the study of nucleic acid interactions and in cancer chemotherapy.

3.1.1. Underivatized Dinucleosides

In order to evaluate any new methods developed, it was decided that conventional HPLC and mass spectral analyses would be performed for comparison. Davis and coworkers (31) have used phosphate buffer (pH 5.07, 10 mM)/methanol (94:6, v/v) as mobile phase for the reverse phase (RP) HPLC analysis of nucleosides. For the dinucleosides, we found that it was necessary to use a gradient elution with an increasing methanol concentration in the mobile phase in order to achieve good separation and a reasonable analysis time (about 15 minutes). The increasing chain length of the methylene bridge increases the retention of the compounds on a reverse phase column due to their increasing lipophilicity. Figure 2 shows the resulting high performance liquid chromatogram of the five dinucleosides. While this is an excellent separation for the routine quantitative analysis of these compounds, it is not suitable for combined LC-MS due to
Figure 2: High performance liquid chromatogram of underivatized dinucleosides (1) 5U(CH₂)₄U (XI), (2) 5U(CH₂)₄T (XII), (3) T(CH₂)₄T (XIII), (4) T(CH₂)₂T (XIV), (5) T(CH₂)₆T (XV).

Conditions: Octyl silane, 4.6 mm I.D. x 250 mm column; 2mL/min phosphate buffer (10mM, pH 5.10)/methanol as mobile phase (methanol gradient from 10 to 70% at 2% min.); UV detection at 254 nm.
the polarity and low volatility of the compounds and the presence of the phosphate buffer.

3.1.2. HPLC-Collection-Derivatization-Mass Spectrometry.

In order to gain any qualitative information on these compounds by mass spectrometry after they had been separated by the above procedure, it was necessary to convert them to derivatives because it was found that they decomposed thermally in the direct insertion probe to give a mass spectrum of their pyrolysis products. The hexamethylene-bridged dinucleoside (XV) fraction was collected from the HPLC and evaporated under vacuum. The residue was extracted with three 5mL portions of acetone. The acetone was evaporated under nitrogen stream and the extract was trimethylsilylated with 100 µl BSTFA. The TMS derivative gave a mass spectrum with an observable molecular ion at m/z 854. Although the method works, it is time-consuming; each fraction has to be evaporated and extracted before derivatization or else the water, methanol and phosphate in the eluent interfere with the derivatization.

3.1.3. TBDMS Derivatives

Derivatization of the dinucleosides prior to HPLC analysis with the TBDMS reagent according to published procedures (25-27, 56) produced the TBDMS derivatives as shown in Scheme 3 for the hexamethylene-bridged dinucleoside. A mixture of the five TBDMS derivatives was analysed by reverse phase and normal phase HPLC. Separation is easily achieved with the reverse phase column, but difficult to achieve with the normal phase column. Normal phase chromatography is most useful for compounds having different functional groups, but these derivatives have the same functional groupings and are separated best by the differences in their lipophilicities. Figure 3 shows the reverse phase liquid
Scheme 3: Silylation of \( N,N' \)-hexamethylene-bridged dinucleoside.
Figure 3: High Performance Liquid Chromatogram of TBDMS derivatives of
(1) $\text{du(CH}_2\text{)du(XI)}$, (2) $\text{du(CH}_2\text{)T(XII)}$, (3) $\text{T(CH}_2\text{)T(XIII)}$,
(4) $\text{T(CH}_2\text{)_2T(XIV)}$, (5) $\text{T(CH}_2\text{)_6T(XV)}$

Conditions: 1mL/min 100% acetonitrile as mobile phase; 4.6 mm I.D. x 250 mm
octyl silane reverse phase column; UV detection at 254 nm
chromatogram of the derivatives using 100% acetonitrile as the mobile phase. It is clearly seen from figures 2 and 3 that the separation of the derivatives is not as good as that for the underivatized compounds. However, isocratic conditions could be used, and most importantly, the mobile phase, 100% acetonitrile, is a relatively volatile solvent that would allow combined LC-MS. The derivatization appeared to be quantitative and the derivatives showed no signs of decomposition during work-up and chromatography. This was evidenced by the lack of other peaks in the chromatogram that might be attributed to underivatized material or even partially silylated derivatives.

Combined LC-MS was performed, using the reverse phase separation, with the SP-8000 high performance liquid chromatograph coupled to the VG 7070 mass spectrometer via a VG Micromass LC-MS interface. Figures 4 and 5 show the LC-MS total ion chromatogram (TIC) along with the UV detector chromatogram and some reconstructed ion chromatograms (RIC) for the five derivatives. The molecular ions are observed in the mass spectra of all the derivatives whose molecular weights are between 924 and 1022 amu. The technique is sensitive enough to easily detect the molecular ions with about 1-5 ug of each derivative injected into the chromatograph. The electron-impact induced mass spectra are shown in figures 15-19. The fragmentations and rearrangements will be discussed in the next chapter.

3.2. NUCLEOSIDES

3.2.1. The Underivatized Nucleosides

The liquid chromatographic analysis of the underivatized nucleosides has most often been performed with ion exchange chromatography. However, reverse phase is becoming increasingly popular due to the higher separation efficiencies
Figure 4: Liquid Chromatography-Mass Spectrometry total ion chromatogram and UV detector chromatogram of TBDMS derivatives of (1) $\text{du(CH}_2\text{)du(XI)}$, (2) $\text{du(CH}_2\text{T(XII)}$, (3) $\text{T(CH}_2\text{T(XIII)}$, (4) $\text{T(CH}_2\text{)}_2\text{T(XIV)}$, (5) $\text{T(CH}_2\text{)}_6\text{T(XV)}$.

Conditions: Octyl Silane, 4.6 mm I.D. x 250 mm column; 0.9 mL/min 100% acetonitrile as mobile phase; VG Micromass LC-MS interface.
Figure 5: Liquid Chromatogram-Mass Spectrometry reconstructed ion chromatogram of the [M-57]$^+$ ions for the TBDMS derivatives of the dinucleosides.

Conditions: Octyl silane, 4.6 mm I.D. x 250 mm column; 0.9 mL/min 100% acetonitrile as mobile phase
VG micro mass LC-MS interface.
that can be attained. We have studied the method of Davis and coworkers (31) for the reverse phase separation of nucleosides in order to be able to comparatively evaluate any new methods that we develop. The deoxyribonucleosides separated well as shown in Figure 6. However, for the ribonucleosides, it was necessary to use a gradient elution (see figure 7) in order to separate 5-methyluridine and guanosine. These two compounds do not separate with isocratic analysis, which is contrary to what Davis and coworkers found. The difference in observation may have resulted from the ways in which the mobile phases were prepared, and differences in the column packings and the lengths of the columns. Davis and coworkers prepared their mobile phase for daily use by diluting an appropriate aliquot of the stock buffer with distilled-in-glass water to 200 mL or more, followed by the addition of the methanol, and then completed the dilution to 1.00 liter with water. We, on the other hand, diluted an aliquot of the stock buffer to 1.00 liter with water and mixed the two solvents with the SP8000 liquid chromatograph mixing system. They used 4 x 300 mm μ Bondapak/C18 columns.

This method is not amenable to combined LC-MS for the same reasons that were stated earlier for the underivatized dinucleosides. Here too chemical derivatization promises to allow the use of non-polar, volatile solvents that would be compatible with the LC-MS.

3.2.2. TBDMS Derivatives

It was mentioned earlier, in chapter one, that the TMS derivatives of nucleosides are unstable to hydrolysis and that the more stable sterically crowded silyl derivatives have been used in the synthesis, chromatography and mass spectrometry of nucleosides (53-55). The silylation reactions of the purine and pyrimidine nucleosides are illustrated in Scheme 4. The nucleosides containing the primary amino function are silylated to form N,O-persilyl and O-persilyl
Figure 6: High Performance Liquid Chromatogram of (1) deoxycytidine (VI), (2) deoxyuridine (I), (3) deoxyguanosine (VII), (4) thymidine (III), (5) deoxyadenosine (VIII).

Conditions: Octyl silane, 4.6 mm I.D. x 250 mm column; 2 mL/min phosphate buffer (pH 5.10)/methanol (94:6, v/v) as mobile phase; uv detection at 254 nm.
Figure 7: High Performance Liquid Chromatogram of (1) cytidine (V), (2) uridine (II), (3) 5-methyluridine (IV), (4) guanosine (IX), and (5) adenosine (VII).

Conditions: Octyl silane, 4.6 mm I.D. x 250 mm column; 2 mL/min. phosphate buffer (pH 5.10)/methanol as mobile phase; UV detection at 254 nm.
Scheme 4: Silylation of purine and pyrimidine nucleosides.
products resulting in a mixture of the two products. The reactions of the four major ribonucleosides and deoxyguanosine were examined with thin-layer chromatography (TLC). The thin-layer chromatograms showed two products for the purine and cytosine nucleosides and one product for uridine and thymidine. The TLC data are given in Table 1.

HPLC analysis of these reaction mixtures after Sephadex LH-20 clean-up also showed the presence of the two derivatives of each of the nucleosides mentioned above. Unfortunately, quantitative N,O-persilylation cannot be achieved; an equilibrium between the N,O-persilyl and O-persilyl derivatives appears to exist in the reaction mixture. These derivatives have been studied previously by both TLC and mass spectrometry (56).

HPLC analysis of the O-silylated derivatives of both the ribo- and deoxy-ribonucleosides, prepared on a large scale and isolated by TLC so that there were no N,O-persilyl derivatives present, was performed with both reverse and normal phase columns. Unlike the dinucleoside derivatives, the derivatives of the nucleosides separate easily on the normal phase column but not on the reverse phase column. While the derivatives of the dinucleosides are separated by reverse phase liquid chromatography according to the differences in their lipophilicities, those of the nucleosides are separated by normal phase liquid chromatography according to their different functional groups. The liquid chromatography of the deoxy-and ribonucleoside derivatives are given in figures 8 and 9. Although these O-silyl derivatives separate quite well, the N, O-persilyl derivative formation would interfere in any procedure based on this separation.
Table 1

TLC Data\(^a\) on TBDMS Derivatives of Ribo- and Deoxyribonucleosides.

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Derivative</th>
<th>(R_f) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>2',3',5'-O-tris-TBDMS</td>
<td>0.68</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2',3',5'-O-tris-TBDMS</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>2',3',5'-O-tris-TBDMS-(N^6)-TBDMS</td>
<td>0.81</td>
</tr>
<tr>
<td>Cytidine</td>
<td>2',3',5'-O-tris-TBDMS</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>2',3',5'-O-tris-TBDMS-(N^4)-TBDMS</td>
<td>0.61</td>
</tr>
<tr>
<td>Guanosine</td>
<td>2',3',5'-O-tris-TBDMS</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>2',3',5'-O-tris-TBDMS-(N^2)-TBDMS</td>
<td>0.56</td>
</tr>
<tr>
<td>Thymidine</td>
<td>3',5'-O-bis-TBDMS</td>
<td>0.65</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>3',5'-O-bis-TBDMS</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>3',5'-O-bis-TBDMS-(N^6)-TBDMS</td>
<td>0.79</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>3',5'-O-bis-TBDMS</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>3',5'-O-bis-TBDMS-(N^4)-TBDMS</td>
<td>0.56</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>3',5'-O-bis-TBDMS</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>3',5'-O-bis-TBDMS-(N^2)-TBDMS</td>
<td>0.49</td>
</tr>
</tbody>
</table>

\(^a\)TLC on 182-F silica gel; Developing solvent: methylene chloride/methanol(19:1, v/v).
Figure 8: High Performance Liquid Chromatogram of TBDMS derivatives of (1) thymidine (III), (2) deoxyadenosine (VIII), (3) deoxycytidine (VI), and (4) deoxyguanosine (X)

Conditions: 4.6mm I.D. x 250 mm PAC column, 2 mL/min. CH$_2$Cl$_2$/MeOH (95:5, v/v) as mobile phase, UV detection at 280 nm.
Figure 9: High Performance Liquid Chromatogram of TBDM derivatives of (1) uridine (II), (2) adenosine (VII), (3) cytidine (V), and (4) guanosine (IX).

Conditions: PAC, 4.6 mm I.D. x 250 mm column; 1.5 mL/min methanol-chloroform as mobile phase with methanol gradient; UV detection at 280 nm.
3.2.3. **DMAM-TBDMS Derivatives**

To overcome the problem of incomplete N-silylation, a method was developed in which the nucleoside were first reacted with the dimethylformamide-dimethyl-acetal (DMF-DMA) to form the N-dimethylaminomethylene (N-DMAM) derivatives of those nucleosides containing the primary amino function. This was followed by O-silylation with the TBDMS reagent. As reported previously (51), N-DMAM derivatives are stable to hydrolysis and do not decompose on TLC plates. They are therefore suitable for column liquid chromatographic analysis of the nucleosides.

At room temperature the deoxynucleosides give a single product each after standing with the (DMF-DMA) reagent for 2.5 hours. Thymidine and uridine do not react under these conditions. At temperatures between 60 and 100°C, however, the latter two nucleosides give the N3-methyl derivatives after 1 hour, and guanosine and deoxyguanosine react further to give the N1-methyl-N2-DMAM derivatives. In addition the ribonucleosides form the 2',3'-O-DMAM derivatives but these are unstable, even to atmospheric moisture. Water was therefore added to the mixture after the first reaction but before evaporation of the excess reagent to hydrolyse the 2',3'-O-DMAM substituent. TLC and HPLC analysis of the reaction mixtures indicated that the N-DMAM derivatives form and that there are no side products. The second step in the derivatization procedure involved O-silylation with the TBDMS reagent. It was originally intended to have a simple addition of the TBDMS reagent after the formation of the N-DMAM derivative without any evaporation procedure. However, it was found necessary to evaporate the excess (DMF-DMA) reagent otherwise it interfered with the TBDMS reagent and silylation failed to proceed. The silylation reaction goes to completion in 1 hour at 80°C. The final step in the derivatization procedure prior to HPLC analysis involved Sephadex LH-20 column chromatographic clean-up to isolate the derivatives from
Deoxyadenosine (VIII)

Scheme 5: Preparation of DMAM-TBDMS derivative of nucleosides containing the primary amino function.
polar reagents and impurities. Scheme 5 summarizes the reaction chemistry and optimized procedures with deoxyadenosine as the example.

Liquid chromatographic data for the DMAM-TBDMS derivatives of the nucleosides are given in Table 2. HPLC analysis of the derivatives indicated that they could not be separated on a reverse phase column as was observed for the TBDMS derivatives of nucleosides. They are well separated by normal phase partition chromatography, however. The chromatogram for the deoxyribonucleoside derivatives is shown in Figure 10. An interesting observation is that only 1-2% methanol in methylene chloride is needed for the separation of the derivatives. In the absence of the methanol the purine nucleoside and cytidine derivatives eluted after 40 minutes. It is quite possible that the silica support still has exposed silanol groups that interact with the more polar derivatives. Thus instead of pure partition chromatography, there is probably adsorption chromatography also taking place on the column.

The DMAM-TBDMS derivatives are less polar and have higher volatility and more enhanced thermal stability than the TBDMS derivatives. They also show more satisfactory electron-impact mass spectra, thus making them better derivatives for combined LC-MS.

The combined LC-MS analysis of the DMAM-TBDMS derivatives was performed with the high performance liquid chromatograph, coupled to the VG7070 mass spectrometer by the VG Micromass LC-MS interface. At a mobile phase flow rate of 0.8 mL/min. and a belt speed of 1 cm/sec, good detection was achieved. At a sample evaporator setting of 80% of full power (temperature cannot be measured), the derivatives were completely evaporated off the belt without any memory effect. Methylene chloride has proved to be quite troublesome for the LC-MS analysis, however; when the interface housing became hot due to the infra red heating, the eluent appeared to evaporate in the transfer line causing sputtering onto the
<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>TLC Value</th>
<th>RF Value</th>
<th>HPLC Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine</td>
<td>0.46</td>
<td>0.39</td>
<td>1.00</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>0.36</td>
<td>1.60</td>
<td>2.24</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>0.30</td>
<td>3.23</td>
<td>1.00</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>0.45</td>
<td>0.34</td>
<td>1.55</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.43</td>
<td>4.30</td>
<td>2.30</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.34</td>
<td>2.30</td>
<td>4.20</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aTLC on silica gel IB2-F; developing solvent: methylene chloride/methanol (49:1, v/v)

bHPLC performed on normal phase (PAC) column (4.6 mm i.d. x 250 mm); mobile phase: 1% methanol in methylene chloride.
Figure 10: High Performance Liquid Chromatogram of DMAM-TBDMS derivatives of (1) thymidine, (2) deoxyadenosine, (3) deoxycytidine, and (4) deoxyguanosine.

Conditions: PAC, 4.6 mm I.D. x 250 mm Column; 1 mL/min CH₂Cl₂/CH₃OH (99:1, v/v) as mobile phase; UV detector at 280 nm.
Figure 11: Liquid Chromatography-Mass Spectrometry Total Ion Chromatogram of the DMAM-TBDMS derivatives of (1) thymidine, (2) deoxyadenosine, (3) deoxycytidine, and (4) deoxyguanosine. Conditions: PAC, 4.6 mm i.d. x 250 mm column; 1% CH3OH in CH2Cl as mobile phase; VG Micromass LC-MS interface.
Figure 12: Liquid Chromatography-Mass Spectrometry Reconstructed Ion Chromatogram of the molecular ions of DMAM-TBDMS derivatives of (A) deoxyadenosine, (B) deoxycytidine, and (C) deoxyguanosine.

Conditions: PAC, 4.6 mm I.D. x 250 mm column; 1% CH$_3$OH in CH$_2$Cl$_2$ as mobile phase; VG Micromass LC-MS interface.
belt. Cooling the line helped but this proved inconvenient. Experiments are under way to find a better solvent system for the LC-MS analysis of the derivatives. The LC-MS total ion chromatogram and reconstructed ion chromatograms of the molecular ions of the DMAM-TBDMs derivatives are shown in Figures 11 and 12. The mass spectra of the derivatives are given in the next chapter.

3.2.3.1. Quantitative Studies of the DMAM-TBDMs Derivatization

Preliminary quantitative studies on the DMAM-TBDMs derivatization method have been carried out by derivatizing varying amounts of each of the three nucleosides containing the amino function using the optimized conditions illustrated in Scheme 5. After Sephadex LH-20 column clean-up, the derivatives were analysed by normal phase HPLC and detected by a UV absorption detector at 280 nm. Analysis of each sample was performed in triplicate. The area for each peak was measured by the SP 8000 data system. Plots of average area versus amount of each nucleoside derivative are shown in Figure 13. For all three derivatives there appears to be a good correlation between area and amount of nucleoside indicating that there is almost 100% conversion of nucleoside to derivative and also that there is nearly 100% recovery of the product during clean-up and chromatography.

One very important step in the preparation of the derivatives is the Sephadex LH-20 clean-up procedure. The polar solvent, DMF, and the imidazole are absorbed by the Sephadex while the non-polar derivatives are eluted. It has been determined that the first 1 mL of the eluent after application of the sample contains all the derivatives. It is very important that the column is not overloaded or else it may lead to sample loss. Also it is important that enough of the eluent is collected but not so much that interfering materials are eluted with the derivatives.
Figure 13: Quantitative studies of DMAM-TBDMS derivatization.
3.2.4. Trifluoroacetyl Derivatives

The use of trifluoroacetyl derivatives for the gas chromatographic and mass spectral analysis of nucleosides and nucleic acid hydrolysates has been investigated (17,56,59). The derivatives are stable on the gas chromatographic column and on TLC plates. We therefore decided to investigate their liquid column chromatographic properties and the possibility of their use for combined LC-MS analysis of nucleosides.

Derivatization of nucleosides with trifluoroactic anhydride produces the N, O-pertrifluoroacetyl derivatives. The derivatives are much easier to prepare, and the reaction time is much shorter than that for the DMAM-TBDMS derivatives. The solvent and excess reagent are volatile and are easily evaporated under a stream of nitrogen. Despite these advantages, the TFA derivatives were found to be not very suitable for combined LC-MS analysis of the nucleosides due to the following disadvantages: (a) the guanine nucleosides need temperatures as high as 150°C which results in some breakage in the glycosidic bonds of the nucleosides (17); (b) adenine nucleosides form a mixture of two derivatives; and (c) the derivatives are more polar than the DMAM-TBDMS derivatives and therefore need more polar solvents for their separation; also the polarity difference between the guanine nucleoside derivatives and the derivatives of the other nucleosides is such that gradient elution is needed to chromatograph a mixture containing the derivatives of all the nucleosides. Figure 14 shows the liquid chromatogram of the TFA derivatives.
Figure 14: High Performance Liquid Chromatogram of TFA derivatives of deoxynucleosides. (1) Bis-TFA-thymidine (2) tetrakis-TFA-deoxyadenosine (3) tris-TFA-deoxyadenosine (4) tris-TFA-deoxycytidine (5) Tris-TFA-deoxyguanosine. Conditions: PAC column; 2mL/min CH$_2$Cl$_2$/CH$_3$OH gradient (5-55% at 2%/min.); UV detection at 254 nm.
CHAPTER FOUR

4. MASS SPECTRAL FRAGMENTATIONS OF NUCLEOSIDE DERIVATIVES

In this chapter the electron-impact induced fragmentation and rearrangement mechanisms of the TBDMS derivatives of the dinucleosides and DMAM-TBDMS derivatives of the nucleosides are presented. The spectra of TBDMS and TFA derivatives of nucleosides have been discussed previously (17,27,56).

4.1. TBDMS DERIVATIVES OF DINUCLEOSIDES

The mass spectra of the five TBDMS derivatives of the dinucleosides are shown in figures 15-19. One very important fragmentation process that occurs in the spectra of TBDMS derivatives is the elimination of the very stable \( t \)-butyl radical, \((\text{CH}_3)\text{C}^*\), leading to the \((M-\text{tBu})^+\) ion, \( e \). For the dinucleoside derivatives the peaks corresponding to this ion are quite intense. The fragmentation behavior of silyl ether derivatives can be explained generally; as shown in Scheme 6, by assuming an initial charge localization on the ether oxygen, followed by an \( \alpha \)-cleavage either in the substrate skeleton (a,b, or c,) or in the silyl group with expulsion of an alkyl radical (d or e). For TBDMS derivatives, process e is the most favoured since the tert-butyl radical is very stable and its elimination relieves steric crowding around the silicon.

Scheme 6:
Figure 6: Mass spectrum of TBDMS derivative of dU(CH₂)₅T (XII)
Figure 17: Mass spectrum of TBDMS derivative of T(CH₂)₂T (XIII).
Figure 18: Mass spectrum of TBDMS derivative of $T(CH_2)_2T(XIV)$. 
Figure 19: Mass spectrum of TBDMS derivative of $T(CH_2)_6T$ (XV).
Surprisingly, molecular ions are observed (although at low abundance), whereas in the spectra of TBDMS derivatives of nucleosides, the molecular ion is usually not observed (60).

The fragmentation behavior of the derivatives is illustrated in Scheme 7 and the ion assignments are given in table 3. This fragmentation behavior is elaborated in Scheme 8 for the hexamethylene-bridged dinucleoside. The fragmentations shown in Scheme 8 are supported by the observation of metastable transitions (table 4). The observed metastable transitions indicate that the $(M-tBu)^+$ ion, e, is the main precursor for most of other ions.

One significant ion, $(e-tBuMe_2SiOH)^+$, h, results from elimination of $tBuMe_2SiOH$ from one of the sugar moieties of ion e. Evidence presented in previous work on the TBDMS derivatives of nucleosides (56,60) indicates that it is the 3'-silyloxy group that is eliminated and that a 5'-silyloxonium ion is required for this to occur. Therefore, it is proposed in Scheme 8 that loss of the $tBuMe_2SiOH$ occurs from that sugar containing the silyloxonium ion at the 5' position.

Another significant ion resulting directly from decomposition of e is ion f. It is proposed that this involves a concerted rearrangement of a 2' hydrogen to the nucleobase's carbonyl oxygen at position 2, breakage of the glycosidic bond, and elimination of the sugar unit that does not carry the the positive charge. Similarly, both ions f and h fragment further to give the very abundant ion k. This ion appears to decompose further through the rearrangement of the silyl group to the nucleobase to give the ion $(a+H+Me_2Si)^+$. Ions of similar structure and origin as these, as well as most of the other ions in table 3, have been proposed in previous work on TBDMS derivatives of nucleosides (27,56,60). The mechanism of formation of other ions unique to these dinucleosides such as $(a+3H)^+$, cannot be proposed with any certainty at this point. However, their assignment is quite certain due to the "labelling"
<table>
<thead>
<tr>
<th>Proposed Assignment&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-dU(CH&lt;sub&gt;2&lt;/sub&gt;)dU&lt;sup&gt;(XI)&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-dU(CH&lt;sub&gt;2&lt;/sub&gt;)T&lt;sup&gt;(XII)&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-dU(CH&lt;sub&gt;2&lt;/sub&gt;)T&lt;sup&gt;(XII)&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-T(CH&lt;sub&gt;2&lt;/sub&gt;)T&lt;sup&gt;(XIII)&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-T(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;T&lt;sup&gt;(XIV)&lt;/sup&gt;</th>
<th>TBDMS&lt;sub&gt;4&lt;/sub&gt;-T(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;T&lt;sup&gt;(XV)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td>SiX&lt;sub&gt;3&lt;/sub&gt;</td>
<td>75</td>
<td>75</td>
<td>101(56.6)</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>81</td>
<td>81</td>
<td>81(100)</td>
<td>81</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>X&lt;sub&gt;2&lt;/sub&gt;SiOH</td>
<td>89</td>
<td>89</td>
<td>115(52.4)</td>
<td>89</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>tBuSiX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>115(17.0)</td>
<td>115(21.0)</td>
<td>141(12.7)</td>
<td>115(18.0)</td>
<td>115(17.0)</td>
<td>115(21.0)</td>
</tr>
<tr>
<td>b + 2H</td>
<td>113(7.0)</td>
<td>127(8.0)</td>
<td>113(19.8), 127(6.3)</td>
<td>127(6.0)</td>
<td>127(6.0)</td>
<td>127(10.0)</td>
</tr>
<tr>
<td>X&lt;sub&gt;2&lt;/sub&gt;Si + C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>129(16.2)</td>
<td>129(17.0)</td>
<td>155(20.6)</td>
<td>129(16.5)</td>
<td>129(15.0)</td>
<td>129(9.0)</td>
</tr>
<tr>
<td>b + H + CH&lt;sub&gt;2&lt;/sub&gt;, q</td>
<td>125(8.0)</td>
<td>125(5.0)</td>
<td>125(6.7)</td>
<td>139(9.0)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>X&lt;sub&gt;2&lt;/sub&gt;SiHOSiX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>133(9.0)</td>
<td>133(8.5)</td>
<td>159(11.9)</td>
<td>133(8.5)</td>
<td>133(2.5)</td>
<td>133(6.0)</td>
</tr>
<tr>
<td>tBuX&lt;sub&gt;2&lt;/sub&gt;SiOCH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>145(42.0)</td>
<td>145(58.0)</td>
<td>171(31.8)</td>
<td>145(63.5)</td>
<td>145(60.0)</td>
<td>145(53.3)</td>
</tr>
<tr>
<td>s-H-tBuX&lt;sub&gt;2&lt;/sub&gt;SiOH</td>
<td>155(36.2)</td>
<td>155(28.0)</td>
<td>238(20.8)</td>
<td>155(23.7)</td>
<td>155(20.0)</td>
<td>155(26.5)</td>
</tr>
<tr>
<td>s - tBuX&lt;sub&gt;2&lt;/sub&gt;SiOH</td>
<td>156(42.0)</td>
<td>156(80.9)</td>
<td>—</td>
<td>156(88.4)</td>
<td>156(57.0)</td>
<td>156(53.6)</td>
</tr>
<tr>
<td>bH + SiX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>169(10.0)</td>
<td>169(6.0), 183(6.0)</td>
<td>209(3.4)</td>
<td>183(11.0)</td>
<td>183(2.0)</td>
<td>183(2.3)</td>
</tr>
</tbody>
</table>

Table 3
Correlation of m/z values for prominent fragment ions of TBDMS and TMTBS derivatives of dinucleosides<sup>a</sup>
<table>
<thead>
<tr>
<th>Proposed Assignment&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>m/z&lt;sup&gt;a&lt;/sup&gt; (RI)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBEM&lt;sub&gt;4&lt;/sub&gt;-dU(CH&lt;sub&gt;2&lt;/sub&gt;)dU&lt;sup&gt;XII&lt;/sup&gt;</td>
<td>TBEM&lt;sub&gt;4&lt;/sub&gt;-dU(CH&lt;sub&gt;2&lt;/sub&gt;)T&lt;sup&gt;XII&lt;/sup&gt;</td>
</tr>
<tr>
<td>a + 2H</td>
<td>236(4.0)</td>
</tr>
<tr>
<td>a + 3H, f</td>
<td>237(37.0)</td>
</tr>
<tr>
<td>s-tBu - C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;3&lt;/sub&gt;</td>
<td>261(10.2)</td>
</tr>
<tr>
<td>s-H - tBu</td>
<td>287(47.0)</td>
</tr>
<tr>
<td>a + H + SiX&lt;sub&gt;2&lt;/sub&gt;</td>
<td>293(16.3)</td>
</tr>
<tr>
<td>a + 2H + X&lt;sub&gt;2&lt;/sub&gt;SiOH</td>
<td>311(22.0)</td>
</tr>
<tr>
<td>f - tBuX&lt;sub&gt;2&lt;/sub&gt;SiOH, k</td>
<td>391(92.0)</td>
</tr>
<tr>
<td>e - (s - H), f</td>
<td>523(14.0)</td>
</tr>
<tr>
<td>e - tBuX&lt;sub&gt;2&lt;/sub&gt;SiOH, h</td>
<td>735(5.5)</td>
</tr>
<tr>
<td>M - tBu, e</td>
<td>867(4.5)</td>
</tr>
<tr>
<td>M&lt;sup&gt;e&lt;/sup&gt;</td>
<td>924(2.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mass spectra obtained on VG7070 mass spectrometer at 70 eV.<sup>b</sup>Positive ions; signs left out for simplification.<sup>c</sup>X = Me for all derivatives except for the TMTBS derivative where X<sub>2</sub> = cyclotetramethylene(C<sub>4</sub>H<sub>8</sub>).<sup>d</sup>Ri of masses below m/z 100 not measured except for the TMTBS derivative. <sup>e</sup>Not observed.
Scheme 8: Partial fragmentation behavior of T8OS derivative of hexamethylene-bridged dinucleoside(X).

(*) Metastable transition observed in mass spectrum.
<table>
<thead>
<tr>
<th>Metastable Transition</th>
<th>Observed</th>
<th>Calculated</th>
<th>Parent Ion</th>
<th>Daughter Ion</th>
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<tbody>
<tr>
<td>719</td>
<td>.71906</td>
<td>965</td>
<td>833</td>
<td></td>
</tr>
<tr>
<td>399.5</td>
<td>399.63</td>
<td>965</td>
<td>621</td>
<td></td>
</tr>
<tr>
<td>385</td>
<td>385.06</td>
<td>621</td>
<td>489</td>
<td></td>
</tr>
<tr>
<td>312.5</td>
<td>312.64</td>
<td>489</td>
<td>391</td>
<td></td>
</tr>
<tr>
<td>287</td>
<td>287.06</td>
<td>833</td>
<td>489</td>
<td></td>
</tr>
</tbody>
</table>
provided by the structural variations in the series of compounds examined. Such ions could prove useful in any structural work on compounds of a similar nature in the future.

The two most important ions in the mass spectrum are the molecular ion and one from which the molecular weight can be determined. Other important ions include those that are indicative of the bridged base such as \((a + 3H)^+\) and \((a + H + SiMe_2)^+\), and those that are indicative of the sugar moiety such as \((S-tBu-C_2H_3)^+\) and \((S-H-tBu)^+\) at m/z 261 and 287, respectively. The ion at m/z 287 is in fact very important in all the TBDMS derivatives of deoxyribonucleosides; it clearly indicates the presence of the deoxyribose moiety with its two silyl groups.

4.2. DMAM-TBDMS DERIVATIVES OF NUCLEOSIDES.

The mass spectra of the DMAM-TBDMS derivatives of the deoxynucleosides and riboadenosine are shown in figures 20-24. The mass spectrum of the thymidine derivative shows that it is simply the TBDMS derivative and that there is no base methylation - the \((M-57)^+\) peak is observed at m/z 413. A full interpretation of the mass spectrum of this derivative is discussed elsewhere (56). The mass spectra of the derivatives of the ribonucleosides are not discussed.

A correlation of the m/z values for important fragment ions of the DMAM-TBDMS derivatives is given in table 5. For these derivatives, as for other compounds containing the TBDMS group, an intense \((M-57)^+\) peak is observed. However, the molecular ion is also very intense; in fact the molecular ion for the guanine nucleoside derivatives is more abundant than the \((M-57)^+\) ion. The abundance of the molecular ion is due to accommodation and stabilization of the positive charge on the base. The dimethylaminomethylene grouping increases the number of nitrogen atoms in the base and also stabilizes the positive charge on the base by extending the conjugation. These features also result in very
Figure 20: Mass spectrum of TBDMS derivative of thymidine (III).
Figure 21: Mass spectrum of DMAM-TBDMS derivative of deoxyadenosine (VIII).
Figure 22: Mass spectrum of DMAM-TBDMS derivative of adenosine(VII).

Molecular ion not observed due to difficulties in calibration above 650 amu.
Figure 23: Mass spectrum of DMAM-TBDMS derivative of deoxyctidine (VI).
Figure 24: Mass spectrum of DMAM-TBDMS derivative of deoxyguanosine(x).
Table 5

Correlation of m/z values for important fragment ions of TBDMS and DMAM-TBDMS derivatives of deoxynucleosides.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Proposed Assignment\textsuperscript{b}</th>
<th>m/z (RI)%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T (III)</td>
</tr>
<tr>
<td>Me\textsubscript{3}Si</td>
<td>73</td>
</tr>
<tr>
<td>C\textsubscript{3}H\textsubscript{5}O\textsubscript{2}</td>
<td></td>
</tr>
<tr>
<td>Me\textsubscript{2}SiOH</td>
<td>75</td>
</tr>
<tr>
<td>CH\textsubscript{2}\begin{array}{c} \text{O} \end{array}</td>
<td>81</td>
</tr>
<tr>
<td>Me\textsubscript{2}HSiOH</td>
<td>89</td>
</tr>
<tr>
<td>tBuMe\textsubscript{2}Si</td>
<td>115(49.5)</td>
</tr>
<tr>
<td>bH-C\textsubscript{3}H\textsubscript{7}N\textsubscript{2}</td>
<td>—</td>
</tr>
<tr>
<td>b-HNCO</td>
<td>82</td>
</tr>
<tr>
<td>Me\textsubscript{2}Si+C\textsubscript{3}H\textsubscript{5}O\textsubscript{2}</td>
<td>129(22.0)</td>
</tr>
<tr>
<td>Me\textsubscript{2}SiOSiCH\textsubscript{2}Me</td>
<td>131(16.8)</td>
</tr>
</tbody>
</table>

continued...
<table>
<thead>
<tr>
<th>Proposed Assignment</th>
<th>T(III)</th>
<th>dC (VI)</th>
<th>dA (VIII)</th>
<th>dG (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-C$_2$H$_4$N$_2$</td>
<td>—</td>
<td>—</td>
<td>133(27.1)</td>
<td>149(8.1)</td>
</tr>
<tr>
<td>bH-C$_2$H$_4$N$_2$</td>
<td>—</td>
<td>—</td>
<td>134(55.6)</td>
<td>150(4.3)</td>
</tr>
<tr>
<td>b+2H-C$_2$H$_4$N$_2$</td>
<td>—</td>
<td>—</td>
<td>135(20.1)</td>
<td>151(3.0)</td>
</tr>
<tr>
<td>tBuMe$_2$SiOCH$_2$</td>
<td>145(100)</td>
<td>145(27.5)</td>
<td>—</td>
<td>145(11.5)</td>
</tr>
<tr>
<td>Me$_3$SiOSiMe$_2$</td>
<td>147(44.6)</td>
<td>147(42.2)</td>
<td>147(27.7)</td>
<td>147(34.2)</td>
</tr>
<tr>
<td>e-bH-tBuMe$_2$SiOH</td>
<td>155(54.5)</td>
<td>155(48.9)</td>
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<td>155(56.2)</td>
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<tr>
<td>bH+C$_2$H$_3$-C$_2$H$_6$N</td>
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<td>149(28.0)</td>
<td>173(1.8)</td>
<td>189(8.4)</td>
</tr>
<tr>
<td>bH-Me</td>
<td>111(9.4)</td>
<td>151(12.9)</td>
<td>175(73.4)</td>
<td>191(8.8)</td>
</tr>
<tr>
<td>b+H</td>
<td>126(12.9)</td>
<td>166(52.7)</td>
<td>190(83.0)</td>
<td>—</td>
</tr>
<tr>
<td>b+2H</td>
<td>127(11.7)</td>
<td>167(69.9)</td>
<td>191(62.3)</td>
<td>207(17.5)</td>
</tr>
<tr>
<td>b+CH$_2$O</td>
<td>169(5.4)</td>
<td>208(33.0)</td>
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<td>—</td>
</tr>
<tr>
<td>s-H-tBuMe$_2$SiOH</td>
<td>212(4.6)</td>
<td>212(6.3)</td>
<td>212(2.6)</td>
<td>212(7.9)</td>
</tr>
<tr>
<td>bH+C$_2$H$_3$</td>
<td>153(3.1)</td>
<td>193(20.4)</td>
<td>217(100)</td>
<td>233(23.7)</td>
</tr>
<tr>
<td>s-tBu-C$_2$H$_3$</td>
<td>261(24.5)</td>
<td>261(3.2)</td>
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Table 5 (continued)

<table>
<thead>
<tr>
<th>Proposed Assignment b</th>
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<tr>
<td></td>
<td>T(III)</td>
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<tr>
<td>b+Me₂Si</td>
<td>183(19.9)</td>
</tr>
<tr>
<td>M-tBu-bH</td>
<td>287(65.3)</td>
</tr>
<tr>
<td>bH+tBuMe₂Si</td>
<td>241(13.7)</td>
</tr>
<tr>
<td>b+C₂H₃+tBuMe₂Si</td>
<td>—</td>
</tr>
<tr>
<td>e-tBuMe₂SiOH</td>
<td>281(87.4)</td>
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<tr>
<td>M-tBu, e</td>
<td>413(16.6)</td>
</tr>
<tr>
<td>M</td>
<td>470(c)</td>
</tr>
</tbody>
</table>

\(^a\)Mass spectra acquired on VG7070 mass spectrometer at 70 eV. RI of masses below m/z100 not measured.

\(^b\)Positive ions.

\(^c\)Not observed.
abundant \((b + H)^+\) and \((b + 2H)^+\) ions.

The molecular ion and \((M-tBu)^+\), are the two main precursors for other fragment ions. The fragmentation behavior of the DMAM-TBDMS derivatives of nucleosides is illustrated in Scheme 9 for the deoxyadenosine derivative. This is elaborated in Scheme 10.

In the molecular ion, rupture of the glycosidic bond with charge retention on base plus single or double proton rearrangement results in the \((b + H)^+\) and \((b + 2H)^+\) ions. On the other hand if the charge is on an ether oxygen of the sugar, \(\alpha\)-cleavage occurs with the elimination of the \(tBu^+\) radical which produces the \((M-tBu)^+\) ion (see scheme 6). Rupture of the glycosidic bond and elimination of \((b + H)\) from \((M-tBu)^+\) results in the \((S-H-tBu)^+\) ion. The mass spectral data for the DMAM-TBDMS derivative of deoxyadenosine are given in table 6. The fragmentation behavior in schemes 9 and 10 is supported by high resolution measurement for the fragment ions (table 6) and by observation of metastable transitions (table 7).

Another feature observed in the mass spectra of the derivatives is that the \((b + 2H)^+\) ion is more abundant for the ribose than the corresponding deoxyribose nucleoside derivative, and it is more pronounced in the purines. This is clearly illustrated by the mass spectra of the adenine nucleoside derivatives (figures 21 and 22). The double hydrogen rearrangement is related to the group at the 2’ position, and the electron-rich purine is much more capable of accommodating the second hydrogen atom than the pyrimidine moiety.

4.3. TRIFLUOROACETYL DERIVATIVES OF NUCLEOSIDES

Figure 25 shows the mass spectrum of the trifluoroacetyl (TFA) derivative of deoxyguanosine(X). The mass spectra of the TFA derivatives are not as satisfactory as those of the DMAM-TBDMS derivatives: the mass spectrum in figure
Scheme 10: Partial fragmentation behavior of DMAM-TBDMS derivative of deoxyadenosine.
<table>
<thead>
<tr>
<th>Proposed Assignment</th>
<th>Elemental Composition</th>
<th>Exact Mass</th>
<th>Nominal m/z</th>
<th>Relative Int., %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C — H — N — O — Si</td>
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<td></td>
<td></td>
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<tr>
<td>tBuMe₂Si</td>
<td>6 15 0 0 1</td>
<td>115.098</td>
<td>115</td>
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<tr>
<td>[a+H]</td>
<td>5 4 4 0 0</td>
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<tr>
<td>Me₂Si+C₃H₆O</td>
<td>5 9 0 2 1</td>
<td>129.040</td>
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<td>Me₂SiOSiCH₂Me</td>
<td>4 11 0 1 2</td>
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<td>Me₂HSiOSiMe₂</td>
<td>4 13 0 1 2</td>
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<tr>
<td>[a+Me]</td>
<td>6 6 4 0 0</td>
<td>134.061</td>
<td>134</td>
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<tr>
<td>Me₃SiOSiMe₂</td>
<td>5 15 0 1 2</td>
<td>147.069</td>
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<tr>
<td>e-bH-tBuMe₂SiOH</td>
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<td>b-HCN</td>
<td>7 8 5 0 0</td>
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<td>bH-Me</td>
<td>7 7 6 0 0</td>
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<td>b+H</td>
<td>8 10 6 0 0</td>
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</tr>
<tr>
<td>b+2H</td>
<td>8 11 6 0 0</td>
<td>191.105</td>
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<td>191</td>
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<td>s-H-tBuMe₂SiOH</td>
<td>11 20 0 2 1</td>
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Table 6
Mass Spectral Data for DMAM-TBDMS Derivative of Deoxyadenosine (VIII)².
<table>
<thead>
<tr>
<th>Proposed Assignment</th>
<th>Elemental Composition</th>
<th>Exact Mass&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Nominal</th>
<th>Relative&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C  H  N  O  Si</td>
<td>Calc'd.</td>
<td>Meas'd</td>
<td>m/z</td>
</tr>
<tr>
<td>bH+C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;3&lt;/sub&gt;</td>
<td>10 13 6 0 0</td>
<td>217.120</td>
<td>217.120</td>
<td>217</td>
</tr>
<tr>
<td>n-tBu</td>
<td>13 27 0 3 2</td>
<td>287.153</td>
<td>287.152</td>
<td>287</td>
</tr>
<tr>
<td>e-tBuMe&lt;sub&gt;2&lt;/sub&gt;SiOH</td>
<td>15 21 6 2 1</td>
<td>345</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-tBu, e</td>
<td>21 37 6 3 2</td>
<td>477</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>25 46 6 3 2</td>
<td>534</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Data obtained on VG7070 mass spectrometer at 70eV.

<sup>b</sup>Positive ions; signs left out for simplification.

<sup>c</sup>m/z above 287 amu not measured due to difficulties in calibration.
Table 7

Metastable Transitions Observed for the DMAM-TBDMS Derivative of Deoxyadenosine (VIII).

<table>
<thead>
<tr>
<th>Metastable Transition</th>
<th>Observed</th>
<th>Calculated</th>
<th>Parent Ion</th>
<th>Daughter Ion</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>534</td>
<td>477</td>
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<tr>
<td>426</td>
<td>426.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>249.53</td>
<td></td>
<td>477</td>
<td>345</td>
</tr>
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<td>161</td>
<td>161.18</td>
<td></td>
<td>190</td>
<td>175</td>
</tr>
<tr>
<td>154</td>
<td>154.25</td>
<td></td>
<td>534</td>
<td>287&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>94.5</td>
<td>94.51</td>
<td></td>
<td>190</td>
<td>134</td>
</tr>
</tbody>
</table>

<sup>a</sup>A double transition involving: $M^+ \rightarrow [M-tBu]^+ \rightarrow [S-H-tBu]^+$
Figure 25: Mass spectrum of trifluoroacetyl derivative of deoxyguanosine (X).
25 shows a weak molecular ion; peaks diagnostic of the base moiety are not observed and it has many abundant ions of low mass with fewer ions in the high mass region. A comparison of the mass spectra of the DMAM-TBDMS and TFA derivatives clearly indicates the value of the silyl derivatives for mass spectrometry of the nucleosides. The TFA derivatives are more polar than the DMAM-TBDMS derivatives and therefore need slightly higher temperatures for their evaporation into the ion source of the mass spectrometer. Combined with their liquid chromatographic requirements the above observations suggest that the TFA derivatives are unsuitable for the combined LC-MS analysis of the nucleosides.
CHAPTER FIVE

5. CONCLUSION

The results presented in this thesis clearly indicate that chemical derivatization, combined with combined liquid chromatography - mass spectrometry is a useful method for the analysis of nucleosides. The advantages of the method have been discussed and include good chromatographic behavior, enhanced volatility and thermal stability, and very favorable electron-impact induced mass spectral fragmentations and rearrangements for the derivatized compounds.

Despite these advantages, the DMAM-TBDMS derivatives may still not be acceptable for routine analysis of the nucleosides due to one disadvantage - the derivatives require a long reaction and work-up time. This is due to the requirement that excess DMF/DMA reagent from the first derivatization step be removed by evaporation prior to the addition of the TBDMS reagent. Further experimentation aimed at shortening the preparation time could study the use of solvents such as pyridine, acetonitrile, or methanol for the DMF/DMA-nucleoside reaction. These solvents are more volatile than DMF, which is presently being used, and could therefore be evaporated faster. A second problem to overcome is the use of methylene chloride as eluting solvent in the LC-MS analysis; although the solvent is very good for the normal phase HPLC separation of the nucleoside derivatives, it is so volatile that it evaporates in the transfer line to the LC-MS interface due to heating by the infra-red heater thus producing noisy signals. Less volatile solvents such as Hexane/isopropanol could be investigated as an alternative mobile phase for LC-MS.
Thusfar the DMAM-TBDMS and TFA derivatives have been studied. The TFA derivatives were found to be less acceptable compared to the DMAM-TBDMS derivatives. Another type of derivatization method that could be examined for combined LC-MS is the formation of N, O-permethyl derivatives. These derivatives could be prepared by the method of De Leenheer and Gelijks (19a). The permethyl derivatives are reported to have the general advantages of low molecular weight, ease of preparation, high chemical stability over a period of time without special precautions, and good volatility for mass spectrometry (19). They also give satisfactory electron-impact mass spectra. Because of the possibility of keto-enol and amino-imino tautomerism in the base, permethylation of nucleosides produces a variety of isomeric derivatives. Each of the purine and cytosine nucleosides have been shown to produce two isomeric derivatives (19). Ideally, single derivatives are preferred, but if they are unattainable it must be ensured that all the derivatives formed separate from each other on the liquid chromatographic column. These derivatives would be compared to the DMAM-TBDMS derivatives to determine which would be better for LC-MS analysis of the nucleosides.

The application of chemical derivatization, particularly DMAM-TBDMS derivatization, and LC-MS to specific problems in nucleic acid research, such as nucleoside-carcinogen adducts, should demonstrate the feasibility of this approach.
REFERENCES

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