"PYRROLIDINE DERIVED NOVEL DNA BINDING AGENTS: CHIRAL POLYAMINES AND CONSTRAINED PNA ANALOGS"

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IN

CHEMISTRY

BY

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PUNE - 411008

JUNE 2001

Dedicated to
My Parents, Aunt and Husband

CERTIFICATE

This is certify that the work presented in the thesis entitled **"PYRROLIDINE DERIVED NOVEL DNA BINDING AGENTS: CHIRAL POLYAMINES AND CONSTRAINED PNA ANALOGS"** submitted by D. Nagamani was carried out by the candidate at the National Chemical Laboratory Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

June 2001

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CANDIDATE 'S DECLARATION

I here by declare that the thesis entitled **"PYRROLIDINE DERIVED NOVEL DNA BINDING AGENTS: CHIRAL POLYAMINES AND CONSTRAINED PNA ANALOGS"** submitted for the degree of Doctor of Philosophy in Chemistry to the University of Pune has not been submitted by me to any other university or institution. This work was carried out at the National Chemical Laboratory, Pune, India.

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1. Pyrrolidyl Polyamines: Branched, Chiral Polyamine Analogues that stabilize DNA Duplexes and Triplexes Nagamani, D.; Ganesh, K. N. Org. Lett. 2001, 3, 103-106.
2. Chiral Dendritic Polyamines and their interactino with Plasmid DNA (manuscript under preparation).
3. Neutral, Conformationally constrained aminomethylprolyl Peptide Nucleic Acid analogs (to be communicated).

ABSTRACT

The thesis entitled **"PYRROLIDINE DERIVED NOVEL DNA BINDING AGENTS: CHIRAL POLYAMINES AND CONSTRAINED PNA ANALOGS"** is divided into two Sections **A** and **B** as follows.

***SECTION A*: Chiral, Branched Pyrrolidyl Polyamines: Synthesis and Bio-physical studies**

Chapter 1: ***Introduction: Polyamines Biological role and analogs***

Linear polyamines putrescine, spermidine and spermine are important constituents of almost all prokaryotic and eukaryotic cells. They have diverse role in physiological process viz., protein synthesis, cell division and cell growth. Polyamine analogs are also emerging as therapeutic options for the development of potent new antidiarrheals in AIDS related cases. They also have shown promise as anticancer agents as they are able to kill the cells and inhibit cell growth. Natural polyamines favor triplex DNA formation at neutral pH and could be important in stabilizing triplex DNA in chromosomal structures. Since the negative charge density of triplex is higher than that of duplex, polyamines should bind more strongly to the triplexes and shift the equilibrium in favor of triplex formation. These diverse roles played by polyamines in several biological processes have evoked recent interest in the syntheses of various modified analogs of polyamines to improve their properties.

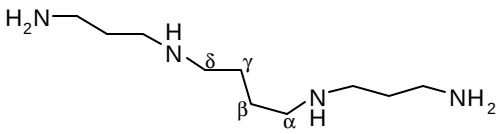
Most of the structural modifications involved terminal N-substitutions, rigidification via interconnection of secondary amines based on cycloputrescine core or by introducing aromatic moiety in the polyamine backbone and are achiral. Recently there have been reports on modification of linear analogs by introduction of chirality. These modified analogs were found to be superior in their activity over their achiral counterparts.

This chapter gives a brief review on the synthesis and biological properties of various polyamine analogs. In this work chiral and branched pyrrolidyl polyamines are designed.

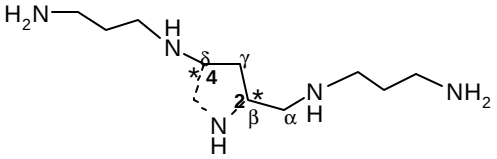
Chapter 2: ***Synthesis of Chiral, Branched Pyrrolidyl Polyamines***

In context of above polyamine application potential, we have employed conformational constrain on linear spermine. Since many of the polyamine receptor sites eg. nucleic acids and membranes are chiral in nature we envisaged that the introduction of chirality in the polyamine would be beneficial in selective binding. A new

strategy of designing polyamine analogs involved conformational restriction, which has been quite successful in the area of peptidomimetics. It involved the introduction of a -CH_2NH- bridge between the α and δ carbon atoms of central tetramethylene fragment of spermine **I** leading to a five membered pyrrolidine ring **II**. (Figure 1) with two asymmetric centers C2 and C4 with an additional nitrogen atom. Such analogs exhibited considerable improvements over spermine in selective stabilization of DNA triplexes. These molecules are elaborated in this work by addition of amino propyl chains leading to four chiral and branched pyrrolidyl polyamines with increased charge content. (**III**-**VI**)



I



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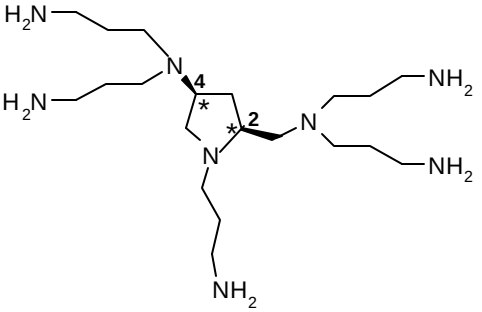
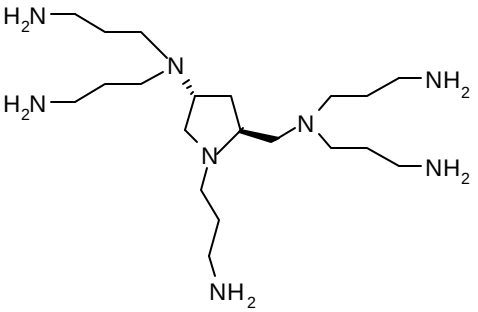
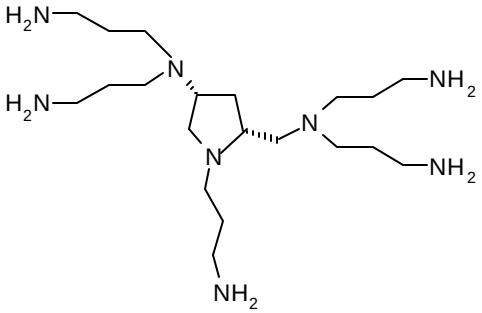


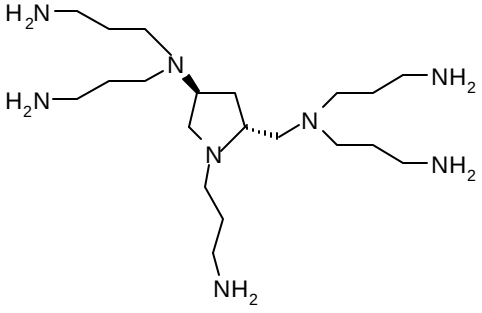
III (2*S*,4*S*)-L-*cis*



IV (2*S*,4*R*)-L-*trans*



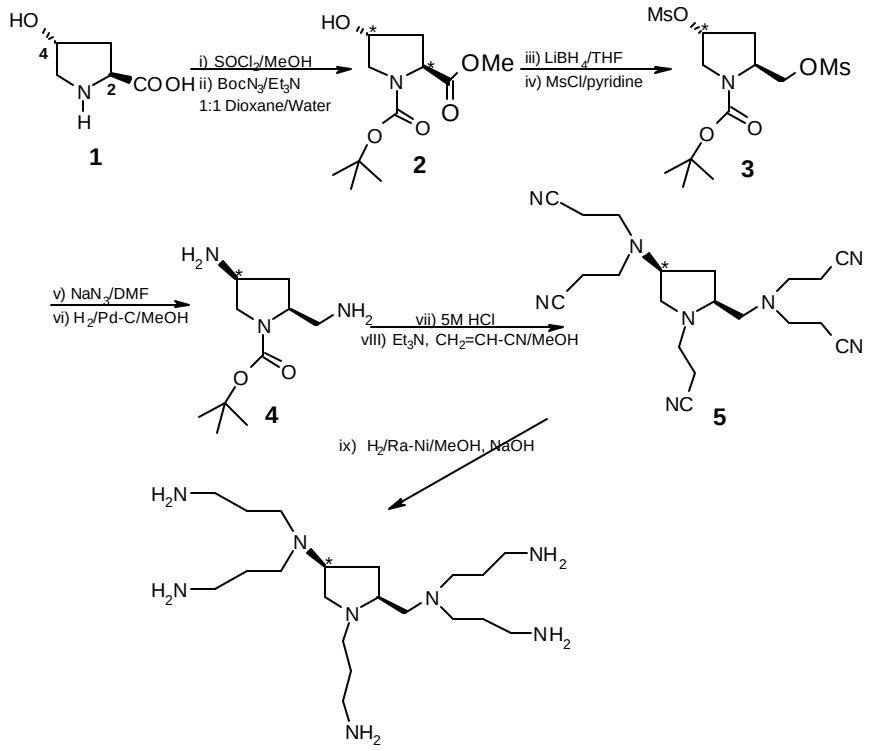
V (2*R*,4*R*)-D-*cis*



VI (2*R*,4*S*)-D-*trans*

Figure 1

This chapter describes the synthesis of all the four diastereomeric polyamines **III**-**VI** and their analogs**,** starting from commercially available *trans*-4-hyrdoxy-L-proline by a general synthetic route as described for polyamine **III** in Scheme 1. Final



Scheme 1

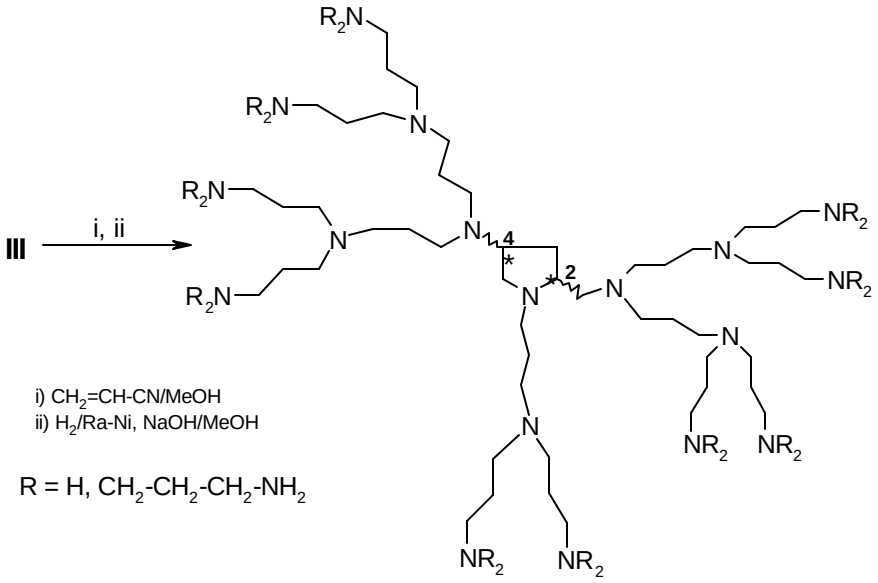
octamines were purified by ion-exchange chromatography. All the octamines were characterized by spectral analysis and structures were confirmed by FAB mass. 2*S*,4*R*-L-*trans* octamine **IV** was obtained from **2** by inversion of the secondary OH by a Mitsunobu reaction following similar sequence of reactions as in Scheme 1. 2*R*,4*R*-D-*cis* octamine **V** and 2R,4*S*-D-*trans* octamine **VI** were obtained from D-cis hydroxy proline, which was obtained from trans-hydroxy-L-proline by epimerisation reaction at C2.

As the surface charges on the polyamine increases the ability to stabilize DNA also increases due to electrostatic interaction. Hence, in the present work we

envisaged that growing the polyamine molecules in a divergent manner by repeated addition of aminopropyl chains to the chiral pyrrolidine core could lead to polycationic molecules. These kind of molecules interact with anionic phosphate groups of DNA and form micellar polyionic complexes and may have utility in DNA transfection. With this rationale molecules of type VII are synthesized. (Scheme 2)

Chapter 3:Bio-physical studies of Chiral, Branched Pyrrolidyl Polyamines:

In this chapter, Interaction of polyamines **III-VII** with DNA is described using various techniques like U.V, Fluorescence and CD spectroscopy. Oligonucleotide sequences **6***, **7, 8** required for this study were synthesized on an automated DNA synthesizer. Comparative stability of duplexes (**7:8)** and triplex **6*:7:8)** in the absence and presence of spermine **I** and branched pyrrolidyl polyamines **III-VI** were determined by U.V absorbance-temperature plots. U.V T_m was measured over a range of



Scheme 2

polyamine and salt concentrations and compared with spermine under identical conditions. Thermal denaturation studies showed that these molecules remarkably stabilize DNA at as low a concentration as 0.01 mM. Enhanced stabilization was predominantly due to strong electrostatic interactions of positive charges on amino groups with anionic DNA. This was also supported by salt and pH dependent studies.

The relative strength of DNA binding by these polyamines was also analyzed by Fluorescence assay using an intercalator Ethidium bromide. This assay was carried out individually with spermine and pyrrolidyl polyamines. The data indicated that the concentrations of polyamines required for a 50% displacement of ethidium bromide (C_{50}) were as follows: spermine (16.4 μM), (2*S*,4*S*)-L-*cis* **III** (0.96 μM), (2*S*,4*R*)-L-*trans* **IV** (1.1 μM), (2*R*,4*R*)-D-*cis* **V** (2.55 μM), (2*R*,4*S*)-D-*trans* **VI** (2.9 μM). Thus (2*S*,4*S*)-L-*cis* **III** seems to bind the DNA duplex strongest followed by the polyamines **IV**, **V**, **VI.** Results from this experiment showed similar trend as in case of U.V T_m and all the polyamines bind duplex DNA at least 8-15 times as strong as spermine under identical conditions.

The synthetic potential of proline in designing novel DNA binding agents such as constrained chiral pyrrolidyl polyamine analogs described above was extended to design and study constrained PNA analogs. This study will be presented in **Section B**.

Oligonucleotide sequences

6* d T T C T T T T T T C T T T T T C T

7 d G C C A A G A A A A A A G A A A A A G A C G C

8 C G G T T C T T T T T T C T T T T T C T G C G

SECTION B: Constrained Peptide Nucleic Acid analogs

Chapter 4: *Introduction: PNA analogs and derivatives*

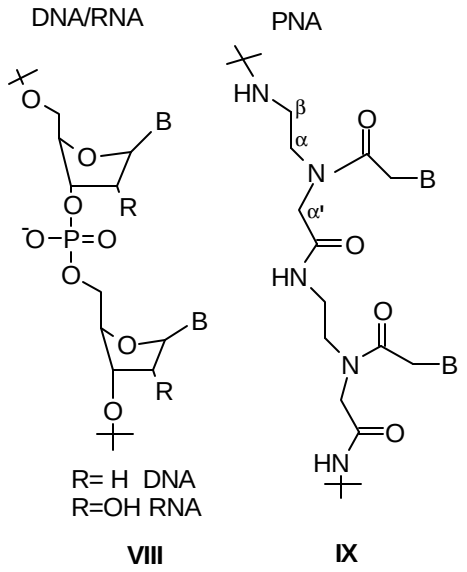
Interest in the potential of DNA analogs as gene-targeted drugs (antisense, antigene) has led to synthesis and evaluation of a number of its analogs. A knowledge of properties of these analogs with DNA and RNA could provide better understanding of structure of natural DNA, which is an important aspect to know its chemical and biological properties.

Among the various analogs studied to date Peptide Nucleic Acid (PNA) a new DNA analog has gained considerable interest. In PNA, the phosphodiester backbone has been replaced by an achiral polyamide backbone, with repeating units of N-(2-aminoethyl) glycine to which nucleobases are attached with an acetamide linkage. (Figure 2) PNA is homomorphous with DNA and homopyrimidine PNA oligomers bind very strongly to complementary DNA [$(PNA)_2$-DNA] by Watson-Crick, Hoogsteen

bonding to form triple helices that are much more stable than the DNA-DNA hybrids. They also bind to duplex DNA by strand displacement and form both parallel and antiparallel (N-terminus of PNA to 5' end of the DNA; C-terminus to 5' end respectively) complexes.

In contrast to homopyrimidine sequences, mix sequences of PNA bind to complementary DNA/RNA with 1:1 stoichiometry to form duplexes. Here, binding can be either parallel or antiparallel mode, antiparallel PNA-DNA or PNA-RNA having higher stability than parallel hybrids with high sequence specificity and affinity.

PNA backbone is uncharged and the increased thermal stability of PNA-DNA hybrids compared to DNA-DNA hybrids is ascribed due to the lack of electrostatic repulsion between the two strands. Inspite of the resistance to cellular enzymes such as nucleases and proteases, they do suffer from major limitations in their applications



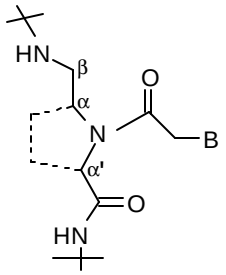
VIII **IX**

Figure 2

because of the poor cellular uptake, solubility problems and lack of selectivity in binding. To overcome these drawbacks, several modifications have been attempted eg. introduction of chiral amino acids in the PNA backbone, attachment of polylysine, polycationic spermine to the C-terminus of the PNA and the search for functional synthetic analogs is continuing. This section briefly reviews the recent advancements in the area of peptide nucleic acids.

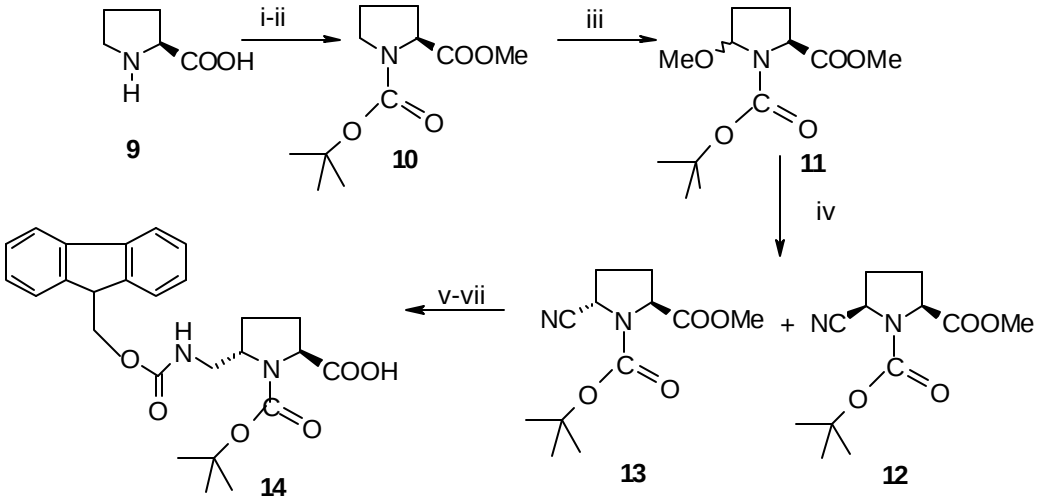
Chapter 5 : Synthesis and Bio-physical studies of conformationally constrained PNA analogs

There is a considerable interest in the chemical modification of PNA backbone to influence its properties such as binding affinity, specificity and cellular uptake. In this context, introduction of chirality and rigidity in the backbone by bridging the α-carbon



5-aminomethyl prolyl PNA

Figure 3



Reagents: i) $SOCl_2$/MeOH; ii) $BocN_3$/Et_3N-1:1 DMF/H_2O; iii) MeOH, Bu_4NBF_4; iv) TMS-CN, TMSTf/CH_2Cl_2,-35°C; v) H_2/$Pd(OH)_2$-MeOH; vi) 2N NaOH; vii) Fmoc Cl/10% Na_2CO_3

Scheme 3

of ethylene diamine with α'-carbon of glycine unit (Figure 3) by an ethylene units is an interesting approach. This gives rise to a five membered pyrrolidine ring with two chiral centers at C2 and C5.

The monomers of this type can be derived from either pyroglutamic acid or L/ D-proline. We have chosen the synthesis starting from L/D proline as described in Scheme 3, with the rationale that it would a provide a general route for the functionalization of hydroxy proline derivatives as well. A new functionality was introduced in at C5 in intermediate **10** by anodic methoxylation to get the product **11** as a diastereomeric mixture. Cyanation of this mixture was carried out using TMS-CN and catalytic amount of TMSTf at -35°C to obtain the 5-CN derivatives **12** and **13**, which were separated by flash chromatography. Catalytic reduction of the cyano compound yielded the 5-amino methyl derivative. Ester hydrolysis and protection of the primary amino group as Fmoc-carbamate gave the required monomer **14**, for the synthesis of modified PNA oligomers.

Oligomers (**15-20**) were synthesized on solid support using Boc-Fmoc strategy using Merrifield's standard solid phase peptide synthesis (SPPS). A single cycle using the modified monomer **14** is described in Scheme 4. Nucleobase was attached to the

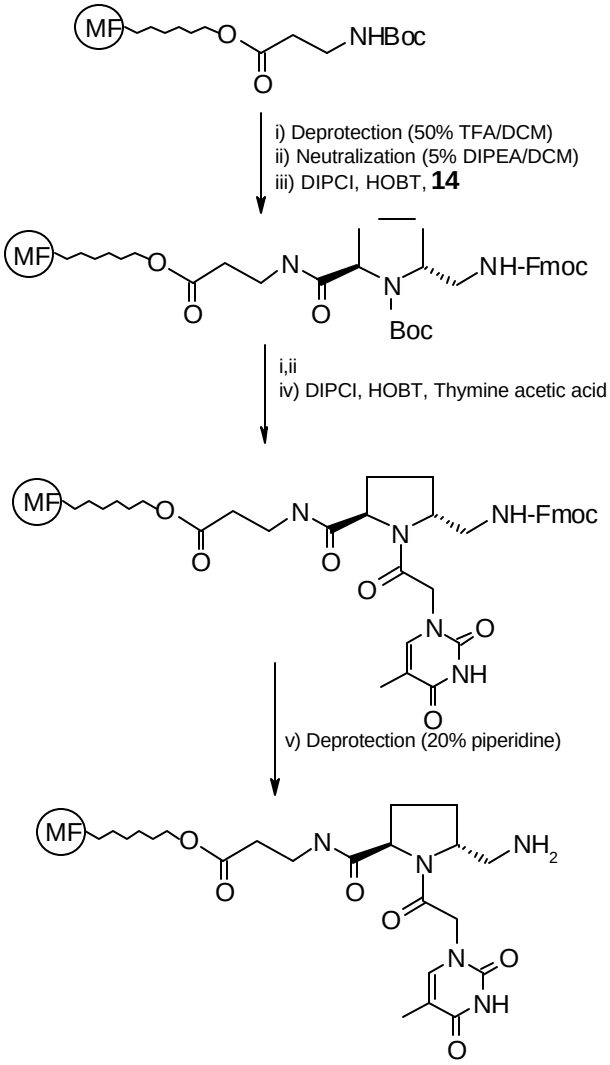
Table 1

	PNA sequences
15	H- T T T T T T T T- β- ala-OH
16	H- t T T T T T T T- β- ala-OH
17	H- T T T T T T T t- β- ala-OH
18	H- T T T t T T T t- β- ala-OH
19	H- G t A G A t C A C T-β-ala- OH
20	H- G T A G A T C A C T-β-ala- OH
21	5' d C G A A A A A A A A C G
22	5' d A G T G A T C T A C

ring nitrogen on the solid support. β-alanine was used as the first amino acid. At the end of the synthesis oligomers were cleaved from the solid support using trifluromethane sulfonic acid in TFA and purified on a C8 RP FPLC column. The bio-

physical studies of these oligomers (Table 1) and their complexing with complementary DNA (**21-22**) will be presented using various techniques like UV, CD spectroscopy and gel electrophoresis.

Solid phase peptide synthesis of amp PNA analogs
Boc/Fmoc strategy



Scheme 4

-----X-----

ABBREVIATIONS

A	Adenine/absorbance
amp	aminomethylprolyl
aeg	aminoethyl glycine
aq	aqueous
ala	alanine
ap	antiparallel
Ac_2O	Acetic anhydride
ß-ala	ß-alanine
cm	centimeter
CH_3CN	Acetonitrile
$CHCl_3$	Chlorofom
CH_2Cl_2	Dichloromethane
C	Cytosine
CHEN	Cycloheptenyl
CHEXN	Cyclohexenyl
CPEN	Cyclopropenyl
CPG	Controlled pore glass
CBz	Benzyloxy carbonyl
CD	Circular Dichroism
DCC	Dicyclohexyl carbodiimide
DCM	Dichloromethane
DCU	Dicyclohexyl urea
DIAD	Diisopropyl azodicarboxylate
d	deoxy
dG	2'-deoxy guanosine
DCE	Dichloroethane
dA	2'-Deoxy adenosine
DNG	Deoxynucleoguanidine
DE	diethyl
dc	decarboxylated/decarboxylase
dcSAM	decarboxylated S-adenosylmethionine
dC	2'-Deoxy cytidine
dT	2'-deoxythymidine
DIPCDI	Diisopropylcarbodiimide
DMAP	4-N,N' Dmethylaminopyridine
DMT	Dimethoxytrityl chloride
DIPEA	Diisopropylethylamine
DMF	N,N'-dimethylformamide
DNA	2'-deoxyribonucleic acid
DC-CHOL	(3-β-(N4-spermine carbamoyl)cholesterol

ds	Double stranded
eda	ethylene diamine
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAB	Fast Atom Bombardment
Fmoc	9-Fluorenylmethoxycarbonyl
FPLC	Fast Protein purification Liquid Chromatography
g	gram
G	Guanine
gly	Glycine
GNA	Glucosaminenucleic acid
GDEPT	Gene directed enzyme prodrug therapy
h	hour
HOBt	1-Hydroxybenzotriaziole
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IR	InfraRed
Lys	lysine
LNA	Locked Nucleic Acids
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MF	Merrifield Resin
mg	milligram
min	Minute(s)
MHz	Megahertz
µM	micromolar
mL	millilter
mM	millimolar
MS	Masspectrometry/Mass Spectrum
mmol	millimole
M	molar
MeOH	methanol
MTA	5'-Methylthioadenosine
N	Normal
NMDA	N-methyl-D-aspartatel
nm	nanometer
NMR	Nuclear Magnetic Resonance
NAP	Nucleic acid purification
ODC	Ornithinedecarboxylase
OPAC	ODN/PNA assisted affinity capture
ODN	deoxyoligonucleotide
PCR	Polymerase Chain Reaction

Pip	piperidyl
POM	Pyrrolidineamide oligonucleotide mimic
pyr	pyridyl
PPh_3	Triphenylphosphine
PNA	Peptide Nucleic Acid
Pro	Proline
p	parallel
PLL	Poly-L-Lysine
PEI	Polyethyleneimine
PEG	Polyethyleneglycol
PAMAM	Polyamidoamine
PhTx	Philanthotoxin
PLC	Phospholipidase
psi	per square inch
R	Rectus
RNA	Ribonucleic Acid
RNAse	Ribonuclease
r.t	room temperature
ROS	Reactive oxygen species
RP	reverse phase
S	Sinister
ss	Singlestrand/single stranded
SAMDC	S-adenosylmethionine decarboxylase
spm	spermine
SPPS	Solid Phase Peptide Synthesis
T	Thymine
tert-Boc	tert-Butoxy carbonyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
TR	Trypanothione Reductase
THF	Tetrahydrofuran
TEAA	Triethylammonium acetate
TRAP	Telomere Repeat Amplification Protocol
TMSTf	Trimethylsilyl Trifluoromethane sulfonate
TLC	thin layer chromatography
TMSCN	Trimethyl silyl cyanide
UV-vis	Ultraviolet-Visible

SECTION A

CHIRAL, BRANCHED PYRROLIDYL POLYAMINES: SYNTHESIS AND BIOPHYSICAL STUDIES

CHAPTER 1

INTRODUCTION

POLYAMINES BIOLOGICAL ROLE AND ANALOGS

1.1. INTRODUCTION

Biogenic polyamines (Figure 1) such as putrescine **1**, cadavarine **2**, spermidine **3** and spermine [N,N'-bis(aminopropyl)diamino]butane] **4** are widely distributed in the plant and animal kingdom.[i] These linear polyamines, which are aliphatic bases, possess a wide diversity in their biological and pharmacological activities[ii] both when free and on conjugation with other biomolecules such as sugars,[iii] steroids,[iv] alkaloids,[v] phospholipids[vi] and peptides.[vii] The linear polyamines are the natural products of amino

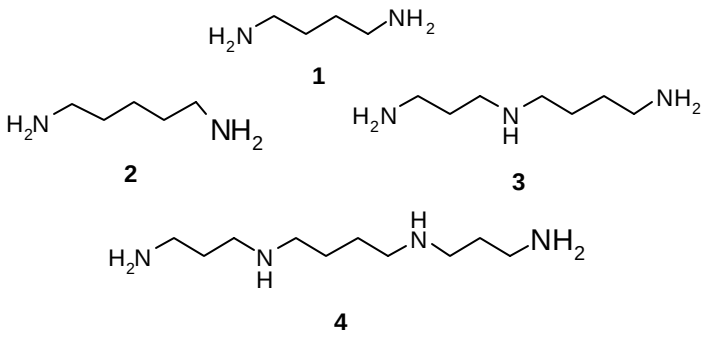
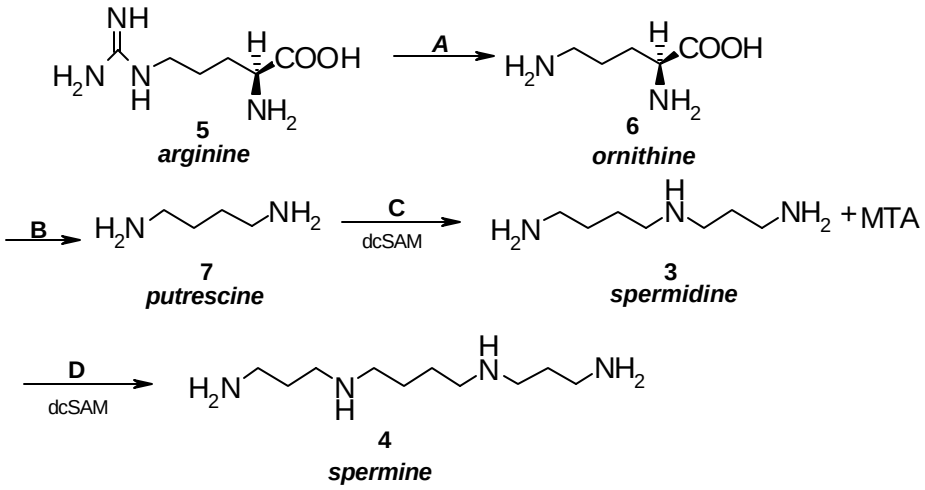


Figure 1. Biogenic Polyamines

acid metabolism and are ubiquitously found in all cells in substantial amounts. Although Leeuwenhoek[viii] first reported the isolation of spermine in 1678 as spermine phosphate crystals, only over the past few decades greater attention has been focussed on the naturally occurring polyamines and their analogs due to their potential biological properties and pharmacological applications.[ia,ix] Since many of the biological phenomena involving polyamines are not well understood there is a great need for newer synthetic analogs, conjugates and evaluation of their biological properties. This chapter presents an overview on polyamine literature, in relation to contents of Section A of this thesis, which deals with the synthesis of novel chiral, constrained branched polyamine analogs and the study of their interaction with DNA.

1.2. BIOSYNTHESIS OF POLYAMINES

In general, the biosynthetic pathway[x] of polyamines (Scheme **1**) involves conversion of L-arginine **5** to L-ornithine **6**, by enzyme arginase, followed by decarboxylation of ornithine by ornithine decarboxylase (ODC) to the diamine putrescine **1**, the simplest of the natural polyamines. This is then transformed to spermidine **3** by spermidine synthase and further to spermine **4** by spermine synthase. The aminopropyl groups in spermidine **3** and spermine **4** are derived from decarboxylated S-adenosyl methionine dcSAM that is the decarboxylation product of S-adenosyl methionine catalyzed by S-adenosyl methionine decarboxylase.



Enzymes: **A,** arginase; **B**, ornithine decarboxylase;
C, spermidine synthase; **D**, spermine synthase;
dcSAM, decarboxylated S-adenosylmethionine

Scheme 1. Polyamine Biosynthetic Pathway

1.3. BIOLOGICAL ROLE OF POLYAMINES

Although, the precise role of polyamines is not fully understood, many functions of prokaryotic and eukaryotic cells have been shown to be polyamine dependent.

Polyamines are essentially protonated at physiological pH[ixe] (i.e. ammonium ions at pH 7.4) and can interact with anionic species, in particular the nucleic acids. The polyamine biological polycations are essential for cell growth and division.[xi] Their functions range from stabilization/modulation of membrane functions[xii] and mitochondrial activities to facilitating DNA transfection by phage.[xiii] Intracellular polyamines are essential for cell proliferation[xiv] in particular, neoplastic growth and chemical carcinogenesis.[xv,xvi] Their cellular concentration is highly regulated by their metabolic pathway. Spermine and spermidine are known to stabilize chromatin and nuclear enzymes[xvii] and ribonucleases.[xviii] Polyamines are also involved in the modulatory activity of NMDA receptors[xix] and polyamine analogs have potential applications in therapy of neurological diseases such as epilepsy and neurodegenerative diseases.[xx] Polyamines affect the protein synthesis in several ways.[xxi,xxii] In addition, they also interact with phospholipids of biological membranes, inhibit their peroxidation,[xxiii] affect the structure of biological membranes[xxiv] and help defense of the organisms against tumor development.[xxv]

1.3.1. Polyamine-DNA interaction

Polyamine interaction with DNA results in stabilization of DNA conformations *in vitro* and *in vivo,*[xxvi] thus influencing many biological processes such as transcription, regulation and recombination. This occurs mainly via the electrostatic interaction of positive charges on these molecules with the negative charges on phosphates of DNA.[1a,xxvii] Polyamines are thought to bind by bridging the two strands of DNA to effect structural changes.[xxviii] Their strong interaction with the anionic phosphate backbone leads to DNA condensation by continuous charge neutralization.

The polyamines also stabilize double-helical structures like stems and loops in *r*RNA and *m*RNA and stabilize *t*RNA conformation and ribozymes.[xxix] Because of polycationic structures in which the positive charges are distributed at fixed lengths

along a flexible carbon chain, polyamines are able to bridge critical distances.[xxx] The charge distribution in spermine molecule makes it bind strongly to two phosphate groups in each strand or DNA double helix spanning the major and minor grooves.[xxxi] The distal ammonium charges in spermidine and spermine are separated in the extended conformation by 1.1 and 1.6 nm respectively. These features allow specific interactions and functions that are not shared by the metal ions.[xxxii]

Spermine is known to promote the stability of duplex and triplex when added externally,[xxxiii] as well as in conjugation with sugar[xxxiv] or to the nucleobases.[xxxv] Analysis of difference Raman spectra of highly polymerized calf-thymus DNA at different polyamine concentrations for putrescine, spermidine and spermine using natural and heavy water as solvents revealed structural specificity in the interactions of these polyamines. This specificity lead to the preferential binding through the DNA minor grooves for putrescine and spermidine whereas spermine binds by the major groove. On the other hand, spermine and spermidine present interstrand interactions while, putrescine presents intrastrand interaction in addition to exo-groove interactions by phosphate moieties.[xxxvi]

Molecular dynamics simulation studies of polyamine-DNA systems showed that the major groove is the preferred binding site on A-DNA for the polyamines. Putrescine 1 and cadaverine 2 tend to bind to the sugar phosphate backbone of B-DNA, whereas spermidine 3 and spermine 4 occupy different sites including the binding along the backbone and bridging the major and minor grooves.[xxxvii]

The exact mechanism by which polyamines bind to DNA are far from clear, but it is known that they interact with B-DNA and produce conformational changes.[xxxviii] Spermine induces helical bending leading to DNA condensation and toroidal particles. Polyamines are also known to cause B-Z transition and stabilize the Z-DNA.[xxxix] Two distinct modes of spermine complexation to ZDNA hexamer $d(CGCGCG)_2$ have been observed in crystal structure, one type interacting with phosphate backbone and the

other with basepairs.[xl] Crystal structure[xxviiia] of complex of digocation spermine [[N,N-bis(aminopropyl)diamino]butane, $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+$] and the DNA octamer $d(GTGTACAC)_2$ at 2.0Å resolution showed that the octamer adopts a right handed A-DNA structure and the GT/CA block engages in a novel cross-strand bifurcated hydrogen bonding between the purines G and A. In the complex, spermine is bound to the floor of the deep major groove of A-DNA where it interacts only with bases and not with the phosphates.[xxviiia] In another study[xli] at 1.9 Å resolution, spermine was found to bind across the major groove of a B-DNA dodecamer d(CGCGAATTCGCG) in the major groove of a GC base pair. The terminal primary ammonium groups are involved in electrostatic interactions with phosphate groups and the guanine base on opposite strands of the DNA complex.[42] Central secondary ammonium groups are located close to the guanine O-6 and cytosine N-4 substituents. Lower resolution studies of the structure of yeast phenyl alanine *t*RNA showed that, spermine was found to interact with phosphates (Figure **2**) as well as bases across the deep grove.[xlii]

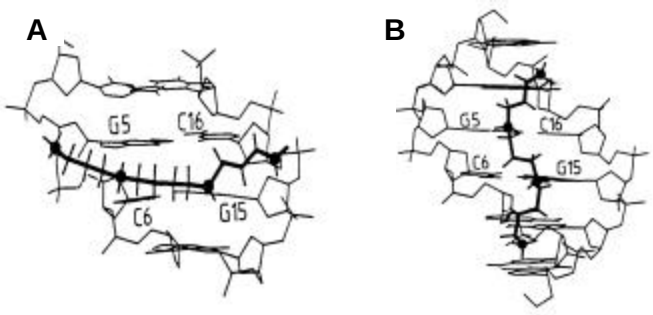


Figure 2. Minimized structures showing the complexation[xxx] of spermine to major groove of $d(CGCGCGCGCGCG)_2$ in the (**a**) cross groove (**b**) down groove binding sites. The spermine molecule is represented by thickened lines with circles indicating N atoms of the protonated amine groups.

Solution studies have implicated a diffused non-specific mode of binding of spermine to the anionic phosphate backbone of DNA.[xliii] Electrical dichroic measurements have indicated that spermine induces a bend in poly (dA-dT) by binding

in the major groove, while stiffening of helix in poly (dG-dC).[xliv] These studies were also supported by the hydrogen-deuterium exchange rate [an enhanced exchange rate in case of poly (dA-dT)] measurements upon spermine complexation.[xlv] Polyamines protect DNA from denaturation due to heat, radiation and chemical reagents. They also guard DNA against strand breakage induced by Cu(II)/H_2O_2 generated reactive oxygen species (ROS) similar to other antioxidants.[xlvi] Depletion of the natural polyamines in cells results in alteration of chromatin and DNA structure and leads to programmed cell death.[xlvii] Polyamines also protect DNA against the formation of radiation induced strand break and cross-linking to proteins. This occurs due to the compaction of DNA structure and consequent reduction in the accessibility of DNA to hydroxyl radicals.[xlviii]

1.4. APPLICATIONS OF POLYAMINES IN CHEMISTRY AND BIOLOGY

The above discussion on the biological role of polyamines underlines the pharmacological importance and therapeutic implications of polyamines. This section briefly outlines their applications in chemistry and biology.

Substituted spermidine and spermine derivatives were found to be potent

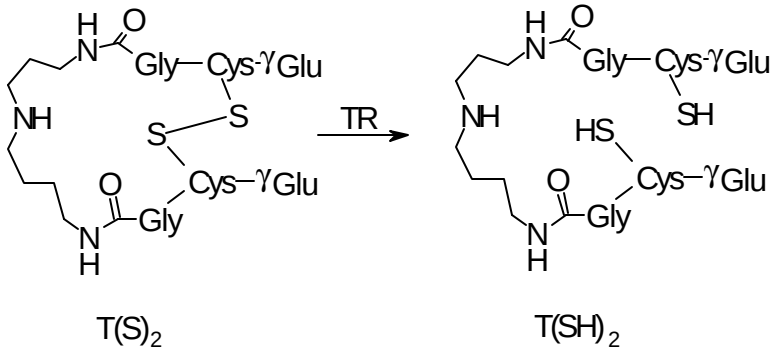


Figure 3. TR mediated transformation of trypanothione to reduced trypanothione

competitive inhibitors of trypanothione reductase (TR) (Figure 3) an enzyme present in trypanosomes,[xlix] which are the causative agents of several diseases like African

sleeping sickness and Kala-azar. Excessive accumulation of the natural polyamines favors malignant transformation of cells. Structural analogs of the polyamines with polyamine-mimetic or antagonist properties are drugs which affect several key steps in polyamine metabolism and are potential candidates for therapeutic prevention of carcinogenesis of the gastrointestinal tract.[I]

The induction of an early increase in polyamine metabolism, termed 'the polyamine response' is recognized to have a critical role in the reaction of neurons to injury and protecting them from dying after the infliction of various types of neurotrauma, including mechanical injuries, neurotoxic insults and ischemia.[Ii]

Several polyamine derivatives attached with or without an intercalative moiety (Figure 4) were found to mimic the active center of pancreatic ribonuclease (RNAse A) and exhibited potent RNAse cleaving activity at near-physiological conditions

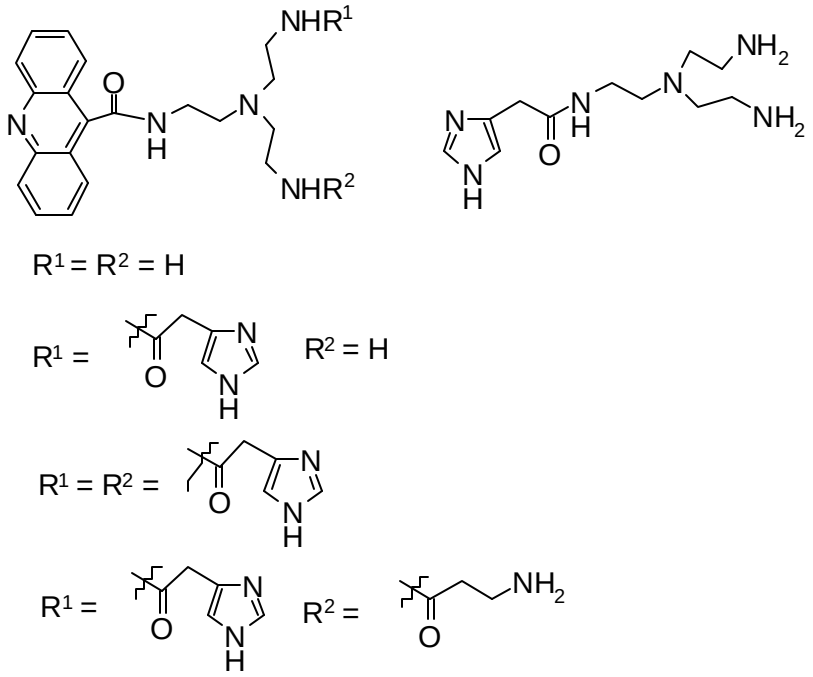


Figure 4: Polyamine-based biomimetic catalysts

emphasizing the existence of nucleic acid binding function in the mimic and the role of 2'-OH group in the substrate to be important factors in showing cleavage activity.[lii]

1.4.1. Gene transfecting agents

Gene transfection is a fundamental technology for molecular biological research and also for gene therapy[liii] in the genetic treatment of several diseases eg. (a) by introduction of genes encoding toxic proteins into tumor cells. (b) by Gene Directed Enzyme Prodrug Therapy (GDEPT) for the treatment of cancer involving introduction of a gene encoding a prodrug-activating enzyme.[liv] Major limitation in the development of gene therapy is the lack of an efficient method of selective delivery of DNA to the nucleus of the target cells using viral or non-viral vectors. To overcome the drawbacks such as immunogenecity, transient duration of gene expression and low capacity for foreign genes associated with the viral vectors, non-viral gene delivery systems[lv] including cationic lipids[lvi] lipid-polyamine constructs[lvii] and other cationic polymers [such as poly-L-Lys (PLL) and polyethyleneimine (PEI)] have been introduced as DNA transfection agents.

The transfer efficiency of complexed DNA into a cell especially for *in vivo* transfer depends on many factors - interaction between the complexes and cell membranes, type of cells involved, cationic components and particularly the ratio of positive charges to negative charges (N/P) of different components of the complex - play an important role in deciding the efficiency. Currently, little is known about the intracellular traffic after endocytosis of the DNA complexes and consequently, there is a need to develop new compounds especially cationic compounds with improved properties. Recently, polyamine-poly(ethylene glycol) constructs (Figure 5) have been designed to form hydrophilic complexes with DNA in which the PEG can form a sheath around DNA for the effective gene delivery. [lviii]

However, there are a number of problems with cationic lipid systems. The DNA-cationic lipid complexes tend to aggregate in aqueous solution, particularly in presence of salts and proteins.[lix] Thus only small amount of DNA is formulated in this way. Water-soluble, polycationic complexes linked to the hydrophilic polymers have been

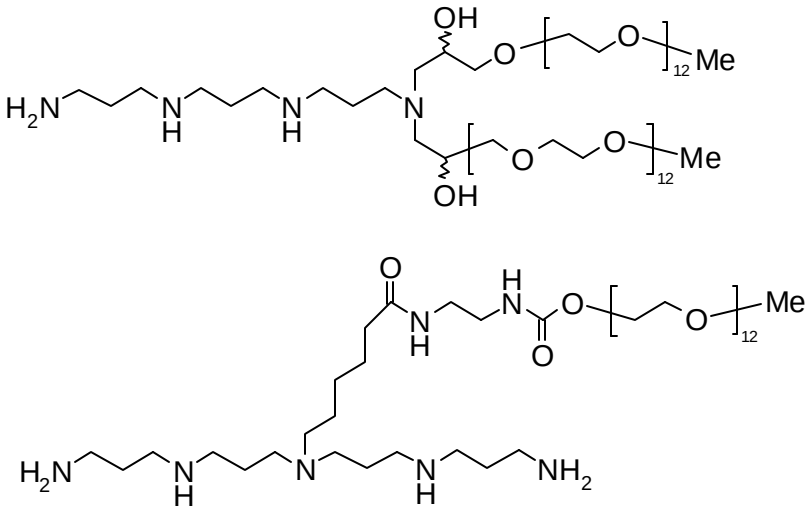


Figure 5. Polyamine-PEG

shown to have better gene transfection efficiency. Among the synthetic DNA delivery agents, cationic lipids based on cholesterol structure (eg. 3-β-[N,(N',N'-dimethylaminoethane)-carbamoyl]cholesterol[lx] (DC-Chol) and (3-β-(N4-spermine-carbamoyl)cholesterol are promising candidates that are already under clinical trials. In these molecules, a carbamate linker that controls the flexibility/rigidity of the molecule links the hydrophobic cholesterol (see page 25) and the hydrophilic sperminyl moieties.

Synthetic non-viral gene transfer vectors, free from the risks associated with viral vectors, will be of considerable potential to the gene-therapy field. Over the past decade, a large variety of polycationic lipids,[lxi] liposomes, lipopolymers and their molecular conjugates for improving cell targeting, cytoplasmic delivery or nuclear transport have been used extensively to deliver genes into a large variety of cell lines

and tissues. Recently, glycosylated polycationic DNA vectors have been introduced as gene vectors to decrease the surface charge density of lipoplexes.[lxii]

1.4.2. Dendrimers for the delivery of antisense agents into cells

The difficulty of getting nucleic acids into cells has hampered efforts to develop both antisense and antigene therapies. Non-viral gene vectors using different cationic liposomes and polymers such as polylysine[lxiii] and PAMAM (polyamidoamine) dendrimers (see Section 1.6.7) have attracted wide attention in gene delivery technology. Early studies using PAMAM dendrimers proved to be very efficient and less toxic than polylysine and are found to be better in stabilizing the oligonucleotides within the cell.[lxiv] Also, De Long *et al*.[lxv] showed that a third-generation PAMAM dendrimer forms stable complexes with phosphorothioate-linked oligonucleotides, which have utility in antisense approach. Recently, bifunctional cationic compounds have been used for efficient delivery of genes to hepatocytes.[lxvi] These compounds consists of a poly(propyleneimine) dendrimer as the DNA binding domain in combination with trivalent galactoside as the cell targeting ligand connected with a hydrocarbon chain. In this class of molecules the dendrimer part compacts the DNA, the galactose residues on the other end of the molecule serve as targeting ligand for the hepatocytes and the hydrocarbon chain in the middle provides a sheath around DNA that protects it against degradation by the biological fluids.[lxvi]

1.5. NATURAL POLYAMINE CONJUGATES

A large number of polyamine conjugates have been isolated from natural sources and found to have potential biological activities as described in the following sections.

1.5.1. Linear polyamine alkaloids

Phenols and phenolic acids are known to play an important role in plants defense repertoire. Some plants contain phenolic acids such as coumaric and ferulic

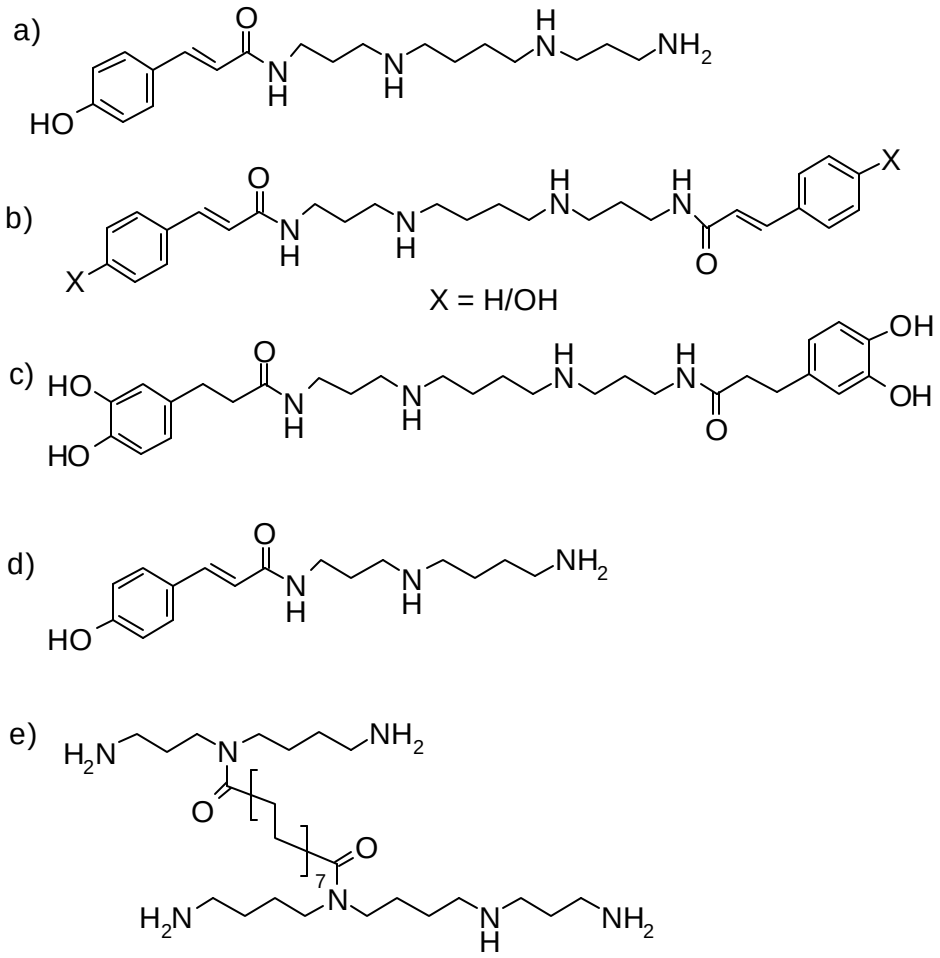


Figure 6. Plant derived polyamine conjugates with hydroxycinnamic acids
a) coumaroyl spermine b) Meytenine c) Kukoamine A d) coumaroyl spermidine
e) Tenuilobine

acid (Figure 6) that are conjugated with polyamines.[lxvii] Polyamine conjugates found in corn and several Solanaceous plants such as tobacco, tomato and potato bear a

strong similarity to acylpolyamines isolated from spider and wasp toxins (see Section 1.5.2). These toxins are known to act on invertebrates by antagonizing ion-gated channel proteins in the muscle membrane.

The conjugate of coumaric acid and spermidine (N8-coumaroyl spermidine) (Figure 6d) is shown to be an antagonist of glutaminergic neuromuscular junctions of crayfish.[lxviii] The alkaloid Tenuilobine (Figure 6e) is the first natural example where both spermidine and spermine are cross-conjugated through a long aliphatic chain.[lxix] Kukoamine A,[lxx] a linear spermine alkaloid containing two hydrocaffeoyl moieties per spermine (Figure 6c) is found to have hypotensive properties and shown to be an inhibitor of tyrypanothione reductase.[lxxi]

1.5.2. Macrocyclic Polyamine Alkaloids

Apart from the linear polyamine alkaloids (Section 1.5.1), several macrocyclic systems[lxxii,lxxiii] (Figure 7) having polyamines as important constituents have been isolated from plants. These include macrocyclic spermidine alkaloids like the

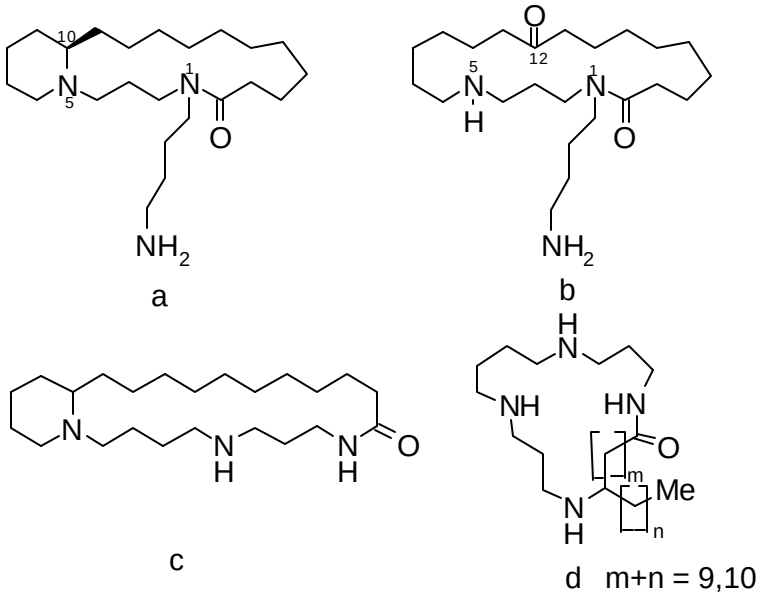


Figure 7. Macrocyclic Oxo Lactams a) Oncinotine b) inandenine-12-one c) isooncinotine d) pithecolobin

oncinotine, (Figure 7a) Isooncinotine (Figure 7c), the inandenines (Figure 7b) and pithecolobins (Figure 7d).

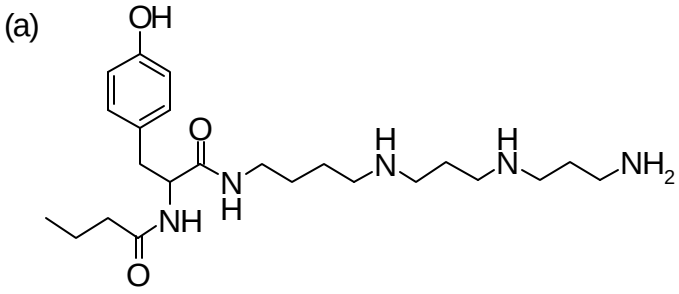
1.5.3. Invertebrate venom toxins from spiders and wasps

A variety of polyamines conjugated to aminoacids such as β-alanine, glycine, lysine, arginine, asparagine, and tyrosine have been isolated from spiders and wasps[lxxiv,lxxv] (Figure 8) and these comprise of philanthotoxin (PhTX-433, Figure 8a) a non-competitive antagonist of certain ionotropic receptors, nephilatoxin 643 (Figure 8b) and α-agatoxin (Figure 8c).[lxxvi] These acyl polyamines are potent antagonists of mammalian neuroexcitatory glutamic acid receptors. Acyl polyamines found in spider venom[lxxvii,lxxviii] (Figure 9) possess an aromatic carboxylic acid and the polyazaalkane chain (α,ω-diaminopolyazaalkane. The different carboxylic acids found as lipophilic head groups constitute, hydroxylated benzoic acids, 3-indolyl acetic acid, and its hydroxylated derivatives as well as 3-indolyl lactic acids. The polyazaalkane chain consists of one to nine aminopropyl-, aminobutyl-, or aminopentyl- units. The aromatic carboxylic acid is characteristically bonded through an amide linkage to the polyazaalkane chain with the help of amino acids like asparagine, aspargic acid, ornithine or ω-methyllysine. Other amino acids like β-alanine and lysine are attached to the polyazaalkane chain as in Joramine and pseudoargiopinine-1 (Figure 9). The N-methylated analogs are found in some of the acyl polyamines like pseudoargiopinine-1 but no N-oxidized compounds are known so far.

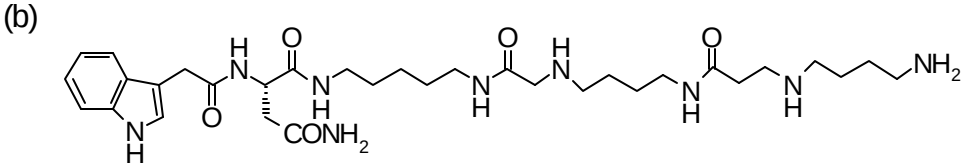
The special feature of these acyl polyamines is that they are very good inhibitors of Ca^{2+} ion channels.[lxxix] These selectively block various glutamate receptors, which are common exciting neurotransmitters in the brain of mammals. Ionotropic glutamate receptors are coupled to an ion channel, which regulates the influx of cations

into cells, which is an important function in signal transduction in the peripheral nervous system in insects.

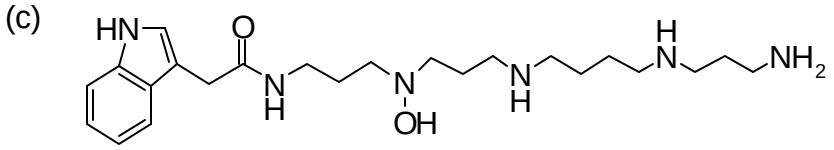
The NMDA receptors play a role in some neurological diseases such as Alzheimer's disease and epilepsy.[lxxx] Spider toxins were the first natural blockers of such receptors. With this varied potential, acyl polyamines serve as lead structures in therapeutics, for new pharmaceuticals and insecticides. Interestingly, all the polyamines do not show the same effects. Even a small structural change leads to a



PhTX(433): a wasp venom from Philanthus trinagulum



Nephilatoxin-643 a spider toxin



AG-448 a funnel-web spider α-agatoxin

Figure 8. Invertebrate venom toxins

varied action as in the case of joramine R = H (Figure 9b) and spidamine R = OH (Figure 9b). The variation in hydroxyl group makes joramine a reversible blocker of neuromuscular synapses whereas, spidamine with a hydroxyl group at C2 of the

aromatic head group is an irreversible blocker. Besides blocking glutamate receptors, acyl polyamines also inhibit the nicotinic acetylcholine receptors. These align in the ion channel with hydrophobic aromatic head group pointing towards the cells and polyazalkane chain blocking the channel. A longer chain leads to a better binding in the receptor as more N-atoms present in the chain interact electrostatically with amino acids of the ion-channel proteins.[lxxi]

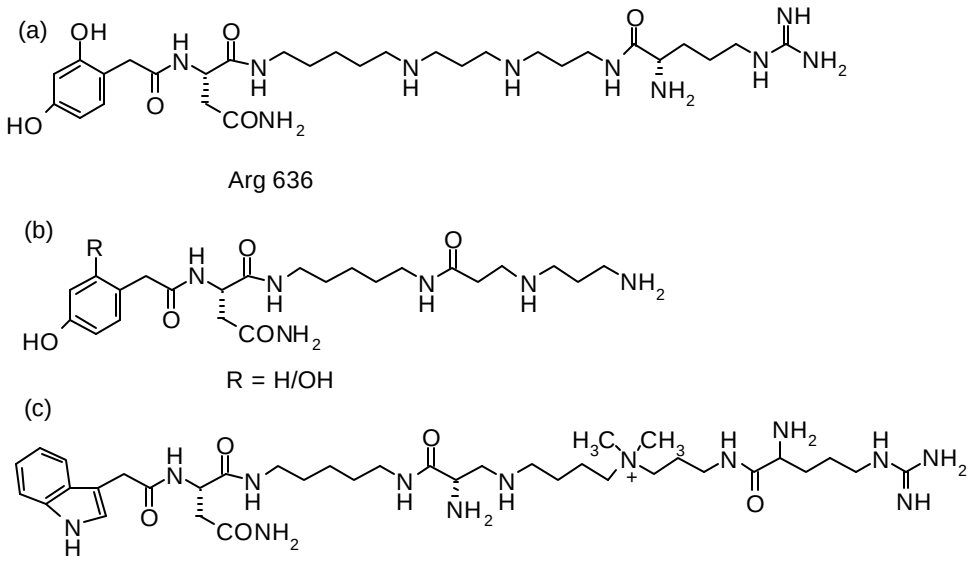


Figure 9: a) Argiopine (Arg 636) b) Joramine/Spidamine c) Pseudoargiopinine

1.5.4. Polyamine alkaloids from marine organisms

Polyamine conjugates have also been isolated from marine sponges. These are found to exhibit interesting biological properties, eg. spermidine conjugates such as pseudokeratidine (Figure 10a) are important components in antifouling paints due to their antimicrobial activity[lxxii,lxxiii] and penaramide is a strong inhibitor of N-type Ca^{2+} channels.[lxxiv] Spermidine metabolites (Figure 10b) isolated from soft coral are also known to exhibit cytotoxic properties[lxxv] and Pitlomycalin A (Figure 10c) a marine

alkaloid has potent antiviral and antimicrobial activity.[lxxvi] Ispidospermidine (Figure11), spermidine alkaloid is a natural inhibitor of PLC, which affects cellular growth, differentiation and cell multiplication.[lxxvii]

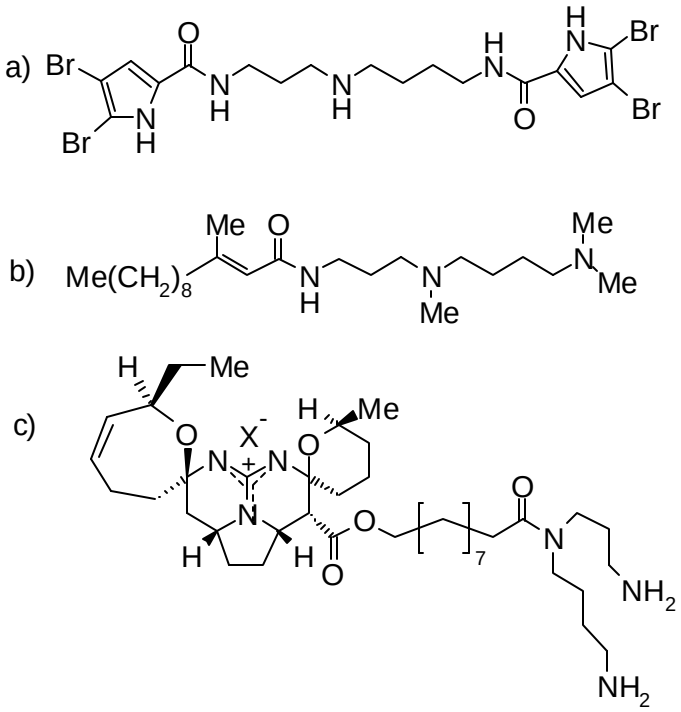


Figure 10. Marine Alkaloids a) Pseudokeratidine c) Pitlomycalin A

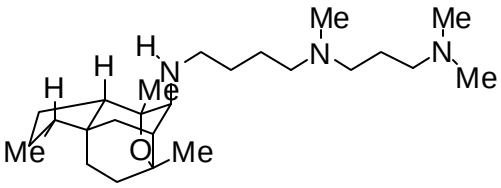


Figure 11

1.6. SYNTHETIC POLYAMINE ANALOGS AND CONJUGATES

Understanding the role-played by natural polyamines and their conjugates in biological systems and elucidation of the structures of various polyamine conjugates found in nature has led to the design and synthesis of various analogs for potential therapeutic applications. The combined effort of biologists and synthetic chemists has led to the design and synthesis of several unnatural analogs of polyamines with varying degrees of anticancer, antitumor, antimicrobial and other important pharmacological activities. Since many of these analogs have shown to possess high therapeutic potential, synthesis and evaluation of various types of analogs is continuing to be an active area of research. Recently, polyamines have attracted attention in material chemistry because of the ease with which dendritic structures can be constructed.[lxxxviii,lxxxix] Such structures are expected to possess unusual physical properties and utility in biological areas such as transfecting exogeneous genes in mammalian cells.[xc] The high concentration of polyamines and higher activity of anabolic enzymes in cancerous cells has led to the design of various analogs. Many analogs were designed and synthesized mainly to inhibit biosynthesis enzymes, such as ornithine decarboxylase and S-Adenosyl-*L*-methionine decarboxylase.[xci] This section overviews the important synthetic analogs of polyamines.

1.6.1. Structural modifications of natural polyamines

Several polyamine analogs have been synthesized in recent past, which are modified natural polyamines. A list of such structural modifications is shown in Figure 12. Most of the modifications involve substitution of the terminal amino functions, eg. spermine and norspermine analogs[xcii] (Figure 12a,b), homologation of the hydrocarbon chain as in homospermine analogs, bisadamantyl homospermines (Figure 12c and 12e) and variation in the number of nitrogen atoms in the chain or introduction of an aromatic moiety into the polyamine backbone[xciii] (Figure 12h). Structure-activity relationship studies of a large number of these analogs[xciv] against tumor cells revealed

that, the cytotoxicity depends on the number of nitrogen atoms, distance between nitrogen atoms, nature of terminal alkyl substituents and the charge density. The terminally alkylated polyamines have been shown to have potent antitumor and antiparasitic activity.[xcv] Weakly charged polyamines like pyridine analogs cannot compete with the natural polyamines for transport through cell membranes.[xcvi] Among the linear polyamines reported so far, polyamines bearing ethyl or benzyl groups were

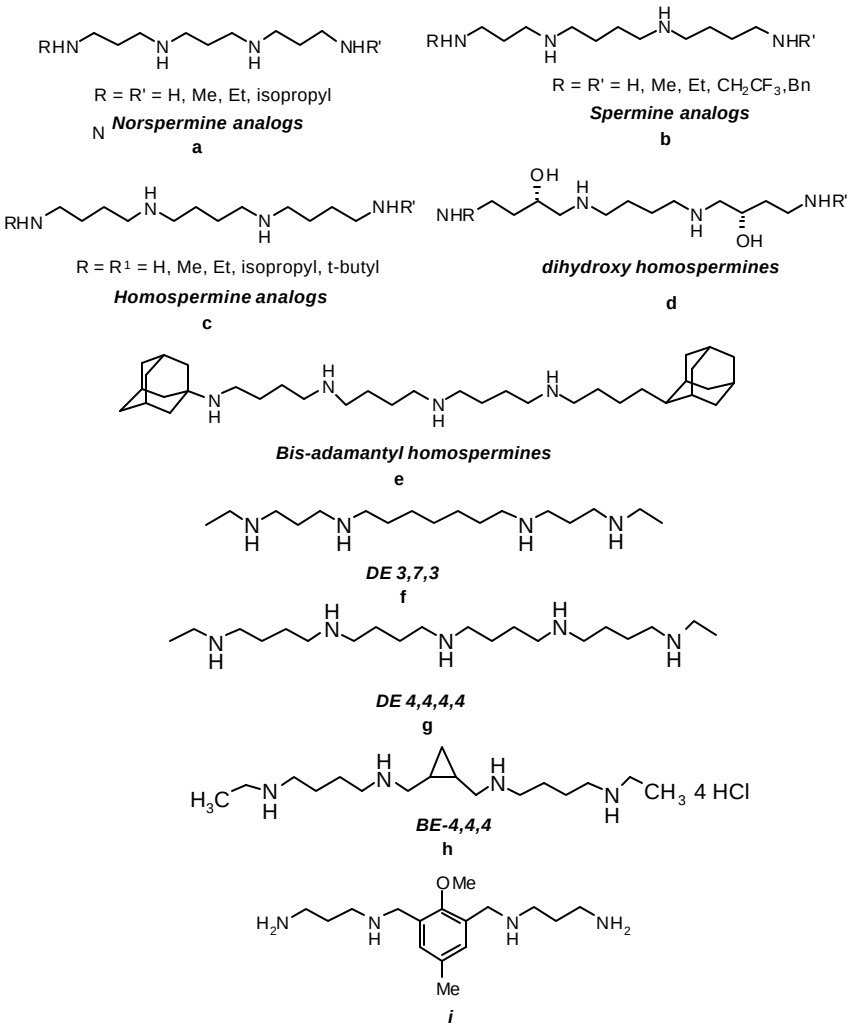


Figure 12. Linear Polyamine Analogs

found to be promising candidates in cancer therapy; the diethyl analogs DE-3,7,3 and DE-4,4,4,4 (Figure 12f,g) are already under the clinical trials.[xcvii] The alkylated tetramine analogs eg. cis cyclopropyl analogs of BE-4-4-4 (Figure 12h) were found to be highly

cytotoxic.[xcviii] The synthesis and evaluation of pentamine analogs of BE 4-4-4-4 (Figure 12g) with the introduction of *cis* double bonds has also been reported. The potency of inhibition of NMDA receptors by N,N' terminal dialkylated homologs of tetramine spermine decrease in the order (a) alkylation: N-terminally alkylated polyamines > terminal primary amines, (b) length of the polyamine backbone: homospermine > nor spermine (c) size of the terminal alkyl groups: adamantly > *tert*-butyl > ethyl > methyl. This emphasizes the potential of tetramine backbone as an important pharmacophore to modulate NMDA receptor channel function. The unsymmetrically alkylated polyamine analogs (Figure 13) have been found to protect DNA against strand breakage induced by Cu(II)/H_2O_2 dependent oxygen-radical generating system.[xcix]

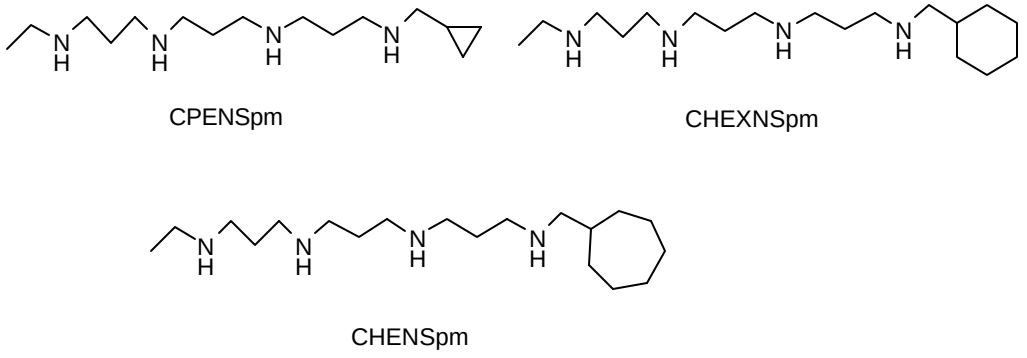


Figure 13. Unsymmetrically Substituted Polyamine Analogs

The alkyl and acyl spermidines and spermines carrying hydrophobic aromatic groups (Figure 14) exhibit a great role as the inhibitors of trypanothione reductase, an enzyme present in trypanosomes.[c]

The replacement of the methylene groups with an isosteric oxygen atom lead to the formation of oxa-isoster of polyamines (Figure 15). The polyamines bearing an aminoxy group have good bacteriostatic properties[ci] and are useful tools in generating novel information on the role of polyamines in biological systems.[cii,ciii]

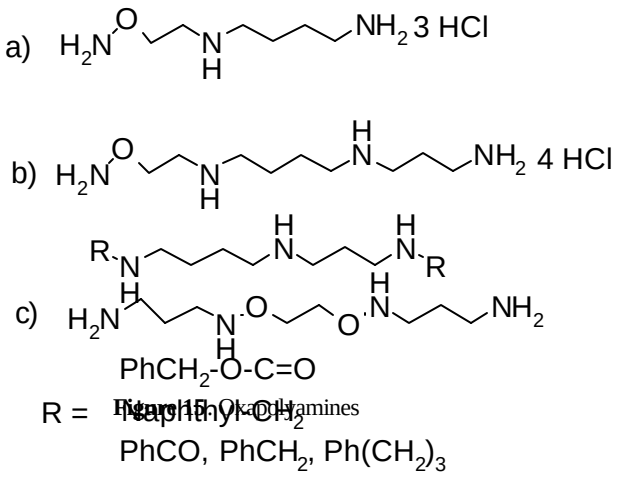


Figure 15. Oxa-polyamines

Figure 14

Bisguanidine analogs of spermine (Figure 16) have been reported as better DNA duplex and triplex stabilizing agents compared to spermine. In these modifications

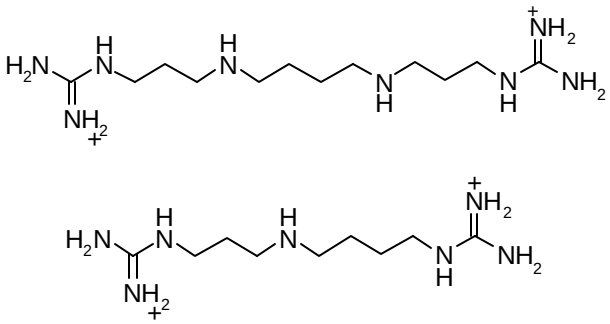


Figure 16. Guanidino Polyamines

additional charges in the form of guanidine group enhances the binding efficiency of these molecules.[civ]

1.6.2. Cyclic polyamines

Conformationally restricted polyamines have been synthesized in which N-C4-N part of the natural polyamine has been locked (Figure 17) with a polymethylene chain of variable length[cv]. Macrocyclic polyamines (Figure 18) have also been prepared to

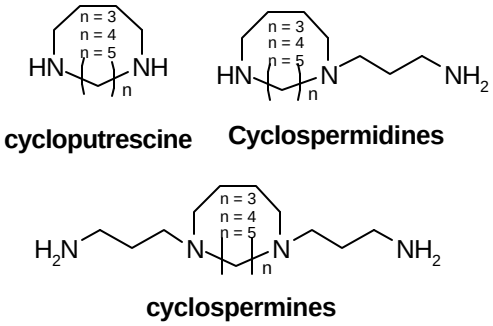


Figure 17. Cyclo polyamines

study their complexation with nucleotidyl phosphates.[cvi] Polyamines of the cyclophane type were found to selectively destablize the folded RNA and form Cu^{2+} complexes.[cvii]

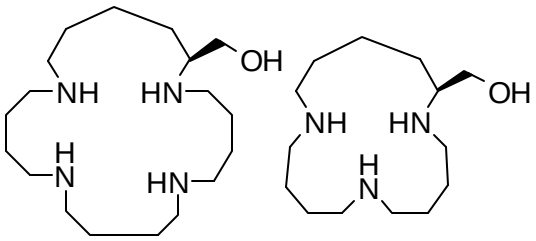


Figure 18. Macrocyclic Polyamines

In another report cyclic analogs of diethylspermines (Figure 19) have been synthesized in which the terminal ethyl groups are tied back into chain forming cyclic

bispiperidines. These and polyamines with terminal aromatic dipyridines were used to study the effect of charge. The piperidine analog forms a tetracation while the pyridine

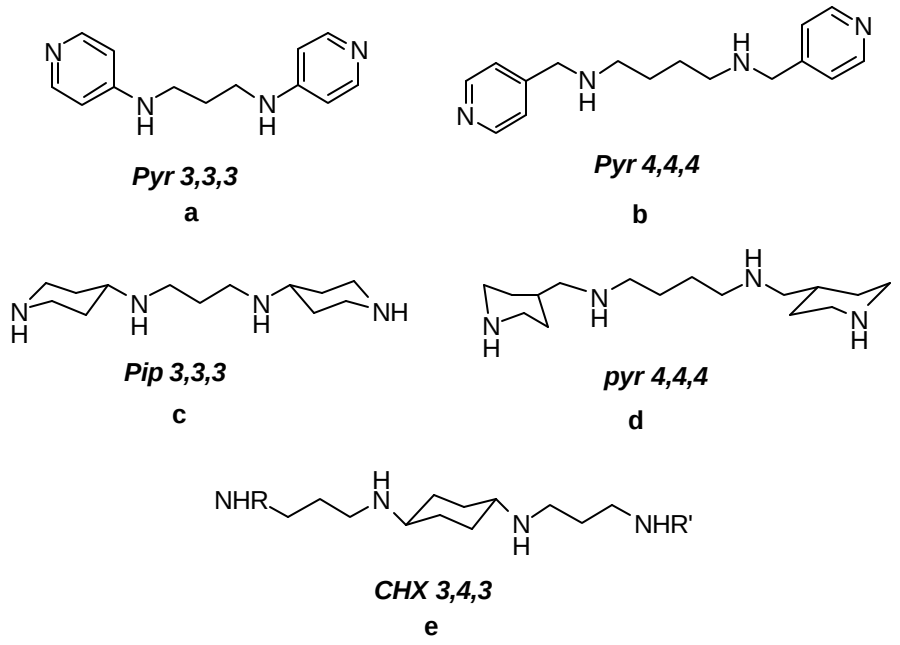


Figure 19. Dipyridyl and Dipiperidyl DENSPM analogs

analog forms only a dication at physiological pH and competes poorly with the linear polyamines.[cvi]

1.6.3. Polyamine-Nucleoside/Nucleotide conjugates

Polyamine conjugated nucleosides and nucleotides have been synthesized and their biological properties have been studied.[cviii] Polyamine oligonucleotide conjugates synthesized using standard solid phase protocol in which the polyamine chain attached to the purine or pyrimidine and suitably protected[xxxv,cix] (Figure 20) are used to study duplex and triplex stabilizing properties. They are useful for studying uptake of nucleosides and modified oligonucleotides in cells. [cx]

1.6.4. Polyamine-peptide conjugates

These constitute another important class of compounds that show promising

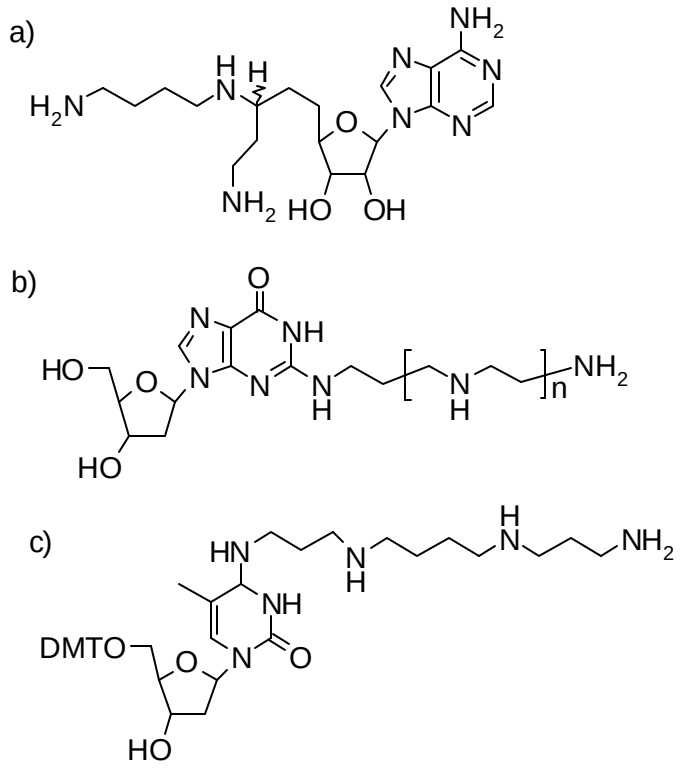


Figure 20. Polyamine-Nucleoside conjugates

biological activity.[cxi] Polyamine-Peptide conjugates containing the phosphinate group within the polyamine chain and a dipeptide moiety (Figure 21) exhibited potent inhibition of glutathionyl spermidine synthase/amidase, a key enzyme that participates

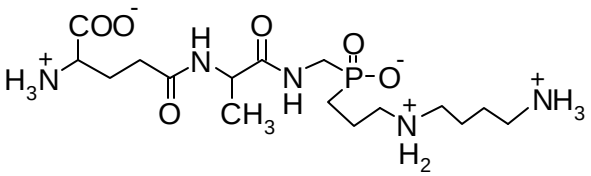


Figure 21

in trypanothione biosynthesis.

1.6.5. Steroidal and lipid polyamine conjugates

Natural polycationic polyamines spermidine and spermine compact DNA through interactions reversible under physiological conditions. Lipopolyamines (Figure 22) in which the hydrocarbon tail is linked to a cationic head group eg. Cholesterol polyamine (Figure 22a),[cxii] Lipospermine (Figure 22b) and steroidal polyamines (Figure 22c) like squalamines[cxiii] binds DNA and were shown to stably condense nucleic acids into discrete nucleolipidic particles.[cxiib,dcxiv] The amphiphillic dihydroxy cholanamide (Figure 23a) derivative synthesized by Blgabrough *et al*[cxv] showed poor binding affinity

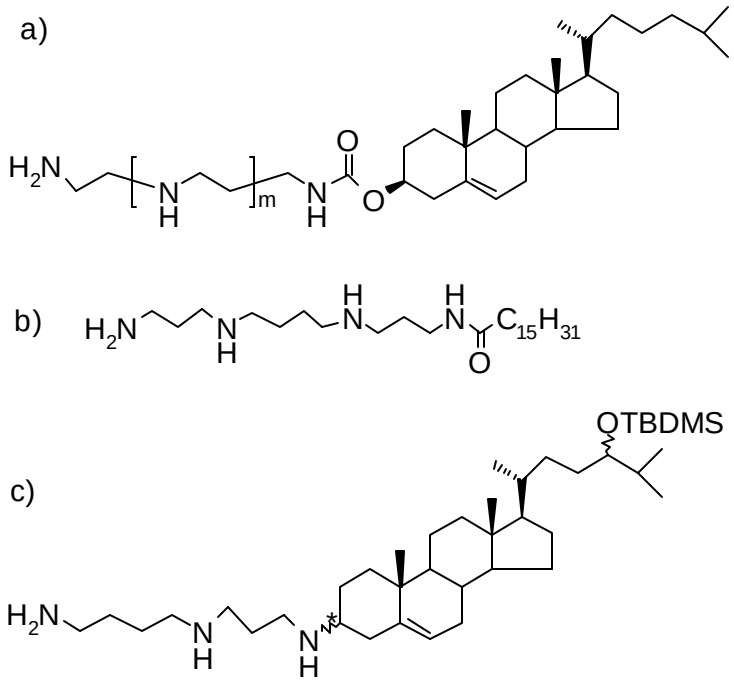


Figure 22. a) Cholesterol-polyamine b) Lipospermine c) α,β-Squalamines

to DNA when compared to the multicationic polylysine.[cxvi] Among the synthetic DNA

delivery agents, cationic lipids based on cholesterol structure (eg. 3-β-[N,(N',N'-dimethylaminoethane)-carbamoyl]cholesterol[cxvii] (DC-Chol) (Figure 23b), (3-β-(N4-spermine-carbamoyl) cholesterol[cxviii] (Figure 23c) were found to be promising

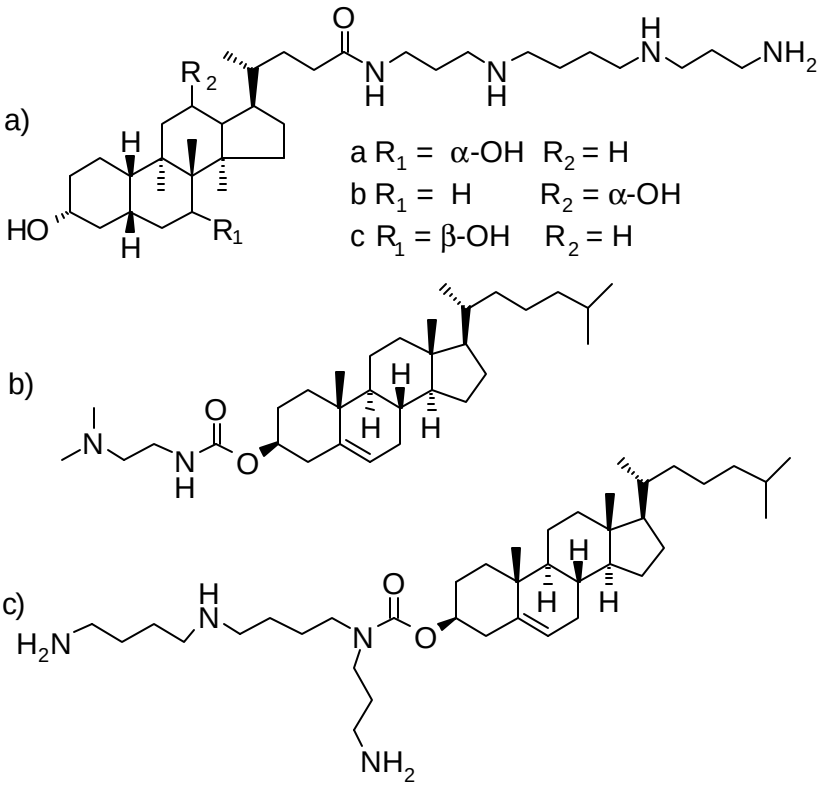


Figure 23. (a) Bile Acid Polyamine Amides (b) DC-CHOL (c) 3-β-(N4-soernube-carbamoyl)cholesterol

candidates and are already under clinical trials. In these steroid-polyamine conjugates, polyamine chain length and steroid structure and space filling were found to influence the gene transfection efficiency.[cxix]

1.6.6. Molecular umbrella-spermine conjugates

Double and tetra walled molecular ubmbrella-spermine conjugates have been synthesized based on covalent coupling of two or more rigid amphiphillic walls[cxx] to the

central scaffold. They cover the attached agent (a mock drug) and shield it from the incompatible environment. These new class of surfactants were hypothesized to enhance the permeability of pendant polar molecules eg. peptides or antisense oligonucleotides across the lipid bilayers by masking their hydrophilicity. In these analogs spermine was specifically chosen as the mock drug on the basis of its affinity towards DNA and also its strong hydrophilicity.[cxxi]

1.6.7. Dendritic polyamines

Polyamines are used in the synthesis of dendrimers that are a new class of highly branched polymers[cxxii] (Figure 24). The interior cavities and multiple peripheral functional groups facilitate potential applications of these molecules in biomedicine and bioorganic chemistry. This class of molecules can self-assemble with DNA and are used for transfecting exogenous genes into the mammalian cells. The synthetic strategies adopted are either convergent or divergent. In the convergent strategy

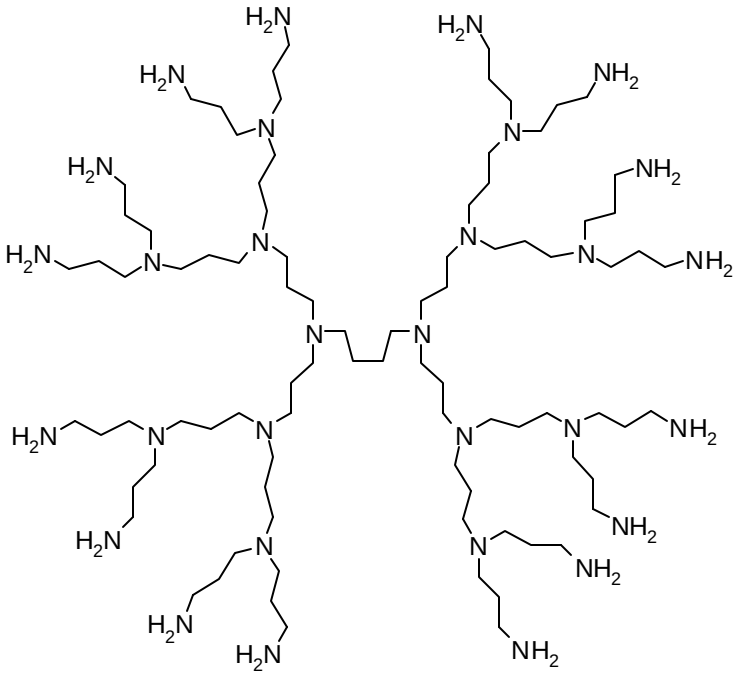


Figure 24. Polyamine dendrimer

smaller fragments are joined in the end of the synthesis and in the divergent strategy the molecule is constructed starting from the core by repeated addition of building units.

1.6.8. Miscellaneous Polyamine-conjugates

Carboranyl analogs (Figure 25) of natural polyamines have been developed as potential agents for boron neutron capture therapy of brain tumors.[cxxiii,cxxiv]

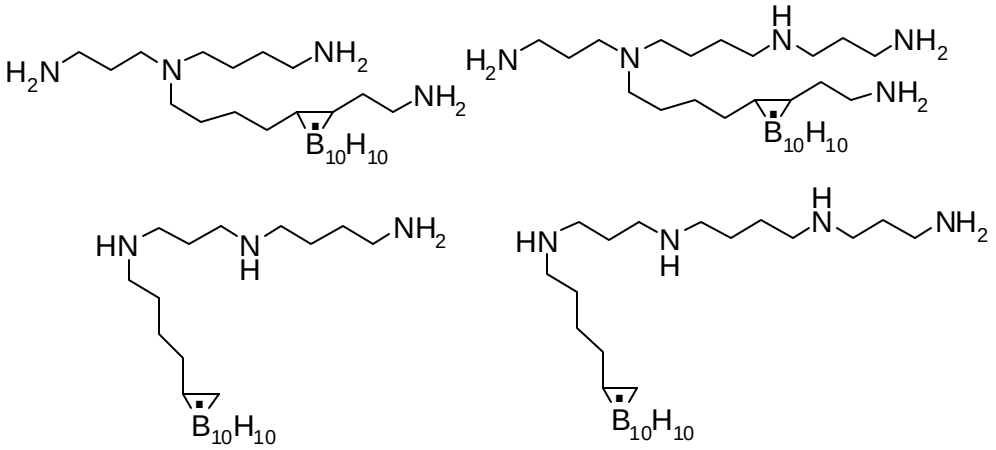


Figure 25. Boranyl polyamines

Conjugation of polyamines with cytotoxic agents like chlorambucil (Figure 26) led to the derivatives, which can cross the cellular membrane to reach the target DNA.

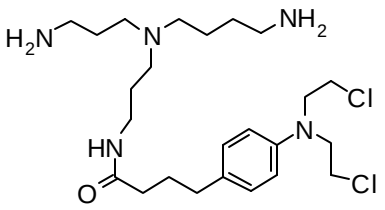


Figure 26

Chlorambucil is known to cross-link the two helices of DNA which is shown to be more effective when conjugated to the polyamine.[cxxv]

Polyamines containing thiophene in the chain (Figure 27a) have been found to be useful in the elucidation of the physiological significance of polyamine regulatory site of NMDA receptor complex.[cxxvi] Branched polyamines such as the tetramine (Figure 27c) have been used in the preparation of iron chelators[cxxvii] and the hexamine (Figure 27b) in the synthesis of dendrimers.[cxxviii]

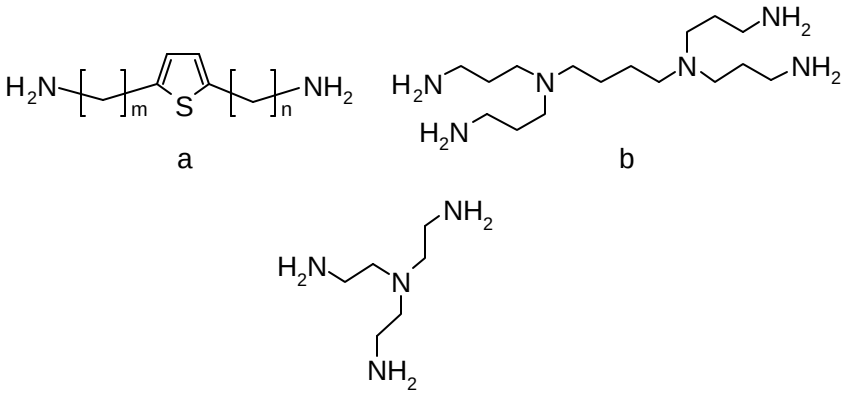
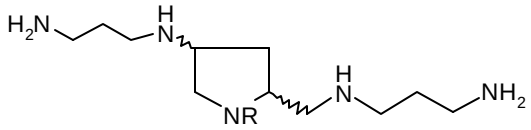


Figure 27

1.6.9. Conformationally constrained polyamines

The modifications discussed so far are achiral and have high degree of conformational freedom, which limits the selectivity of binding in the biological systems.



R = H, Boc

Figure 28. Chiral spermine analogs

To enhance the specificity of binding of polyamines with bioreceptors such as membranes, nucleic acids, which are chiral in nature, several modifications have been introduced recently with a conformational constrain in the existing polyamine backbone structure. A recent development of this approach was presented in the synthesis of chiral analogs of spermine (Figure 28) with rigidity in the N-C4-N region of the natural polyamine and with chirality.[cxxix] The new chiral analogs were found to bind as strong as spermine to duplex DNA or even stronger than spermine in binding to triple helical DNA. This study still leaves much room for further modifications and future studies are required to understand the effect of chirality of the polyamine moiety in stabilizing DNA duplexes and triplexes. As a follow up on this work recently, polyamines with cyclopropyl, cyclobutyl, alkynyl and O-C_6H_4 moieties have been prepared for their use as anticanceragents[cxxx] (Figure 29). This approach has been shown to be very successful in the area of peptidomimetics.[cxxxi]

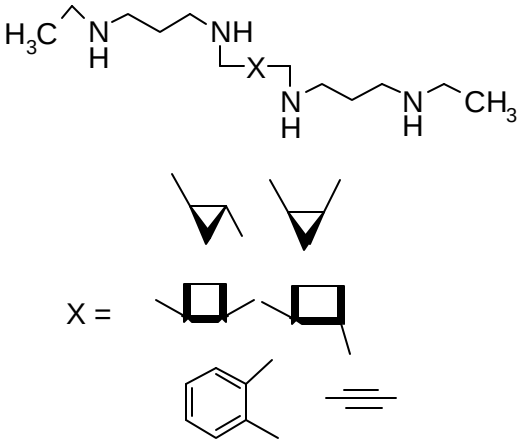


Figure 29. Constrained Polyamines

1.7. PRESENT WORK

As described in the previous sections of this chapter there has been a tremendous development over the past few decades in understanding the chemistry and biochemistry of polyamines and their conjugates, triggered by their potential applications. **Chapter 1** has outlined the various natural and synthetic polyamines, their conjugates, the role of polyamines in the biological process and their potential applications in various fields. The work in this thesis is mainly devoted to the synthesis of a new class of chiral, branched polyamines, resulting from conformational constraints of linear polyamines and their interaction with DNA. The constrain approach is also shown to be applicable to design of novel peptide nucleic acid analogs in Section B (Chapters 4 and 5).

Chapter 2 describes the synthesis of chiral pyrrolidyl polyamines, which are envisaged for the selective interaction with nucleic acids. Due to the presence of the additional aminopropyl side chains, the cationic charge density on these molecules is high for more efficient interaction with DNA. The synthesis of target polyamines from the commercially available *trans*-4-hydroxy-L-proline that has two chiral centers at C2 and C4 is described. Suitable functionalization of these centers lead to the synthesis of all the four possible stereoisomers (2 pairs of diastereomers) required for the study. The synthesis of a second generation chiral dendrimer corresponding to octadecamines is also reported.

Chapter 3 presents studies on the interaction of the synthesized chiral pyrrolidyl polyamines with DNA using UV, CD and fluorescence spectroscopy. The results indicate some selectivity in binding of duplexes and triplexes as a function of chirality.

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CHAPTER 2

SYNTHESIS OF CHIRAL, BRANCHED PYRROLIDYL POLYAMINES

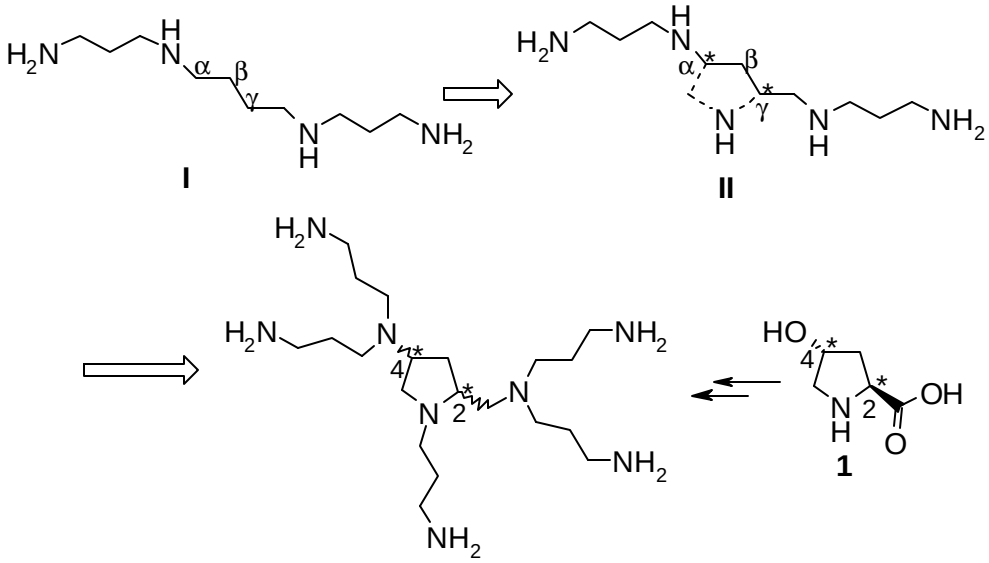
2.1. INTRODUCTION

As described in the introduction section, linear polyamines have ubiquitous biological activity and many polyamines and their conjugates occur in nature. The biological receptors of polyamines such as proteins, nucleic acids and membrane components are chiral in nature, while most of the common natural polyamines are linear and achiral. The many structural/synthetic modifications made to the polyamine backbone have involved variation in the number and distance between nitrogens,[1] terminal N-substitutions of varying size,[2] rigidification via interconnection of secondary amines (cyclopolyamines) based on cycloputrescine core[3] and introduction of aromatic moiety into the polyamine backbone.[4] Due to the chiral nature of many polyamine receptors (nucleic acids and membranes) it was envisaged that the introduction of chirality in the polyamine would be beneficial for their selective interaction. The strategy of designing polypeptides with conformational constriction has been fruitful in the area peptidomimetics.[5] In a previous work from this laboratory, conformationally constrained cyclic, chiral analogs of spermine were reported.[6] These molecules were found to be better than spermine in selective stabilization of DNA triplexes. Following such a strategy, a number of other constrained N,N'-bisethyl spermines were developed and shown to possess interesting biological properties,[7] in particular, considerable growth inhibitory effects on human tumor cell lines. Due to the success of this approach, it was thought to extend it to the higher, homologous polyamines.

2.2. PYRROLIDYL POLYAMINES: RATIONALE AND OBJECTIVES OF THE PRESENT WORK

Pyrrolidyl polyamines are derived from spermine **I** by bridging the α and γ carbon atoms of the central methylene fragment with a $-CH_2NH-$ group, generating a five membered pyrrolidine ring **II** (Scheme 1). This makes the molecule chiral with generation of two asymmetric centers at C2 and C4 resulting in 4 stereomers (2 pairs

of diastereomers / 2 pairs of enantiomers) with an additional nitrogen atom as compared to the tetramine spermine. In the present work, these molecules are elaborated by additional aminopropyl chains leading to chiral and branched pyrrolidyl polyamines **III-VI** with increased charge content. These also form the nuclear structures for the expansion into chiral polyamine dendrimers. Although other chiral dendrimers are known in literature,[8] most polyamine dendrimers[9] are built around a symmetrical core. Synthesis is hence extended to the second generation by repeated acrylonitrile condensation and reduction of the resulting nitrile functionalities to introduce more alkylamino branches leading to chiral dendritic polyamines. In addition to better interaction with DNA due to both charge and structural effects, these newer polyamine analogs and dendrimers may find potential applications as drugs or drug delivery agents and DNA transfection agents.[10] This chapter describes the synthesis of all the four stereoisomeric octamines **III-VI** and their higher analogs starting from the commercially available (2*S*,4*R*)-*trans*-4-hydroxy-L-proline.

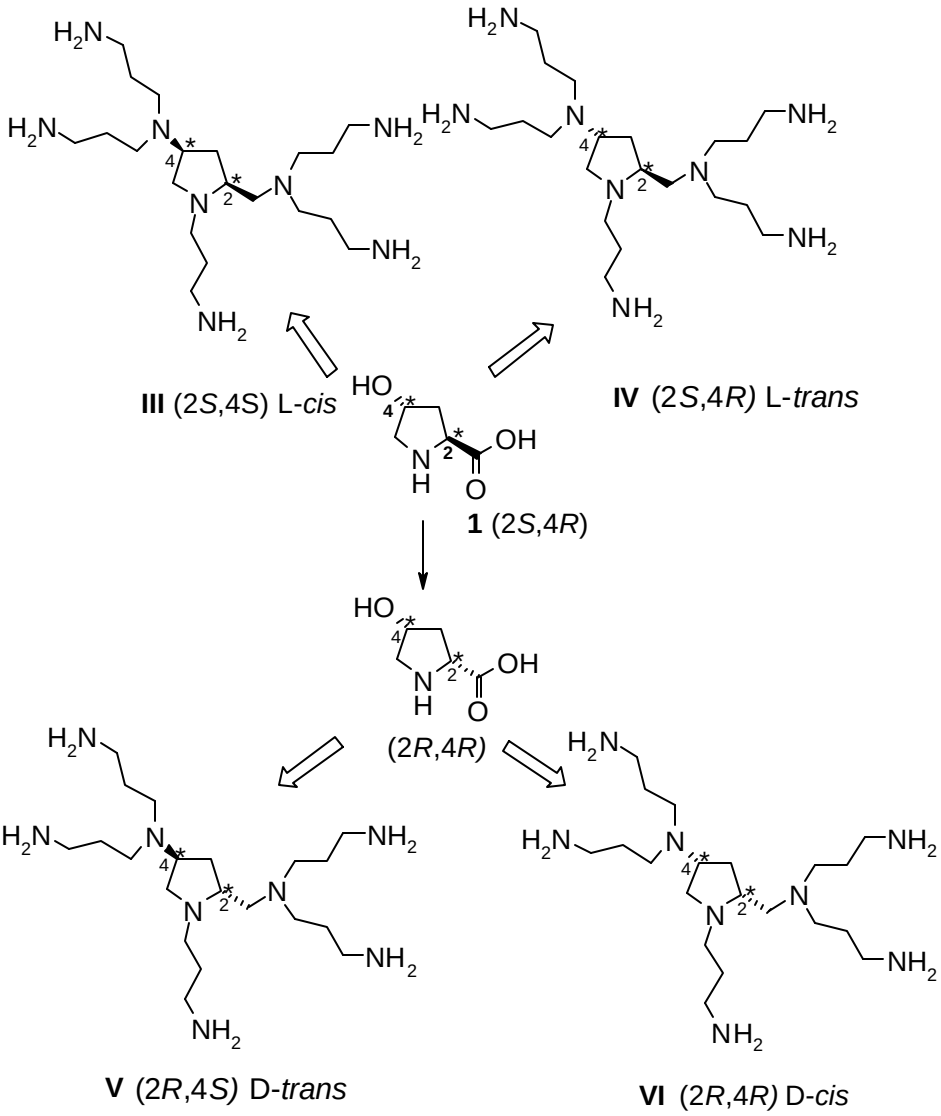


Scheme 1

The objectives of the present chapter are

(a) Synthesis of all the four possible stereoisomers of pyrrolidyl octamines (**III**-**VI**) from *trans*-4-hydroxy-L-proline **1** as in Scheme 2.

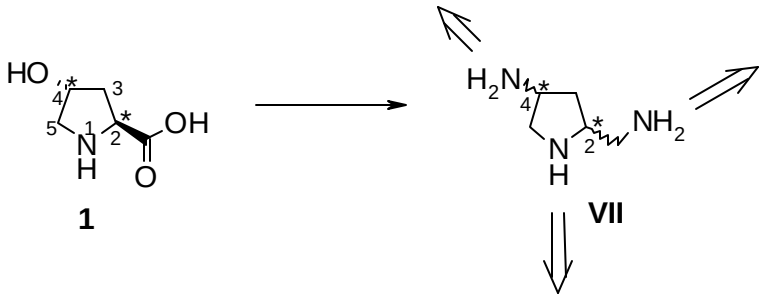
(b) Homologous extension of the end amino groups in polyamines by repeated Michael addition and reduction of the polynitrile functionalities to get the second-generation dendritic polyamines.



Scheme 2

2.3. SYNTHESIS OF PYRROLIDYL POLYAMINES

The commercially available *trans*-4-hydroxy-L-proline **1** is a versatile precursor[11] for functionalization into the required polyamino functionalities. The *trans*-4-amino-L-prolinylamine **VII** synthesisable from **1** is suitable for building chiral-branched polyamines, since it has three amino groups with different reactivities and two different stereocenters (Scheme 3). Such a compound should enable generation of a polyamine library with diversity in structure, charge (number of amino groups) and stereochemistry.

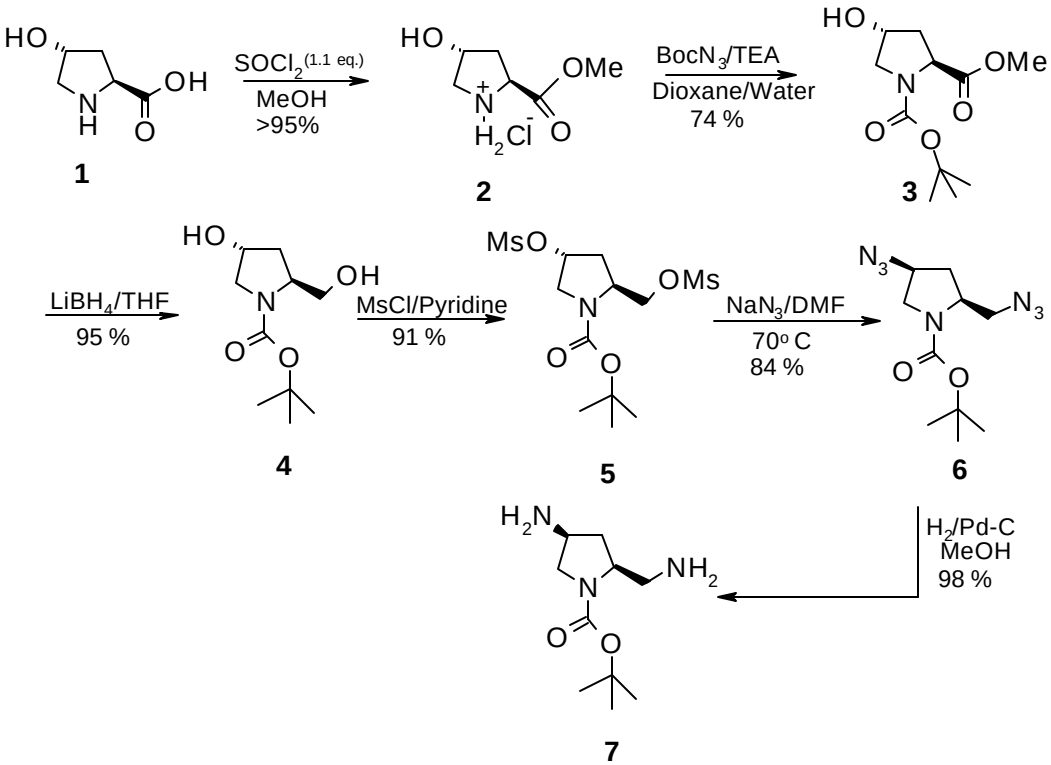


Scheme 3

2.3.1. Synthesis of (2*S*,4*S*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N,N'-bis(3-aminopropyl)amino]-N1-(3-aminopropyl) pyrrolidine (III)

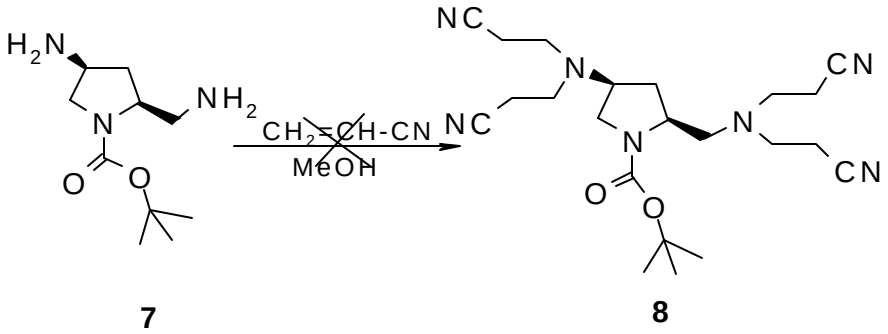
The transformation of *trans*-4-hydroxy-L-proline **1** into the desired triamine **III** was accomplished by subjecting it to a set of reactions as shown in Scheme 4. The starting material **1** was first converted to its 2-carbomethoxy ester **2** by refluxing with thionyl chloride (1.1 eq.) in methanol. The nitrogen of the proline ring (N1) of methyl ester **2** was protected as *tert*-butoxycarbonyl group by reaction with *tert*-butyl carbazide and triethylamine in dioxane/water to get (2*S*,4*R*)-*trans*-4-hydroxy-N1-(*tert*-butoxycarbonyl)-L-proline methyl ester **3**.[12] The ester function in **3** was then reduced to the corresponding alcohol using lithium borohydride in anhydrous THF to yield (2*S*,4*R*)-*trans*-4-hydroxy-L-prolinol **4** in 95% yield.[13] A complete reduction of the ester

to alcohol was confirmed by the disappearance of the ester methyl peak at δ 3.72 and the appearance of $-CH_2OH$ peak at δ 3.60-3.35 in the ^1H NMR spectrum. The (2*S*,4*R*)-L-*trans* diol **4** on mesylation using methanesulfonyl chloride (2.5 eq.) in pyridine gave the (2*S*,4*R*)-*trans* di-*O*-mesylate **5** in quantitative yield. ^1H NMR of the di-*O*-mesylate showed two singlets at δ 2.95 and 3.10 corresponding to the two $-SO_2CH_3$ groups. The *trans* di-*O*-mesylate **5** on treatment with sodium azide in DMF gave the (2*S*,4*S*)-*cis* diazide 6 resulting from an inversion reaction at C4. A characteristic peak appearing at 2100 cm^{-1} in the IR spectrum for (2*S*,4*S*)-L-*cis* diazide **6** and disappearance of $-SO_2CH_3$ peaks in the proton NMR spectrum confirmed the formation of diazide. This was subsequently reduced to the corresponding (2*S*,4*S*)-L-*cis* diamine **7** by catalytic hydrogenation using Pd-C as the catalyst in methanol. The complete reduction of azide



Scheme 4. Synthesis of (**2*S*,4*S***)-L-*cis* Diamine **7**

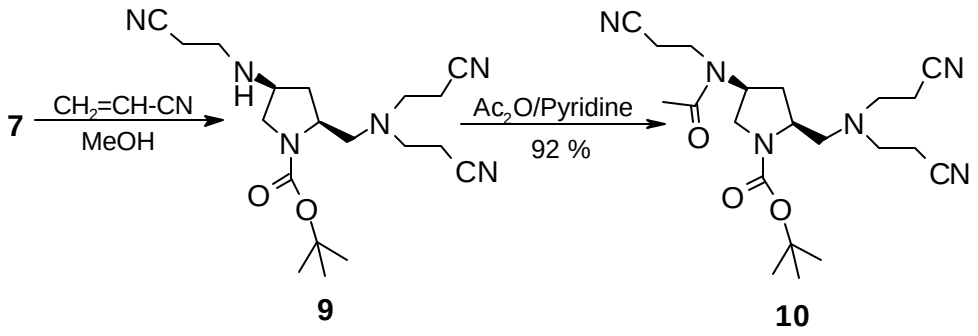
to the amine was indicated by the disappearance of azide peak in IR at 2100 cm^{-1} and appearance of a new peak at 3300 cm^{-1} in the IR spectrum. In the ^{1}H NMR of (2*S*,4*S*)-L-*cis* diamine **7** the H3 appeared at δ 1.55 upfield to the H3 (δ 2.1) of azide **6**, accompanied by an upfield shift of the other protons. The (2*S*,4*S*)-L-*cis* diamine **7** on treatment with excess of acrylonitrile in methanol[14] at 60°C for 72 hours gave the tricyano derivative **9** instead of the expected tetracyano derivative **8** (Scheme 5). This could perhaps be due to the steric influence of the bulky *tert*-butoxycarbonyl group, blocking a second reaction at C4-NH site.



7 **8**

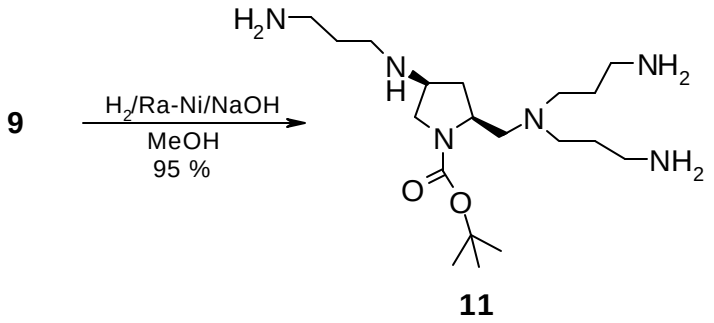
Scheme 5

The structure of the tricyano derivative **9** was confirmed by NMR and mass spectral analysis. The ^{13}C NMR of **9** showed peaks at δ 43.1 (-NH*C*H_2-) and δ 18.5 (-*C*H_2CN) indicating partially cyanoethylated product. FAB mass spectrum of the tricyano derivative **9** showed the molecular ion peak at 375 (page 84) confirming the formation of only a tricyano derivative, since the tetracyano compound **8** will have a mass of 427. Further, the acetylation of **9** (Scheme 6) using acetic anhydride in pyridine gave a monoacetate **10**, whose ^{1}H NMR showed a downfield shift of the H4 indicating the presence of N-acetate at the secondary nitrogen at C4. The tricyano compound **9** was reduced to the pentamine 11 using Ra-Ni as the catalyst and NaOH as co catalyst in



Scheme 6

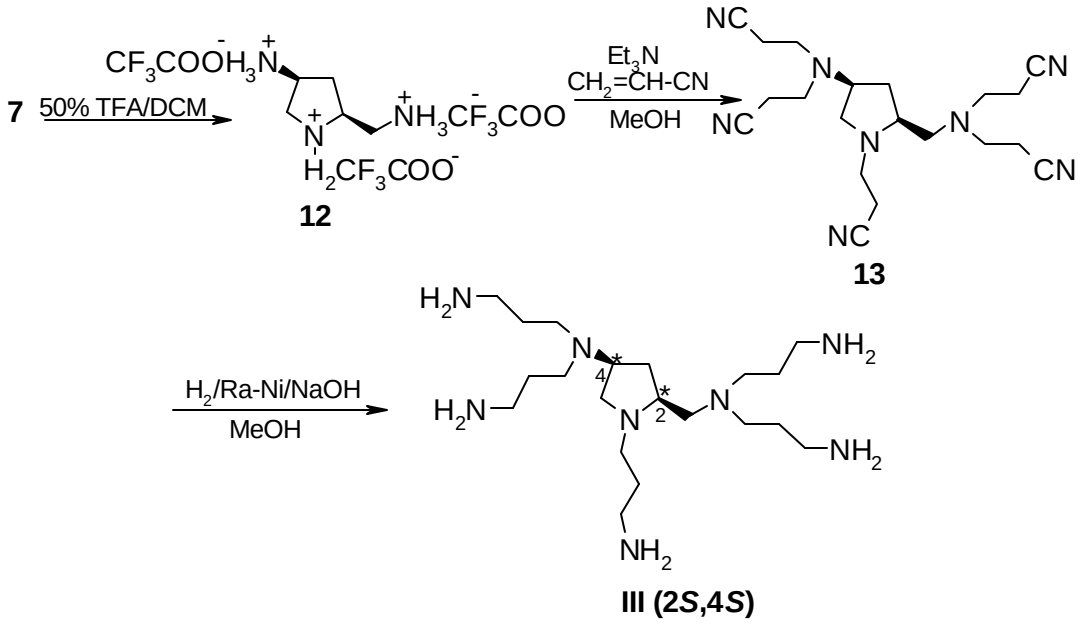
methanol.[15] The successful formation of pentamine **11** (Scheme 7) was characterized by the complete disappearance of cyano peak in the IR spectrum at 2270 cm^{-1} and confirmed by NMR and mass spectral analysis. The absence of peaks at δ 45.1 and 18.7 ($NHCH_2\underline{C}H_2\underline{C}N$) in ^{13}C NMR ruled out the presence of partially cyanoethylated products.



Scheme 7

The deprotection of *tert*-butoxycarbonyl group in **7** using TFA/DCM gave the triamine trifluoroacetate **12** (Scheme 8) which upon neutralization with triethylamine in methanol followed by exhaustive cyanoethylation using 2.5 to 4 eq. of acrylonitrile per amino group gave the pentacyano derivative **13** (Scheme 8). The structure of **13** was confirmed by the characteristic IR peak at 2270 cm^{-1} due to –CN and FAB mass showing a molecular ion peak at 381 (page 91) corresponding to the pentacyano

compound. This was quantitatively hydrogenated in the presence of Raney Nickel/H_2 and NaOH using methanol as solvent at room temperature to give the (2*S*,4*S*)-L-*cis* octamine (**III**) in good yield. Under the reaction conditions, the partially ethanol soluble polynitrile suspension was perhydrogenated to form the ethanol soluble poly (trimethylenimines). The presence of sodium hydroxide as a cocatalyst in ethanol prevents the formation of secondary amines by the attack of amine on the already formed intermediate imine.[15] The polyamine **III** was purified by passing its aqueous solution through amberlite cation exchange resin IR-120 and eluted with water.



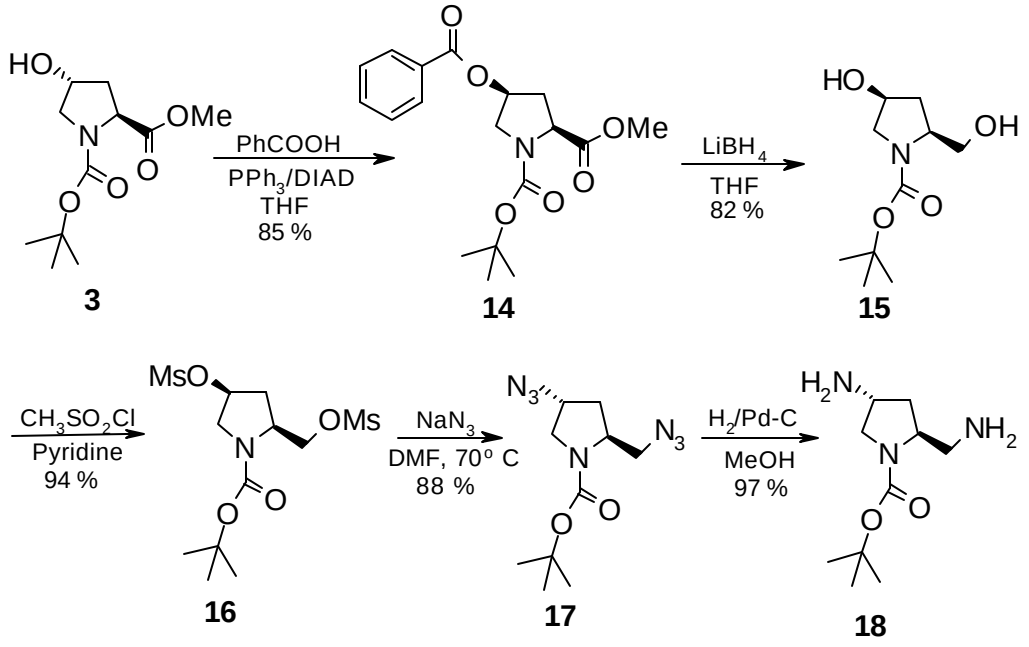
Scheme 8

The complete reduction of the polynitrile was supported by spectroscopic data with the complete disappearance of the C≡N peak at 2273 cm^{-1} in IR spectrum and absence of a peak at 118 ppm due to CN in the ^{13}C NMR spectrum. The structure of the amine **III** was further confirmed by ^1H NMR and FAB mass spectral analysis, which showed a molecular ion peak at 401 (page 114) corresponding to the octamine. This

scheme starting from *trans*-4-hydroxy-L-proline involved one inversion at C4 to get the required (2S,4S)-L-*cis* pyrrolidyl octamine **III**.

2.3.2. Synthesis of (2*S*,4*R*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N,N'-bis-(aminopropyl)amino]-N1-(3-aminopropyl) pyrrolidine (IV)

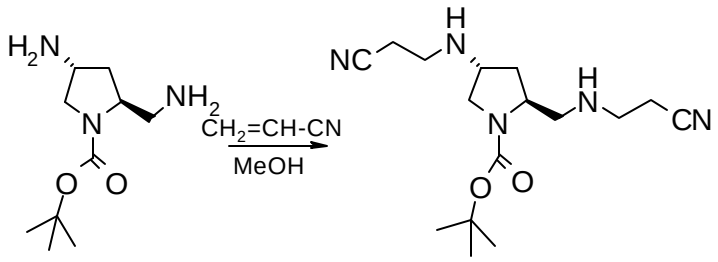
The synthesis of (2*S*,4*R*)-L-*trans* pyrrolidyl polyamine **IV** was achieved starting from (2*S*,4*R*)-*trans*-4-hydroxy-N-(*tert*-butoxycarbonyl)-L-proline methyl ester **3** by double inversion of configuration at C4 (Scheme 9). The first Inversion of stereochemistry at C4 hydroxyl in **3** was done via Mitsunobu reaction[16] using benzoic



Scheme 9. Synthesis of (2*S*,4*R*)-L-*trans* diamine

acid, triphenyl phosphine and diisopropylazodicarboxylate to get (2*S*, 4*S*)-*cis*-4-*O*-benzoyl-N-(*tert*-butoxycarbonyl)-L-proline methyl ester **14**. This on treatment with lithium borohydride in anhydrous THF gave the (2*S*,4*S*)-L-*cis* diol **15** in excellent yield.

The mesylation of the diol **15** using methanesulfonyl chloride in pyridine gave the (2*S*,4*S*)-L-*cis* di-*O*-mesylate **16**. The second inversion of configuration at C4 was effected on treatment with sodium azide in DMF to yield the (2*S*,4*R*)-L-*trans* bisazide **17** to give back the 'α' configuration of the C4 substituent as in starting *trans*-4-hydroxy-L-proline. The diazide **17** on hydrogenation using 60 psi H_2 pressure in presence of Pd-C at room temperature afforded the (2*S*,4*R*)-L-*trans* diamine **18**. Interestingly, the (2*S*,4*R*)-L-*trans* diamine **18** on treatment with excess of acrylonitrile gave the dicyanoderivative **19** (Scheme 10) unlike the (2*S*,4*S*)-L-*cis* isomer which gave the tricyano derivative. This shows that the orientation of the C4 substitutent perhaps interferes in the Michael addition reaction and further work is required to confirm the generality of such observations.

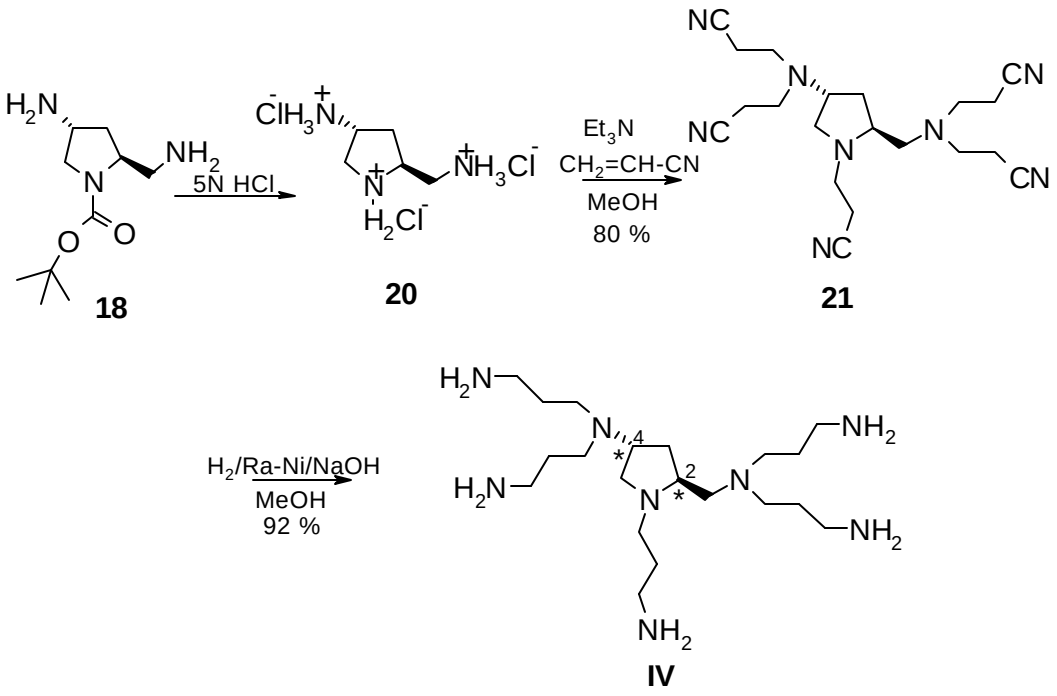


18 **19**

Scheme 10

The deprotection of tert-butoxycarbonyl group in **18** with 5N HCl gave the triamine hydrochloride **20** which was exhaustively cyanoethylated to yield the required (2*S*,4*R*)-L-*trans* pentacyano derivative **21** in good yield (Scheme 11). This on catalytic hydrogenation at room temperature using 300 psi H_2 pressure and Ra-Ni as the catalyst in presence of NaOH gave the desired diastereomer (2*S*,4*R*)-L-*trans* octamine **IV** in good yield. This was purified by following a similar protocol as in the case of (2*S*,4*S*)-L-*cis* isomer **III**. All compounds were completely characterized at all stages by IR, ^{1}H NMR, ^{13}C NMR, mass spectral analysis and by optical rotations (see

experimental). Thus, the two diastereomeric octamines **III** and **IV** were prepared starting from the same intermediate **3** by appropriate configurational inversion reactions.



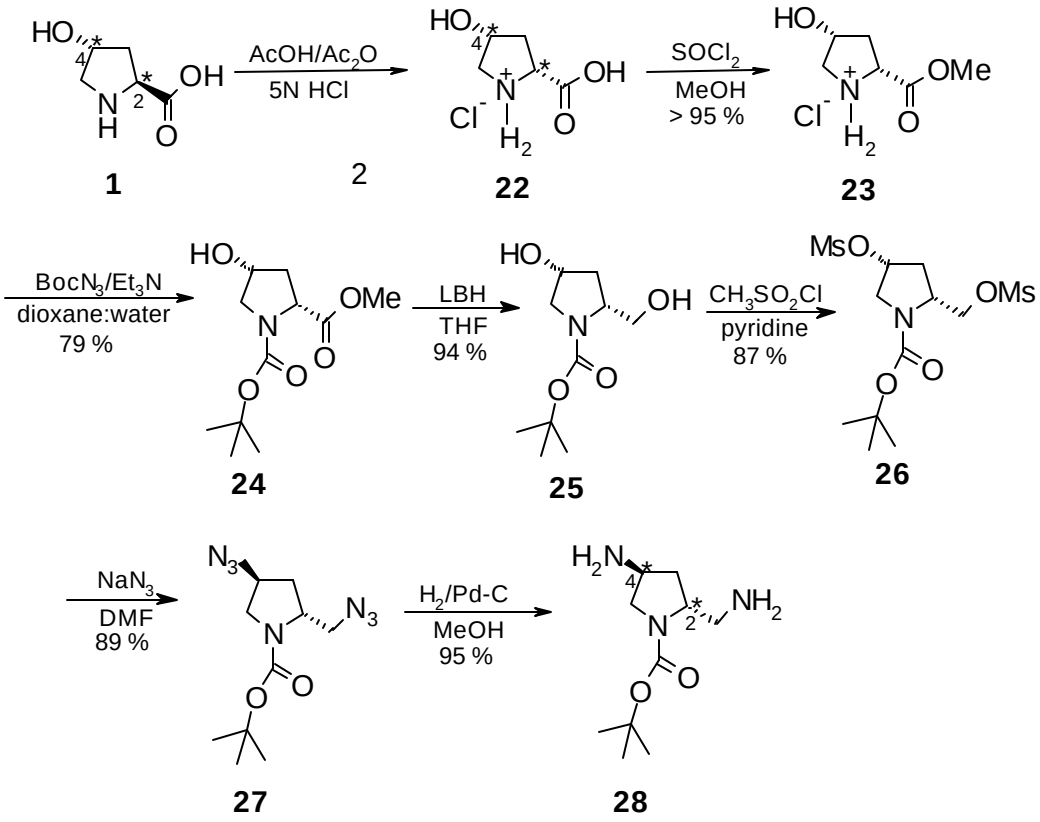
18 **20** **21** **IV**

Scheme 11. Synthesis of (2*S*,4*R*)-*L-trans* octamine

2.3.3. Synthesis of (2*R*,4*S*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4[N,N'-bis-(3-aminopropyl)amino]-N1-(3-aminopropyl) pyrrolidine (V)

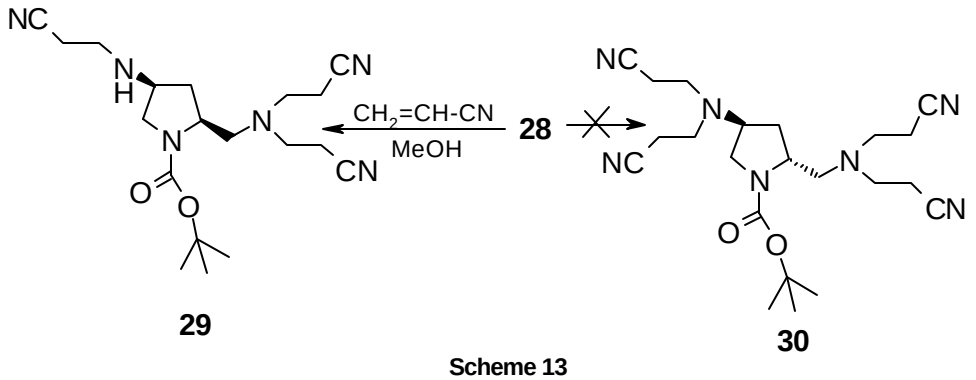
The synthesis of (2*R*,4*S*)-D-*trans* octamine V was carried out starting from *trans*-4-hydroxy-L-proline **1** as follows. Initial epimerisation[17] of the carboxylic function at C2 was done by treatment with acetic anhydride/acetic acid followed by refluxing in 2N aq. HCl to obtain the *cis*-4-hydroxy-D-proline hydrochloride **22** in good yield and high chiral purity as determined by optical rotation. The ^{13}C NMR spectrum of **22** showed single resonance for each of the carbons indicating the enantiomeric purity. Following a similar set of reactions as in Scheme 11, viz., esterification of the 2-

carboxylic acid, protection of the ring nitrogen as *tert*-butoxycarbonyl and reduction of the 2-carboxylic ester **24** using lithium borohydride lead to the (2*R*,4*R*)-D-*cis* diol **25** (Scheme 12).



Scheme 12: Synthesis of (2*R*,4*S*)-*D*-*trans* diamine

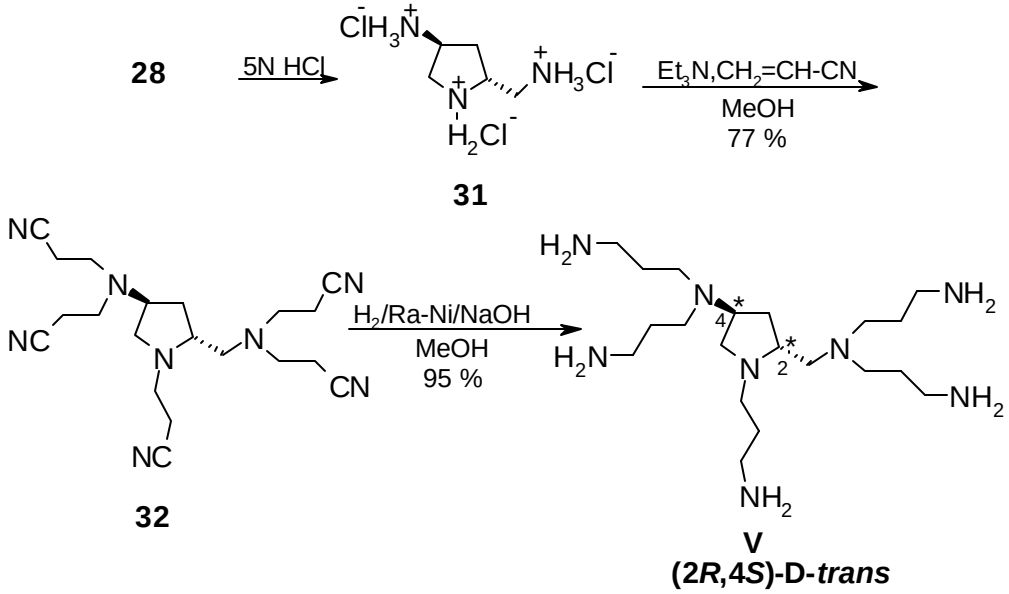
The *cis*-diol **25** on mesylation gave the (2*R*,4*R*)-D-*cis* di-*O*-mesylate **26**, which on treatment with sodium azide in anhydrous DMF gave the (2*R*,4*S*)-D-*trans*-diazide **27**. Hydrogenation in presence of Pd-C in methanol at room temperature gave the (2*R*,4*S*)-D-*trans* diamine **28**. The exhaustive cyanoethylation of the diamine failed to give the expected tetracyano compound **30** (Scheme 13) as mentioned in case of (2*S*,4*S*) diastereomer **7** and instead gave the tricyano compound **29** as confirmed by IR, NMR and mass spectral analysis (see experimental).



29 **30**

Scheme 13

The deprotection of N-*tert*-butoxycarbonyl function using 5N aq.HCl and subsequent neutralization with triethylamine and Michael addition with acrylonitrile gave the (2*R*,4*S*)-D-*trans* pentacyano derivative **32** (Scheme 14). This was characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis (see experimental for data). The pentacyano derivative **32** after hydrogenation using Ra-Ni catalyst in



Scheme 14. Synthesis of (2*R*,4*S*)-D-*trans* octamine

methanol-NaOH afforded the final (2*R*,4*S*)-D-*trans* octamine **V** purified by protocols as described for the polyamine **III**. This scheme involving two inversions one at C2 and

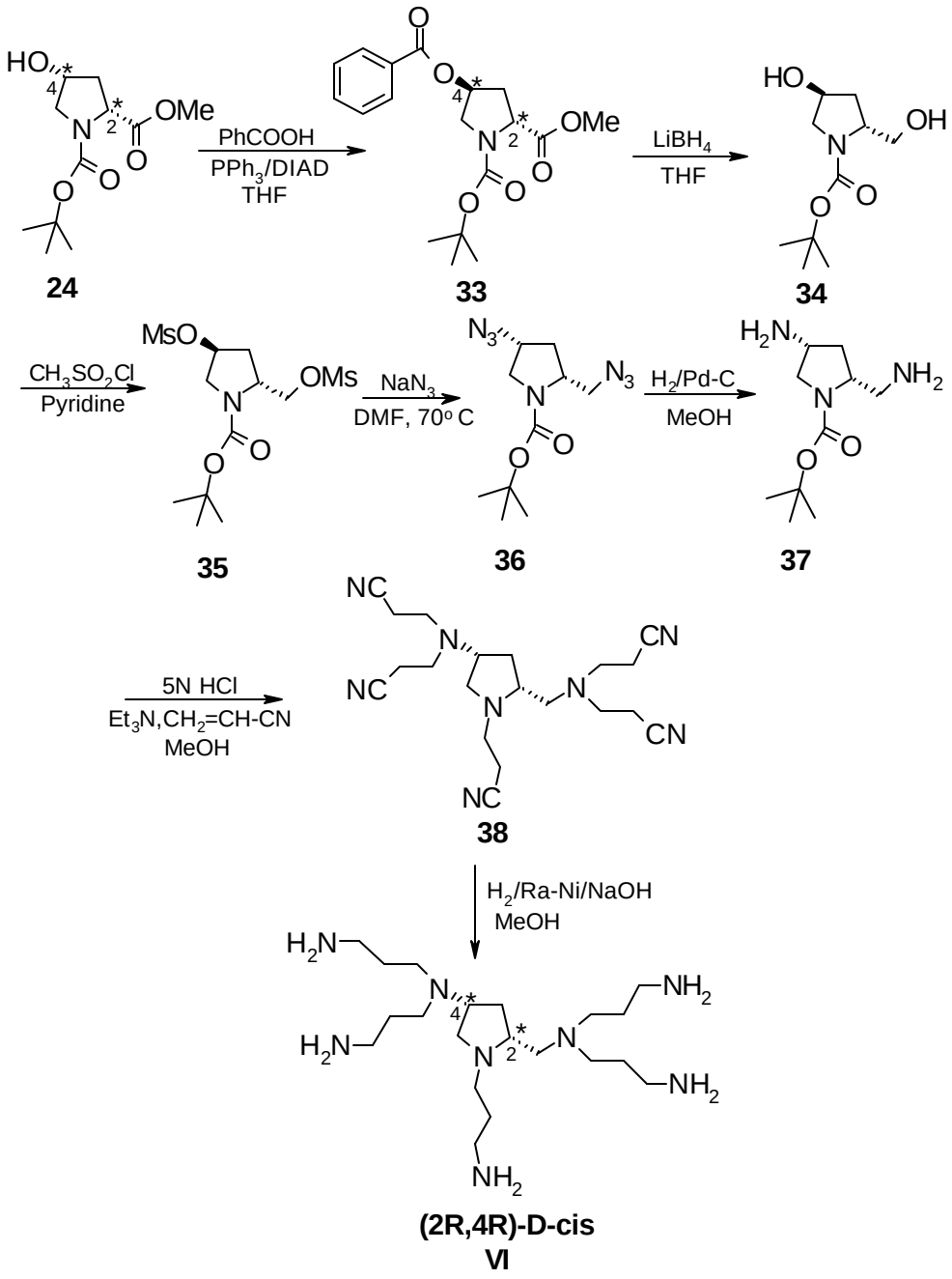
one at C4 gave the desired (2*R*,4*S*)-D-*trans* octamine **V** starting from (2*S*,4*R*)-*trans*-4-hydroxy-L-proline **1**.

2.3.4. Synthesis of (2*R*,4*R*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N,N'-bis-(3-aminopropyl)amino]-N1-(3-aminopropyl) pyrrolidine (VI)

The synthesis of (2*R*,4*R*)-D-*cis* octamine **VI** was carried out starting from (2*R*,4*R*)-*cis*-4-hydroxy-N-(*tert*-butoxycarbonyl)-D-proline methyl ester **24** (Scheme 15). The first inversion at C4 was achieved by using benzoic acid, triphenyl phosphine and diisopropyl azodicarboxylate under Mitsunobu conditions to get the (2*R*,4*S*)-*trans*-4-*O*-benzoyl-N-(*tert*-butoxycarbonyl)-D-proline methyl ester **33**. The *trans*-diester **33** was treated with lithium borohydirde in anhydrous THF to get the (2*R*,4*S*)-D-*trans* diol **34** that on reaction with methanesulfonyl chloride afforded the (2*R*,4*S*)-D-*trans* di-*O*-mesylate **35**. This on treatment with sodium azide in anhydrous DMF gave the (2*R*,4R)-D-*cis* diazide **36** and the reaction was accompanied by another inversion at C4. The *cis* diazide **36** thus obtained was hydrogenated using Pd-C as catalyst to the (2*R*,4*R*)-D-*cis* Diamine **37** that on subsequent deprotection and cyanoethylation gave the (2*R*,4R)-D-*cis* pentacyano derivative **38**. This was reduced to the (2*R*,4*R*)-D-*cis* octamine (***VI***) by hydrogenation using Ra-Ni/NaOH in methanol. All compounds were characterized by spectral data as mentioned in the experimental section.

2.3.5. Synthesis of second generation chiral dendramines

Buchleier et al.[18] first proposed a conceptual approach for synthesis of dendritic polyamines involving a branching reaction sequence through cyanoethylation of amines with acrylonitrile followed by a reduction to double the amine end group

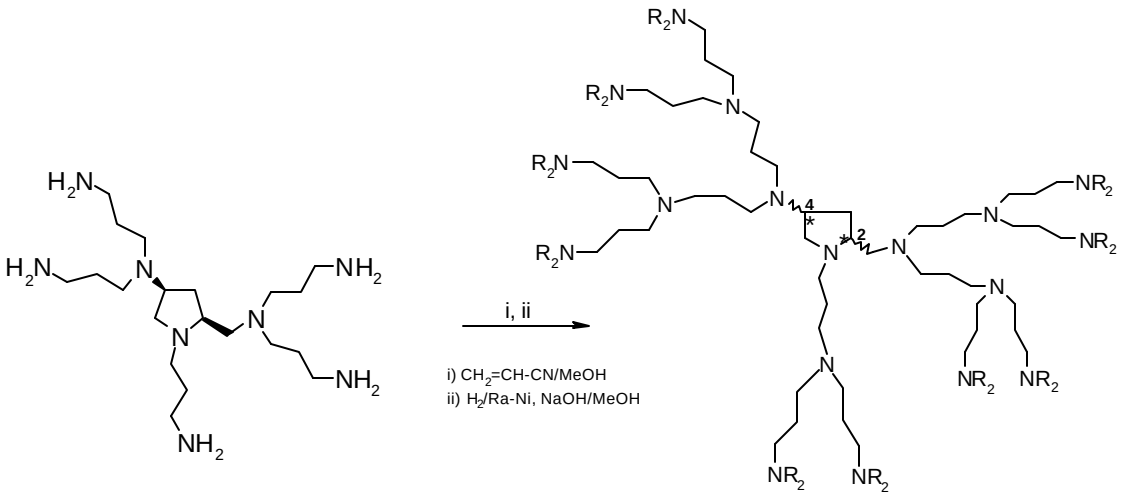


Scheme 15: Synthesis of (2*R*,4*R*)-*D-cis* octamine

functions. A continued stepwise reaction sequence leads to branching and generation of the dendramine. This approach was followed with the present polyamines to afford the next generation pyrrolidyl polytrimethylene imines with a chiral core. Although this

has not been done with all diastereomers **III-VI** in the resent work, the proof of concept and feasibility is illustrated with one of the isomers.

The (2*S*,4*S*) octamine **III** was subjected to Micahel addition (Scheme 16) using acrylonitrile in methanol to obtain a decanitrile **39** which was perreduced by autoclave hydrogenation at high pressure (500 psi) in presence of Ra-Ni/NaOH to yield the dendramine **VIII** (2*S*,4*S*) having 18 amino groups and the amine was characterized by the complete disappearance of the cyano peak in the IR spectrum at 2260 cm^{-1}.



III **VIII** R = H

Scheme 16

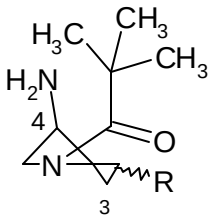
2.4. REACTIVITIES AND PROPERTIES OF PYRROLIDYLAMINES

GENERAL OBSERVATIONS:

During the course of this work, some interesting observations have been made regarding the selective reactivities in pyrrolidine ring system. It is observed that when the ring nitrogen carries a bulky substituent such as Boc, dialkylation (cyanoethylation) does not occur at 4-amino group upon reaction of (2*S*,4*S*)-*cis*-diamine **7** and (2*R*,4*S*)-*trans*-diamine **28** with acrylonitrile (Schemes 6 & 13). However, this was not the case with (2*S*,4*R*)-*trans* diamine **18** and (2*R*,4*R*)-cis-diamine **37** which underwent 4-N-monocyanoethylation. In all the cases, deprotection of N1-*tert*-butoxycarbonyl led to

products with N,N'-dialkylation on 4-amino function. These results suggested that with N1-Boc pyrrolidines, when the 4-amino group has 4*S* stereochemistry, only mono cyanoalkylation was seen but not N,N'-dicyanoethylation. The stereochemistry at C2 had no consequence on these reactions. This perhaps arises as result of steric hindrance caused by Boc group at N1 for a second alkylation reaction at 4*S* amino group. Such a steric block may happen due to a pyrrolidine ring conformation involving a C4 pucker in which the substituent at C4 closely encounters the N1-tert-butoxycarbonyl group, which itself can exist in two rotameric forms (Figure 1). Understanding the exact puckering mode of pyrrolidine ring as a function of substituent requires a detailed analysis of ^{1}H-^{1}H coupling constants of the ring protons. However, it is known in literature[19] that a substituent at C4 forces the pyrrolidine ring for a C4-pucker. Pyrrolidine being a small ring system, the nearby steric effects of substituents on selectivities in the reaction could be significant.

Figure 1. Pyrrolidine ring pucker and clash of C4 and N1 substituents



The nature of pyrrolidine ring pucker as a function of the stereochemistry of the substituent may also have a consequence on the relative conformation of the pendant aminopropyl chains at C4-amino group. One of the effects of this would be on the pattern of distributions of the positive charges on the amino group on the molecular surface. This should be in a way reflected in variations of their pK_a values and hence it was attempted to determine the pK_a values by acid-base titration.

A pH curve was generated by titration of an aqueous solution of the polyamine (2*S*,4*R*)-L-trans hydrochloride with aliquots of aqueous NaOH and pH was measured after each addition. The result is shown in Figure 2 as a plot of pH vs volume of alkali

added and the pK_a values were obtained from the derivative curve.[20] This shows a maxima corresponding to pK_a of 10.5 and a small shoulder at pK_a 11.0. and perhaps represent the pK_a of the primary and tertiary amino groups. However the pK_a differences among the primary amino groups could not be resolved by this experiment and may require careful ^{15}N NMR titration experiments. However, the pK_a values found clearly suggest that these are likely to be predominantly in protonated form at physiological pH. One of the ways to alter the local microenvironment and hence the

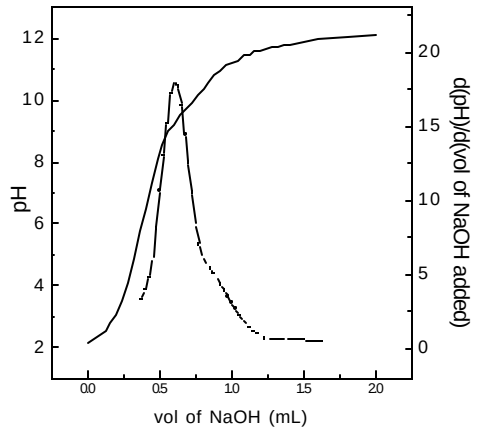


Figure 2. pK_a titration curve

pK_a is to replace some of terminal amino groups with alcohol, ether or other moieties and future synthetic efforts directed towards this objective requires selective protection/deprotection strategies.

2.5. CONCLUSIONS

This chapter describes the chemical synthesis and characterization of the four diastereomeric polyamines (tetra, penta and deca) containing a chiral pyrroilidine core. All polyamines were obtained starting from the commercially available *trans*-4-hydroxy-L-proline. Single inversion at C4 (mesyl to azide) or double inversion (Mitsunobu inversion followed be mesyl to azide) lead to enantiomeric pair at C4, while

epimerization at C2 gave the corresponding enantiomeric pair. Through a combined strategy, all four diastereoisomers are accessed. The pentamines are then elaborated to the decamines by sequential Michael addition using acrylonitrile and perhydrogenation to give polyamines. During the course of reactions, it was interestingly observed that the cyanoethylation of the secondary amine at C4 was affected by the presence of bulky *tert*-butoxycarbonyl substituent on the ring nitrogen. The final polyamine compounds are highly water soluble, hygroscopic and hence are difficult to purify and handle. By this synthetic approach, pyrrolidyl polyamines corresponding to (2*S*,4*S*)-L-*cis*, (2*S*,4*R*)-L-*trans*, (2*R*,4*S*)-D-*trans* and (2*R*,4*R*)-D-*cis* configuration and having different charge contents have been prepared. The synthesized octamines are used in the next chapter for biophysical studies to understand the interaction with DNA.

2.6. EXPERIMENTAL

General: The chemicals used were of either laboratory or analytical grade. All solvents used were purified according to the literature procedures[21] and the reactions were monitored by TLC on silica gel. Usual work-up implies washing of the organic extract with water, brine, drying over anhydrous sodium sulphate and removal of the solvent to dryness by rotary evaporation under vacuum.

Column chromatography was performed for purification of compounds using flash chromatography grade (230-400 mesh) silica gel (SRL, India). The column chromatographic separations were followed by TLC analysis using precoated silica gel TLC plates (Merck Kieselgel 60F_{254}) and developed in petroleum ether-ethyl acetate. The compounds were visualized under UV light, exposure to iodine or by spraying with ninhydrin reagent subsequent to Boc-deprotection (exposing to HCl vapors) and heating. *trans*-4-Hydroxy-L-proline, *tert*-butylcarbazate, DEAD and acrylonitrile were obtained from Aldrich USA. ^{1}H NMR spectra were recorded at 200 MHz/500 MHz ^{13}C

NMR at 50 MHz or 125 MHz on Bruker NMR spectrometers (ACF 200/ DRX 500) fitted with an Aspect 3000 computer. All chemical shifts are referenced to internal TMS for ^{1}H and chloroform-d for ^{13}C and values are quoted in δ (ppm) scale. Optical rotations were measured on JASCO DIP-181 polarimeter. FAB Mass spectra were recorded on

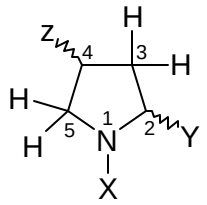


Figure 3. 2,4-disubstituted pyrrolidine. NMR peaks are assigned according to the numbering of pyrrolidine ring as shown in the figure.

Finnigan-Matt mass spectrometer and MALDI-TOF on a Kratos PC Kompact instrument.

(2*S*,4*R*)-*trans*-4-hydroxy-L-proline methyl ester hydrochloride 2

A suspension of *trans*-4-hydroxy-L proline **1** (10 g, 76.3 mmol) in absolute methanol (100 mL) was cooled to 0°C. To this was added thionyl chloride (6.1 mL, 83.6 mmol, 1.1 eq.) dropwise over a period of 15 min. with stirring which was continued at 0°C for 4 h. followed by refluxing for 6 hours. The removal of methanol under vacuum and washing the precipitate with ethyl acetate and diethyl ether followed by drying under vacuum over P_2O_5 gave methyl ester hydrochloride **2** (13.0 g, 94%) as a white solid. This was used for the next step without further purification.

^{1}H NMR (D_2O): δ 4.75-4.55 (m, 2H, H4 & H2), 3.85-3.75 (s, 3H, -OMe), 3.55-3.40 (m, 1H, H5A), 3.40-3.30 (m, 1H, H5B), 2.55-2.35 (m, 1H, H3A), 2.35-2.15 (m, 1H, H3B).

(2*S*,4*R*)-*trans*-4-Hydroxy-N1-(*tert*-butoxycarbonyl)-L-proline methyl ester 3

A mixture of (2*S*,4*R*)-*trans*-4-hydroxy-L proline methylester hydrochloride **2** (8 g, 44.0 mmol), tert-butoxycarbonyl azide (8.0 g, 55.9 mmol), triethylamine (20 mL), water (40 mL) and dioxane (40 mL) was stirred under argon atmosphere at 50°C for 24 h. The reaction mixture was concentrated to a paste by rotary evaporation and the residue was dissolved in ethyl acetate and subjected to usual work up to get the crude product as a thick yellow oil. This was purified by column chromatography on silica gel (100-200 mesh) using 30% ethyl acetate-petroleum ether as an eluant yielding **3** (8 g, 74%) as thick pale yellow oil.

IR (neat, cm^{-1}): 3400, 2980-2900, 1735 and 1670. ^1H NMR ($CDCl_3$): δ 4.55- 4.35 (m, 2H, H2 & H4), 3.72 (s, 3H, -OMe), 3.7-3.4 (m, 2H, H5A & H5B), 2.85-2.40 (bs, 1H, exchangeable), 2.40-2.15 (m, 1H, H3A), 2.15- 1.95 (m, 1H, H3B), 1.50-1.35 (d, 9H, 3 X CH_3 of Boc). $[\alpha]_D^{25^o}$ = -61.5 (c=0.42, $CHCl_3$).

(2*S*,4*R*)-*trans*-4-Hydroxy-N1-(*tert*-butoxycarbonyl)-L-prolinol 4

To an ice-cold solution of (2*S*,4*R*)-L-*trans* ester **3** (5 g, 20.4 mmol) in anhydrous THF (40 mL) was added lithium borohydride (0.67 g, 30.6 mmol) in one portion. The mixture was stirred at 5° C for 1 h. then at an ambient temperature for 4 hours. After the completion of the reaction (monitored by TLC) reaction mixture was concentrated to a paste by rotary evaporation and the residue was subjected to usual work up to get a color less thick oil (4.2 g, 95 %) which was used as such for the next step.

IR ($CHCl_3$, cm^{-1}): 3500-3300, 1670. ^1H NMR ($CDCl_3$): δ 5.30-4.95 (bs, 1H, exchangeable), 4.45-4.25 (bs, 1H), 4.20-3.90 (m, 1H), 3.90-3.35 (m, 4H), 2.80-2.35 (bs, 1H, exchangeable), 2.15-1.90 (m, 1H, H3A), 1.80-1.55 (m, 1H, H3B), 1.45 (s, 9H, 3 x CH_3 of Boc). ^{13}C NMR ($CDCl_3$): δ 157.0 (CO), 80.5 (CMe_3), 69.0 (C4), 66.4 (C5), 58.7 (C2), 55.7 (CH_2), 37.4 (C3), 28.5 (-CH_3). $[\alpha]_D^{25^o}$ = -56.0 (0.56, $CHCl_3$).

(2*S*,4*R*)-*trans*-2-(methanesulphonyloxymethyl)-4-(*O*-methanesulphonyl)-N1-(*tert*-butoxycarbonyl) pyrrolidine 5

A solution of (2*S*,4*R*)-L-*trans* diol **4** (4.0 g, 18.4 mmol) in pyridine (50 mL) was cooled to 0°C. To this was added drop wise methanesulfonyl chloride (4.3 mL, 55.2 mmol) under nitrogen atmosphere. The stirring was continued at 0°C for 2 hours and the reaction flask was kept at 4°C overnight. TLC monitored the completion of the reaction. The solvent pyridine was removed under vacuum at 30°C, the residue was taken in ethyl acetate and subjected to usual work up to get the crude product **5** as an yellow solid. This was purified by column chromatography to get pure **5** as a white solid 6.2 g, Yield 91.2%.

IR (neat, cm^{-1}): 2978, 1692, 1357, 1172. ^1H NMR ($CDCl_3$): δ 5.28-5.10 (m, 1H, H4), 4.75-4.35 (m, 1H, H2), 4.35-4.05 (m, 2H, H5A & H5B), 4.05-3.65 (m, 1H), 3.10-2.95 (d, 6H, $-SO_2\underline{CH_3}$), 2.60-2.20 (m, 2H, H3A & H3B), 1.45 (s, 9H, 3 x CH_3 of Boc).

(2*S*,4*S*)-2-Azidomethyl-4-azido-N1-(*tert*-butoxycarbonyl) pyrrolidine 6

A mixture of (2*S*,4*R*)-L-*trans* di-*O*-mesylate **5** (2.5 g, 6.7 mmol) and sodium azide (1.7 g, 26.8 mmol, 4 eq.) was stirred at 70°C in of DMF (25 mL) followed by usual work up to obtain the (2S,4S)-L-*cis* bisazide **6** (1.5 g, 84%).

IR (neat, cm^{-1}): 1696, 2103. ^1H NMR ($CDCl_3$): δ 4.20-4.05 (m, 1H), 4.05-3.90 (m, 1H), 3.80-3.65 (m, 1H), 3.65-3.55 (m, 2H), 3.40-3.25 (m, 1H), 2.35-2.20 (m, 1H, H3A), 2.10-2.00 (m, 1H, H3B), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 153.4, 79.8, 58.6, 55.5, 52.7, 51.3, 33.5, 27.9. $[\alpha]_D^{25°}$ -9.6° (c=0.52, $CHCl_3$).

(2*S*,4*S*)-2-Aminomethyl-4-amino-N1-(*tert*-butoxycarbonyl) pyrrolidine 7

The diazide **6** (1.2 g, 4.5 mmol) was dissolved in methanol (10 mL) and hydrogenated using Pd-C (0.35 g) and 60 psi H_2 pressure. After the completion of reaction as monitored by TLC, the catalyst was filtered off and the solvent was

evaporated to get the diamine **14** (0.95 g, 98%).

IR (neat, cm^{-1}): 1686, 3180-3400. ^1H NMR ($CDCl_3$): δ 3.95-3.65 (m, 2H), 3.60-3.40 (m, 1H), 3.10-2.75 (m, 3H), 2.45-2.25 (m, 1H), 2.10-2.00 (m, 4H, exchangeable), 1.65-1.50 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.7, 79.3, 59.0, 55.5, 49.8, 45.1, 38.7 and 28.3. *m/e,* 217 (M^++1,10%), 148 (21%), 124 (100%). $[\alpha]_D^{25°}$ -37.8° (c=0.79, $CHCl_3$).

(2*S*,4*S*)-2-[N,N'-bis-(2-cyanoethyl)aminomethyl]-4-[N-(2-cyanoethyl)amino N1-(*tert*-butoxycarbonyl) pyrrolidine 9

The diamine **7** (0.5 g, 2.3 mmol) was dissolved in MeOH (5 mL), treated with acrylonitrile (10 eq. 1.5 mL, 23 mmol) at 0-10°C while stirring, continuing at 0°C for 1 h and was heated for 4 days at 60°C. The reaction mixture was concentrated and the residue was purified by column chromatography on silica gel using EtOAc-petroleumether (75:25) to obtain the tricyano derivative **9** (0.69g, 80%).

IR (neat, cm^{-1}): 3311.5, 2247 and 1686. ^1H NMR ($CDCl_3$): δ 4.00-3.8 (m, 1H, 3.75-3.55 (m, 1H), 3.45-3.25 (m, 1H), 3.25-3.08 (m, 1H), 3.08-2.70 (m, 8H), 2.60-2.45 (m, 6H+ 1H exchangeable), 2.38-2.15 (m, 1H, H3A), 2.05-1.80 (m, 1H, H3B) and 1.5 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 154.2 & 153.9 (Boc CO), 118.6 (CN), 79.3 (CMe_3), 59.9, 56.3, 55.8, 55.1, 52.4, 49.9, 43.1, 35.6, 34.9, 28.1, 20.6, 18.5, 16.4 and 13.8. *m/e* 375 (M^++1, 9%), 301 (35%), 238 (45%), 138 (100%).

(2*S*,4*S*)-2-[N,N'-bis-(2-cyanoehtyl)aminomethyl]-4-[N-(acetyl)-N'-(2-cyanoethyl)amino]-N1-(*tert*-butoxycarbonyl) pyrrolidine 10

The tricyano derivative **9** (0.3 g, 0.84 mmol) was treated with acetic anhydride (0.2 mL, 2.1 mmol, 2.5 eq.) in dry pyridine (3 mL) for 18 h. After completion of the reaction and the usual work up followed by purification over column chromatography on silica gel using 70% ethyl acetate in petroleum ether gave the monoacetate **10** (0.32 g, (92%).

^{1}H NMR ($CDCl_3$): δ 4.35-4.18 (m, 1H, H4), 4.00-3.75 (m, 2H), 3.75-3.45 (m, 2H), 3.30-3.08 (m, 1H), 3.05-2.77 (m, 6H), 2.60-2.45 (t, 5H), 2.35-2.10 (m, 4H), 1.95-1.65 (m, 2H, H3A and H3B) and 1,48 (s, 9H).

(2*S*,4*S*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N-(3 aminopropyl) amino]- N1-(*tert*-butoxycarbonyl) pyrrolidine 11

The tricayno derivative 9 (0.25 g, 0.67mmol) was hydrogenated using Ra-Ni (0.2 mL) and NaOH at 100 psi H_2 pressure in methanol. After the completion of the reaction the catalyst was filtetered and passed through amberlite cation exchange resin to get **11** as pure product.

^{1}H NMR ($CDCl_3$+D_2O): δ 4.00-3.55 (bs, 2H), 3.50-3.00 (m, 2H), 3.00-2.00 (bm, 16H), 2.00-1.50 (m, 6H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 158.2, 82.9, 59.8, 58.9, 55.8, 55.4, 55.1, 49.1, 42.9, 42.7, 35.1, 32.3, 30.9. FAB Mass: 387 (M^++1, 30%), 99 (100%).

(2*S*,4*S*)-2-Aminomethyl-4-amino pyrrolidine tritrifluoroacetate 12

The diamine **7** (0.5 g, 1.1 mmol) was treated with 50% TFA in CH_2Cl_2 (5 mL) at room temperature for 30 minutes to get the triamine trifluoroacetate that was used as such for the next step.

^{1}H NMR (D_2O) : δ 4.15-3.95 (m, 2H), 3.95-3.80 (m, 1H), 3.65-3.45 (m, 3H), 2.95-2.85 (m, 1H), 2.10-1.90 (m, 1H).

(2*S*,4*S*)-2-[N,N'-bis-(2-cyanoethyl)aminomethyl]-4-[N,N'-bis-(2-cyanoethyl) amino]-N1-(2-cyanoethyl) pyrrolidine 13

The triamine trifluoroacetate (0.5 g, 1.1mmol) was neutralized with triethylamine followed by dissolution in MeOH (5 ml) was treated with acrylonitrile (15 eq. 1.1mL, 16.5 mmol) at 0-10°C while stirring, continuing at 0°C for 1 h and heated for 4 days at 60°C. The reaction mixture on work-up and column chromatography on silica gel gave the pentacyano derivative **13** (0.33 g, 80%).

IR (neat, cm^{-1}): 2247. ^{1}H NMR ($CDCl_3$): δ 3.55-3.30 (m, 2H), 3.25-3.15 (m, 1H), 3.10-2.75 (m, 7H), 2.70-2.20 (m, 17H), 1.65-1.50 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 119.1 & 118.9 (CN), 61.5, 60.1; 57.8, 57.5; 50.0, 49.5; 46.9, 33.9, 17.8, 17.6, 16.4. FAB MS: M^{+}+1 381 (39), M^{+}-1 379 (40), 244 (100). $[\alpha]_D$ 25°C -62.5° (c=0.40,$CHCl_3$).

(2*S*,4*S*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N,N'-bis-(3-aminopropyl) amino]-N1-(3-aminopropyl) pyrrolidine (III)

The pentacyano derivative **13** (0.3g, 0.8 mmol) was reduced by catalytic hydrogenation (H_2, 220 psi) using Ra-Ni/NaOH in ethanol for 12 h to ensure complete reduction as followed by TLC and IR spectroscopy. The catalyst was filtered off and the compound was purified by ion-exchange chromatography on Amberlite IR-120 (cation exchanger) using water as an eluant to afford of the octamine **III** (0.29 g, 92%).

IR (neat, cm^{-1}): 3360. ^{1}H NMR (D_2O): δ 3.70-3.45 (m, 1H), 3.10-3.00 (m, 2H), 3.00-2.90 (t, 10H), 2.88-2.70 (m, 3H), 2.70-2.45 (m, 10H), 2.35-2.25 (m, 1H), 2.10-1.95 (m, 1H), 1.90-1.75 (t, 10H). ^{13}C NMR (D_2O): δ 62.5, 58.0, 55.9, 54.7, 52.1, 50.6, 47.6, 38.0, 37.9, 37.8, 33.9, 24.8, 23.7, 23.3. FAB MS: (M^{+}+1) 401 (100), 344 (31), 132 (67). $[\alpha]_D^{25°}$ –23.6° (c=0.75,H_2O)

(2*S*,4*S*)-*cis*-4-O-Benzoyl-N1-(*tert*-butoxycarbonyl)-L-proline methylester 14

To a solution of (2*S*,4*R*)-L-*trans* carbomethoxy ester **3** (5 g, 20.4 mmol), triphenyl phosphine (9.6 g, 36.7 mmol,1.8 eq.) and benzoic acid (2.99 g, 24.5 mmol, 1.2 eq.) in anhydrous acetonitrile (50 mL) at 0°C under nitrogen atmosphere was added diisopropyl azodicarboxylate (7.2 mL, 36.7 mmol, 1.8 eq.). The reaction mixture was stirred at 0°C for 2 h., and then stirred at ambient temperature for 24 hours. After the completion of the reaction, methanol was added to the reaction mixture and concentrated to a paste. The residue was dissolved in a minimum amount of diethyl ether followed by addition of pet ether (40-60°) until the mixture became turbid and then

kept at 5°C overnight. The precipitated triphenyl phosphine oxide was removed by filtration. The residue was washed with diethyl ether-pet-ether mixture and the filtrate was concentrated to dryness, the residue taken in ethyl acetate and subjected to usual work-up. The crude product was purified by column chromatography on 230-400 mesh silica gel using 5% ethylacetate-petroleumether as an eluant and recrystallized to get the pure product **14** (6.03 g, 85%). m.p 87°C.

IR (nujol, cm^{-1}): 2950-2850, 1745, 1710, 1690. ^1H NMR ($CDCl_3$): δ 7.98 (d, 2H, ArH), 7.61-7.40 (m, 3H, ArH), 5.54 (bm, 1H, H4), 4.64-4.58 (m, 0.5 H, H2), 4.51-4.46 (m, 0.5 H, H2), 3.87-3.68 (M, 5H, H5A, H5B and $COOCH_3$), 2.59-2.42 (m, 2H, H3A and H3B), 1.47 (d, 9H, 3 X CH_3 of Boc.). ^{13}C NMR ($CDCl_3$): δ 172.4, 172.1 (ester carbonyl), 165.7 (benzoate carbonyl), 154.1 & 153.7 (Boc CO), 133.3, 129.7, 128.4, 80.3 (CMe_3), 73.5&72.4 (C2), 57.9 & 57.6 (C5), 52.5 (OCH_3), 52.1 (C4), 36.6 & 35.7 (C3), 28.3 (CH_3 Boc.). $[\alpha]_D^{25°}$ –28.0°(c=0.5,$CHCl_3$). FAB mass: 350 (M^++1, 18%), 250 (M^++2-$COOMe_3$, 100%).

(2*S*,4*S*)-*cis*-4-Hydroxy-N1-(*tert*-butoxycarbonyl)-L-prolinol 15

The reduction of the ester **14** (5.5 g, 15.8 mmol) with lithium borohydride (1.03 g, 47.4 mmol) in anhydrous THF (50 mL) followed by usual work up and purification yielded **15** (2.8 g, 82 %) as a white solid. m.p 89-91°C.

IR (nujol): 3300-3200, 2920-2860, 1685 cm^{-1}. ^1H NMR ($CDCl_3$+D_2O): δ 4.4-4.2 (m, 2H, one exchangeable H), 4.15-3.85 (m, 2H), 3.70-3.40 (m, 3H), 2.50-2.20 (bs, 1H, exchangeable), 2.10-1.80 (m, 2H, H3A & H3B) and 1.46 (s, 9H, CH_3 of Boc). ^{13}C NMR ($CDCl_3$): δ 155.7 & 154.9 (Boc CO), 80.0 (CMe_3), 69.6 & 68.9 (C2), 64.0 & 63.4 (C5), 58.5 (C4), 56.6 (CH_2), 38.0 & 37.3 (C3) and 28.5 (CH_3,Boc). $[\alpha]_D^{25°C}$ -44.5° (c=0.40,$CHCl_3$). FAB mass: 218 (M^++1, 32%), 188 [(M^++1)-$COOMe_3$, 100%].

(2*S*,4*S*)-*cis*--2-(methanesulphonyloxymethyl)-4-(O-methanesulphonyl)-N1-(*tert*-butoxycarbonyl) pyrrolidine 16

The *cis*-diol **15** (2.4 g, 11.1 mmol) was treated with mesyl chloride (2.6 mL, 33.3 mmol, 3 eq.) in dry pyridine (25 mL) to yield the (2*S*,4*S*) di-O-mesylate **16** (3.9 g, 94%) after column purification using 50% ethylacetate-pet ether as eluant.

^{1}H NMR ($CDCl_3$): δ 5.35- 5.20 (m, 1H, H4), 4.55-4.48 (m, 1H), 4.36-4.05 (m, 2H), 3.80-3.60 (s, 2H), 3.18-3.00 (d, 6H, 2 x -SO_2CH_3), 2.58-2.25 (m, 2H, H3A & H3B) and 1.50 (s, 9H, 3 x CH_3 of Boc). ^{13}C NMR ($CDCl_3$): δ 153.9 (Boc CO), 80.8 (CMe_3), 68.8 (C2), 54.9, 53.1, 38.6, 37.2 and 28.2.

(2*S*,4*R*)-2-Azidomethyl-4-azido-N1-(*tert*-butoxycarbonyl) pyrrolidine 17

The di-O-mesylate **16** (1.5 g, 4 mmol) and NaN_3 (10eq. 40.2 mmol) were stirred at 70°C in anhydrous DMF for 24 h. and the reaction mixture was then concentrated to a paste. It was extracted with EtOAc (3 x 40ml), washed with water, and concentration of the dried organic extract gave a gum. Purification by column chromatography over silica gel using ethyl acetate-petroleumether (10:90) gave the bisazide **17** as a colorless oil (0.94g, 88%).

IR (neat, cm^{-1}): 2978, 2936, 2882, 2104, 1697. ^{1}H NMR ($CDCl_3$): δ 4.3-3.85 (bm, 2H), 3.85-3.40 (m, 3H), 3.40-3.15 (t, 1H), 2.25-2.05 (m, 2H), 1.5 (s, 9H). $[\alpha]_D^{25°}$ -40.6° (c=0.27, $CHCl_3$).

(2*S*,4*R*)-2-Aminomethyl-4-amino-N1-(*tert*-butoxycarbonyl) pyrrolidine 18

The bisazide **17** (0.8g, 3 mmol) was dissolved in MeOH (8 mL) and treated with Pd-C (10%, 0.16g) and hydrogenated (H_2, 60 psi) for 8h. The catalyst was filtered off and the solvent was evaporated to yield the diamine **18** as thick oil (0.62g, 97%).

IR (neat): 3358, 3296, 2974, 2932, 2874, 1686 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 4.00-3.75 (bs, 1H), 3.65-3.40 (m, 2H), 3.3 5-3.00 (m, 1H), 2.90-2.70 (m,2H), 2.05-1.90

(m,1H), 1.90-1.70 (m,1H), 1.5 (s,9H). *m/e* 215 (M^+<10%), 198 (21%), 185 (100%) 159 (60%). $[\alpha]_D^{25°}$ -35.8°(c=0.32, $CHCl_3$).

(2*S*,4*R*)-2-[N(2-cyanoethyl)aminomethyl]-4-[N-(2-cyanoehtyl)amino]-N1-(*tert*-butoxycarbonyl) pyrrolidine 19

The diamine **18** (0.2 g, 0.9 mmol) was dissolved in methanol (2 mL) and treated with acrylonitrile (10 eq. 0.5 mL, 9 mmol) at 0-10°C for 1 hour followed by heating to 60°C for 4 days to ensure complete cyanoethylation. After the completion of the reaction methanol and excess acrylonitrile were removed and the residue was purified by column chromatography to get **19** as gum. yield 0.19 g.

IR (neat, cm^{-1}): 2247. ^{1}H NMR ($CDCl_3$): δ 4.10-3.80 (bs, 1H), 3.70-3.30 (m, 2H), 3.30-2.70(m,6H), 2.60-2.40 (t, 5H), 2.30-2.10 (m, 1H), 2.00-1.70 (m, 1H), 1.45 (s, 9H).

(2*S*,4*R*)-2-Aminomethyl-4-amino pyrrolidine trihydrochloride 20

The treatment of the diamine **18** (0.6 g) with aq. HCl (5N, 6 mL) at 60°C for 6 hours gave the triamine hydrochloride **20** that was used for the next step without further purification.

^{1}H NMR (D_2O): δ 4.05-3.80 (m, 1H), 3.80-3.50 (m, 1H), 3.50-3.20 (m, 1H), 3.20-2.90 (m, 3H), 2.30-1.90 (m, 2H).

(2*S*,4*R*)-2-[N,N'-bis-(2-cyanoethyl)aminomethyl]-4-[N,N'-bis(2-cyanoethyl) amino]-N1-(2-cyanoethyl) pyrrolidine 21

The trihydrochloride **20** (0.5 g, 2.2 mmol) was neutralized with triethylamine (6 eq. 1.8 ml, 13.2 mmol) followed by removal of excess triethylamine. The residue was dissolved in MeOH (5 ml), treated with acrylonitrile (15 eq. 2.2 ml, 33 mmol) at 0-10°C while stirring, continuing at 0°C for 1 h and was heated for 4 days at 60°C. The reaction mixture was concentrated and the residue was purified by column chromatography on silica gel using EtOAc-petroleumether (75:25) to obtain the pentacyano derivative **21**

(0.67g, 80%).

IR ($CHCl_3$, cm^{-1}): 3018, 2936, 2833, 2247. ^1H NMR ($CDCl_3$): δ 3.65-3.45 (m, 1H), 3.40-3.10 (m,2H), 3.10-2.75 (m,8H), 2.70-2.35 (m,13H), 2.35-2.15 (t, 11H), 2.15-1.65 (m,3H). ^{13}C NMR ($CDCl_3$): δ 118.7 & 118.5 (CN), 61.4, 58.5, 57.7, 56.8, 50.0, 49.6, 47.0, 32.2, 17.3, 16.9, 16.4. FAB MS: 381 (M^++1,100). $[\alpha]_D^{25°}$ -81° (c=0.2, $CHCl_3$).

(2*S*,4*R*)-2-[N,N'-bis(3-aminopropyl)aminomethyl]-4-[N,N'-bis(aminopropyl) amino]-N1-(3-aminopropyl) pyrrolidine IV

The pentacyano derivative **21** (0.5g, 1.3 mmol) was reduced by catalytic hydrogenation (H_2, 220 psi) using Ra-Ni/NaOH in ethanol for 12 h to ensure a complete reduction. The catalyst was filtered off and the compound was purified by ion-exchange chromatography on Amberlite IR-120 (cation exchanger) using water as an eluant to afford of the octamine **IV** (0.48g, 92%).

IR (neat, cm^{-1}): 3360, 2926, 2854. ^1H NMR (D_2O): δ 3.40-3.10 (m, 4H), 3.10-2.10 (m, 22H), 2.00-1.45 (m, 12H), 1.80-1.60 (bs, 7H). ^{13}C (D_2O): δ 62.2, 59.3, 58.8, 58.2, 57.1, 52.6, 52.3, 48.8, 39.4, 35.5, 29.6, 27.4, 27.2, 24.1. FAB MS: 401 (M^++1 30%), 91 (100%). $[\alpha]_D^{25°}$ - 54.5°(c=0.389, H_2O).

(2*R*,4*R*)-cis-4-hydroxy-D-proline 22

A solution of acetic anhydride (76 mL) and acetic acid (153 mL) was heated to 50°C. To this was added *trans*-4-hydroxy-L-proline **1** (10 g, 76.3 mmol) in one portion and the contents were refluxed for 6 h. The mixture was cooled and excess of acetic acid-acetic anhydride were removed under vacuum to get thick oil. The residue was refluxed with 2N HCl (190 mL) for 3 hours. The reaction mixture was treated with charcoal, filtered through celite and the filtrate was concentrated when crystallization began. The crystals of cis-hydroxyproline **22** were filtered under suction, washed with dioxane and dried over P_2O_5 under vacuum (9.95 g, 77.8 %). m.p 149°C. (Lit.[17] 153-

153.5 ^{o}C)

^{13}C NMR (D_2O): δ 172.7, 69.7, 59.1, 54.3 and 37.6.

(2*R*,4*R*)-*cis*-4-hydroxy-D-proline methyl ester hydrochloride 23

The treatment of *cis*-4-hydroxy-D-proline hydrochloride **22** (15 g, 90.1 mmol) with thionyl chloride (7.9 mL, 108.1 mmol) in methanol (100 mL) as mentioned for the L-isomer gave the methyl ester hydrochloride **23** in quantitative yield (15.5 g, 95 %).

^{1}H NMR (D_2O): δ 4.75-4.58 (m, 2H), 3.85 (s, 3H), 3.60-3.45 (2H, M), 2.65 –2.25 (m, 2H).

(2*R*,4*R*)-*cis*-4-Hydroxy-N1-(*tert*-butoxycarbonyl)-D-proline methyl ester 24

The methyl ester hydrochloride **23** (10 g, 55.1 mmol) was treated with *tert*-butoxycarbonyl azide (9.5 g, 66.1 mmol, 1.2 eq.), triethyl amine (19.2 mL, 137.8 mmol) in dioxane and water (1:1,100 mL) at 50^{o}C for 24 hours. Work up and purification as described earlier gave compound **24** (10.7 g, 79%) as a white solid. m.p 80^{o}C.

IR (nujol, cm^{-1}): 3450, 2950-2860, 1730, 1660. ^{1}H NMR ($CDCl_3$):δ 4.39-4.26 (m, 2H), 3.79 (d, 3H, -OMe), 3.66-3.49 (m, 1H, H3A), 2.12-2.00 (m, 1H, H3B), 1.46-1.40 (d, 9H, 3 x CH_3 of Boc.). ^{13}C NMR ($CDCl_3$): δ 174.2 (ester CO), 154.3 & 153.7 (Boc CO), 80.1 (CMe_3 of Boc.), 70.2 & 69.2 (C4), 57.5 & 57.4, 55.0 & 54.4 (C5), 52.2 & 52.1, 38.4 & 37.7 (C3), 28.2 & 28.1 (CH_3). $[\alpha]_D^{25^\circ}$ +20.0° (c=0.50,$CHCl_3$).

(2*R*,4*R*)-*cis*-4-Hydroxy-N1-(*tert*-butoxycarbonyl)-D-prolinol 25

The reduction of methyl ester **24** (5.4 g, 22 mmol) using lithium borohydride (0.72 g, 33 mmol) in anhydrous THF (55 mL) afforded the (2*R*,4*R)*-D-*cis* diol **25** (4.8 g, 94%) as a white solid. m.p 94^{o}C

IR (nujol, cm^{-1}): 3300-3100, 2920-2850, 1670. ^{1}H NMR ($CDCl_3$ + D_2O): δ 4.28 (br, 1H), 4.05-3.99 (bm, 2H), 3.55-3.44 (bm, 3H), 2.37-2.26 (bm, 1H, H3A), 1.94-1.80

(bm, 1H, H3B), 1.46 (s, 9H, 3 x CH_3 of Boc.). ^{13}C NMR ($CDCl_3$): δ 155.2 & 154.7 (Boc. CO), 80.1 ($\underline{C}Me_3$ of Boc.), 69.4 & 68.7 (C2), 63.7 & 63.2 (C5), 58.3 (CH_2), 37.7 & 37.0 (C3), 28.3 (CH_3 of Boc.)

(2*R*,4*R*)-*cis*-2- (O-methanesulhonyloxymethyl)-4-(O-methanesulphonyl)-N1-(*tert*-butoxycarbonyl) pyrrolidine 26

The treatment of the diol **25** (5 g, 23 mmol) with mesylchloride (5.3 mL, 69 mmol, 3 eq.) in dry pyridine (50 mL) and usual work up and purification gave the di-O-mesylate **26** (7.5 g, 87%).

IR ($CHCl_3$, cm^{-1}): 3022, 1693, 1396, 1365, 1217, 1174.6. ^1H NMR ($CDCl_3$): δ 5.32-5.28 (m, 1H, H4), 4.46-4.44 (m, 1H), 4.42-4.16 (bm, 2H), 3.71-3.69 (m, 2H), 3.10 (s, 3H, $-SO_2CH_3$), 3.06 (s, 3H, $-SO_2CH_3$), 2.47-2.37 (bm, 2H, H3A & H3B) and 1.48 (s, 9H, 3 x CH_3 of Boc.)

(2*R*,4*S*)-2-Azidomethyl-4-azido-N1-(*tert*-butoxycarbonyl) pyrrolidine 27

The diazide **27** was prepared from **26** (2.19gm, 5.87 mmol) following a similar procedure as mentioned for the compound **17**. Yield: 1.4g, (89%).

IR (neat, cm^{-1}): 2978, 2936, 2104 (azide), 1697 (carbonyl). ^1H NMR ($CDCl_3$): δ 4.25-3.85 (m, 2H), 3.80-3.40 (m, 3H), 3.40-3.25 (t, 1H), 2.20-2.05 (m, 2H, H3A & H3B), 1.50 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 153.8, 80.1, 58.7, 55.2, 53.4, 51.2, 35.1, 34.1, 28.0. *m/e* 268(M^++1,6%), 211(37%), 155(52%), 111(57%), 57(100%). $[\alpha]_D^{25^\circ}$ +39.7° (c=0.22,$CHCl_3$).

(2*R*,4*S*)-2-Aminomethyl-4-amino-N1-(*tert*-butoxycarbonyl) pyrrolidine 28

The compound **28** was obtained from **27** (1.2g, 4.5 mmol) by hydrogenation under similar conditions as mentioned for compound **4**. yield: 0.92gm, (95%).

IR (neat, cm^{-1}): 3450, 1745. ^1H NMR ($CDCl_3$): δ 3.95-3.85 (bm, 1H), 3.65-3.40

(m, 3H), 3.20-3.00 (m, 4H exchangeable) 2.85-2.70 (m,2H), 1.95-1.80 (m, 1H), 1.80-1.7-0 (m,1H), 1.5 (s, 9H). ^{13}C NMR δ ($CDCl_3$): 153.7, 77.9, 57.5, 53.9, 47.8, 43.9, 36.9 and 27.2. *m/e* (M^++1) 261.1 (50), 144.1 (100). $[\alpha]_D^{25^\circ}$ +36.8° (c=0.32, $CHCl_3$).

(2*R*,4*S*)-2-[N,N'-bis(2-cyanomethyl)aminomethyl]-4-[(2-cyanoethyl) amino]-N1-(*tert*-butoxycarbonyl) pyrrolidine 29

The diamine **28** (0.3 g, 1.4 mmol) was dissolved in MeOH (3 mL), treated with acrylonitrile (10 eq. 0.9 mL, 14 mmol) at 0-10°C for 1 hour and for 4 days at 60°C. The reaction mixture on work up and purification by column chromatography on silica gel gave ticyano derivative **29** (0.37g, 71 %).

IR ($CHCl_3$, cm^{-1}): 3415, 2250, 1681. ^{1}H NMR ($CDCl_3$): δ 4.05-3.90 (m, 1H), 3.65-3.40 (m, 2H), 3.08-2.65 (m, 7H), 2.60-2.45 (q, 6H),2.40-2.12 (m, 4H), 1.45 (s, 9H). *m/e* 375 (M^++1).

(2*R*,4*S*)-2-Aminomethyl-4-aminopyrrolidine trihydrochloride 31

The diamine **28** was treated with (1.2g, 4.5 mmol) 5N aq. HCl under similar conditions as mentioned for compound **18** and used as such for the next step.

^{1}H NMR ($CDCl_3$): δ 4.45-4.35 (m, 2H), 4.15-3.95 (m, 1H), 3.70-3.40 (m, 3H), 2.70-2.52 (m, 1H), 2.52-2.40 (m, 1H).

(2*R*,4*S*)-2-[N,N'-bis-(2-cyanoethyl)aminomethyl]-4-[N,N'-bis-(2-cyanoethyl) amino]-N1-(2-cyanoethyl) pyrrolidine 32

The trihydrochloride salt **31** (0.5 g, 2.22 mmol) was neutralized with triethylamine and cyanoethylated as mentioned for the compound **21** to yield **32** (0.65 g, 77%).

IR (neat, cm^{-1}): 2955, 2835, 2247. ^{1}H NMR δ ($CDCl_3$): 3.60-3.40 (m,1H), 3.45-3.1-0 (m,2H), 3.05-2.75 (m,10H), 2.70-2.35 (m,11H), 2.30-2.15 (m,2H), 2.15 -2.10 (m,2H). ^{13}C NMR δ ($CDCl_3$): 119.0, 118.9, 61.6, 58.8, 58.0, 57.1, 50.2, 49.9, 47.2, 32.5,

17.4, 17.1, 16.7. FAB MS: 381(M^++1,30%), 379(30%), 244(100%). $[\alpha]_D^{25°}$ +71.9° (c=0.35,$CHCl_3$).

(2*R*,4*S*)-2-[N,N'-bis-(3-aminopropyl)aminomethyl]-4-[N,N'-bis(3-aminopropyl) amino]-N1-(3-aminopropyl) pyrrolidine V

The pentacyano derivative **11** (0.5 g, 1.31 mmol) was reduced by catalytic hydrogenation (H2, 210 psi) using Ra-Ni/NaOH in EtOH for 12 hours. The catalyst was filtered and the compound was purified by ion-exchange chromatography on Amberlite IR-120 (cation exchange resin) using water as an eluant to get the (2*R,*4*S*)-*tran*s octamine **V** (0.5 g, 95%).

IR (neat, cm^{-1}): 3420. ^{1}H NMR (D_2O): δ 3.55-3.10 (m, 4H), 3.05-2.25 (m, 22H), 2.20-1.50 (bm, 12H). ^{13}C NMR δ (D_2O): 74.5, 71.5, 71.0, 64.5, 60.9,51.3, 47.4, 47.3, 39.6, 37.6, 36.4. $[\alpha]_D^{25°}$ +54.8°(c=0.22, H_2O). *m/e,* 401.3(M^++1, 42%), 344.2(70%), 287.2(58%), 132.1(100%).

(2*R*,4*S*)-4-O-Benzoyl-N1-(*tert*-Butoxycarbonyl)-D-proline methyl ester 33

The compound **24** (3.5 g, 14.3 mmol) was treated with triphenyl phosphine (4.9 g, 18.6 mmol, 1.3 eq.), diisopropyl diazodicarboxylate (3.7 mL, 18.6 mmol, 1.3 eq.), benzoic acid (2.26 g, 18.6 mmol, 1.3 eq.) in anhydrous THF (35 mL), usual work up and purification gave the product 33 as a white solid (4.4 g, 89.7%). m.p 91°C.

IR (nujol, cm^{-1}): 2950-2850, 1745, 1715, 1670. ^{1}H NMR ($CDCl_3$): δ 8.04-8.00 (m, 2H,ArH), 7.65-7.52 (m, 1H, ArH), 7.52-7.4 (m, 2H, Ar<u>H</u>), 5.60-5.50 (bm, 1H, <u>H</u>4), 4.68-4.40 (m, 1H, <u>H</u>2), 3.90-3.85 (m, 1H, <u>H</u>5A), 3.80 (s, 3H, -OC<u>H</u>$_3$), 3.70-3.60 (m, 1H, <u>H</u>5B), 2.7-2.45 (m, 1H, <u>H</u>3A), 2.45-2.25 (m, 1H, <u>H</u>3B) and 1.5 (s, 9H, 3 x CH_3 of Boc.). ^{13}C NMR ($CDCl_3$): δ 173.1 & 172.8 (ester carbonyl), 165.9 (benzoate carbonyl), 154.4 & 153.6 (Boc. CO), 133.4, 129.7, 128.5 (Ar-<u>C</u>), 80.5 (<u>C</u>Me$_3$ of Boc.), 73.3 & 72.6 (<u>C</u>2), 58.1 & 57.7 (<u>C</u>5), 52.4 (O<u>C</u>H$_3$), 52.2, 36.7 & 35.7 (C3), 28.3 (CH_3 of Boc.). $[\alpha]_D^{25°}$

+49.5°(c=0.40, $CHCl_3$).

(2*R*,4*S*)-*trans*-4-hydroxy-N1-(*tert*-Butoxycarbonyl)-D-prolinol 34

The reduction of the ester **33** (3 g, 8.6 mmol) with lithium borohydride (0.56 g, 25.8 mmol, 3 eq.) in anhydrous THF (30 mL) and usual work up afforded the diol **34** (1.4 g, 76%).

IR ($CHCl_3$, cm^{-1}): 3420-2200, 3010-2960 and 1660. ^1H NMR ($CDCl_3$): δ 4.38 (bs, 1H), 4.25-3.90 (bm, 1H), 3.90-3.30 (m, 4H), 3.20-2.50 (bs, 1H, exchangeable), 2.20-1.90 (m, 1H, H3A), 1.85-1.60 (bs, 1H, H3B), 1.50 (s, 9H, 3 x CH_3 of Boc.).

(2*R*,4*S*)-*trans*-)-2-(O-methanesulphonyloxymethyl)-4-(O-methanesulphonyl)-N1-(*tert*-butoxycarbonyl) pyrrolidine 35

The trans diol **34** (1.2 g, 5.5 mmol) in dry pyridine (15 mL) was treated with methanesulfonyl chloride (1.7 mL, 22 mmol, 4 eq.) at 0°C to get the dimesylate **35** (1.9 g, 93%).

IR (neat, cm^{-1}) 2935, 1682, 1410, 1348 and 1172. ^1H NMR ($CDCl_3$): δ 5.35-5.18 9 (m, 1H, H4), 4.78-4.55 (m, 0.5 H), 4.55-4.40 (m, 0.5 H), 4.35-4.10 (m, 2H), 4.05-3.68 (m, 1H), 3.68-3.50 (m, 1H), 2.60-2.20 (m, 2H, H3A & H3B), 1.48 (s, 9H, 3 x CH_3 of Boc.)

(2*R*,4*R*)-2-Azidomethyl-4-azido-N1-(*tert*-butoxycarbonyl) pyrrolidine 36

The dimesylate **35** (1.5 g, 4.02 mmol) on treatment with sodium azide (1.04 g, 16.1 mmol, 4 eq.) in of DMF (15 mL) gave the diazide **36** (0.88g, 82.2%).

IR (neat, cm^{-1}): 2104, 1697. ^1H NMR ($CDCl_3$): δ 4.25-3.85 (m, 2H), 3.85-3.65 (m, 1H), 3.65-3.25 (m, 3H), 2.35-2.15 (m, 1H), 2.10-1.95 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.1, 80.6, 59.2, 56.1, 53.7, 52.8, 51.9, 33.7, 28.5, 28.1. $[\alpha]_D^{25}$ +11.2° (c=0.52, $CHCl_3$).

(2*R*,4*R*)-2-Aminomethyl-4-amino-N1-(*tert*-butoxycarbonyl) pyrrolidine 37

The bisazide **36** (0.75 g, 2.81 mmol) on hydrogenation (H_2, 60 psi) over Pd-C (0.22 g) as a catalyst in methanol (10 mL) gave the diamine **37** in quantitative yield (0.58 g, 97 %).

IR (neat, cm^{-1}): 1682, 3250-3500. ^1H NMR ($CDCl_3$): δ 4.20-3.80 (s, 4H, exchangeable), 3.75-3.50 (m, 2H), 3.40-3.10 (m, 2H), 2.85-2.65 (m, 1H), 2.45-2.25 (m, 1H), 1.95 (m, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 154.8, 79.9, 63.6, 57.6, 54.4, 45.9, 34.2, 28.4. $[\alpha]_D^{25^\circ}$ +13.7° (c=0.35,MeOH).

(2*R*,4*R*)-2-[N,N'-bis(2-cyanoethyl)aminomethyl]-4-[N,N'-bis-(2-cyanoethyl) amino]-N1-(2-cyanoethyl) pyrrolidine 38

IR (neat, cm^{-1}): 2247. ^1H NMR ($CDCl_3$): δ 3.45-3.10 (m, 3H), 3.10-2.75 (m, 7H), 2.70-1.90 (m, 17H), 1.70-1.45 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 119.2 & 119.1 (cyano), 61.9, 58.4, 58.2, 58.0, 50.4, 49.8, 47.4, 34.3, 18.2, 16.8. FAB MS: M^++1, 381 (100%). $[\alpha]_D^{25^\circ}$ = +143.1° (c=0.11, $CHCl_3$).

(2*S*,4*S*) Decanitrile 39

To a cold solution of the (2*S*,4*S*)-L-cis octamine **III** (0.8 g, 2 mmol) in methanol (10 mL) was added acrylonitrile (25 eq. 3.3 mL,50 mmol) at 0-10°C over a period of 25 minutes. After stirring at 10°C for 1 hour and 4 days at 60°C quantitative bis cyanoethylation was obtained. Excess of acrylonitrile and methanol were removed and the residue was purified by column chromatography on 230-400 silica gel using incremental amount of methanol in ethyl acetate to get the decanitrile **38**. Yield 1.4 g (75.3%).

IR (neat, cm^{-1}): 2247(cyano). ^1H NMR ($CDCl_3$) : δ 3.45-3.25(m, 1H), 3.10-2.95 (m, 1H), 2.95-2.75 (t, 20H), 2.75-2.30 (m, 42H), 2.25-2.00(m, 5H), 1.75-1.50 (m, 10H). MALDI TOF Mass 932 (M^++1, 100%).

(2*S*,4*S*)-L-*cis* octadecapolyamine VIII

The hydrogenation was carried out in a stainless steel autoclave () To 25 mL of a 1.4N NaOH in EtOH/H_2O (95:5) decanitrile **39** (1g, 1.1 mmol) in methanol was added. Under nitrogen the mixture was treated with Raney-nickel (0.6 g) (slurry in water. Aldrich) and the mixture was hydrogenated at 1800 rpm at 600 psi H_2 pressure for 10 hours at room temperature. The catalyst was filtered and washed with EtOH/H_2O(95:5). The solvents were removed by rotary evaporation and the residue was purified by passing through amberlite IR-120 cation exchange resin and the product was eluted with water. Yield 0.97 g, 93.3 %.

IR (neat, cm^{-1}): 3350. ^1H NMR (D_2O) : δ 3.55 –3.45 (bs, 1H), 2.90(s, 3H), 3.00-2.60 (m, 28H), 2.60-2.40 (m, 28H), 2.60-2.40 (m, 34H), 2.40-2.10 (m, 3H), 1.90 (s, 2H), 1.80-1.55 (m 26H).

pK_a determination of polyamines

An aqueous solution of the (2S,4R)-L-trans polyamine (**IV**) (as its hydrochloride salt) 2.5 mg/mL was titrated with aq. NaOH (0.1 N) by addition of aliquots of 40 µL at 25^oC, with constant stirring. The pH after each addition was measured by a Digital pH meter (model DPH 504).

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- Compound **9** ^{13}C NMR Page 83
- Compound **9** Mass Page 84
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- Compound **11** Mass Page 87
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- Compound **13** ^{13}C NMR Page 90
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- Compound **IV** ^{1}H NMR Page 115
- Compound **IV** ^{13}C NMR Page 116
- Compound **IV** Mass Page 117
- Compound **V** ^{1}H NMR Page 118
- Compound **V** ^{13}C NMR Page 119
- Compound **V** Mass Page 120
- Compound **39** ^{1}H NMR Page 121
- Compound **39** Mass Page 122
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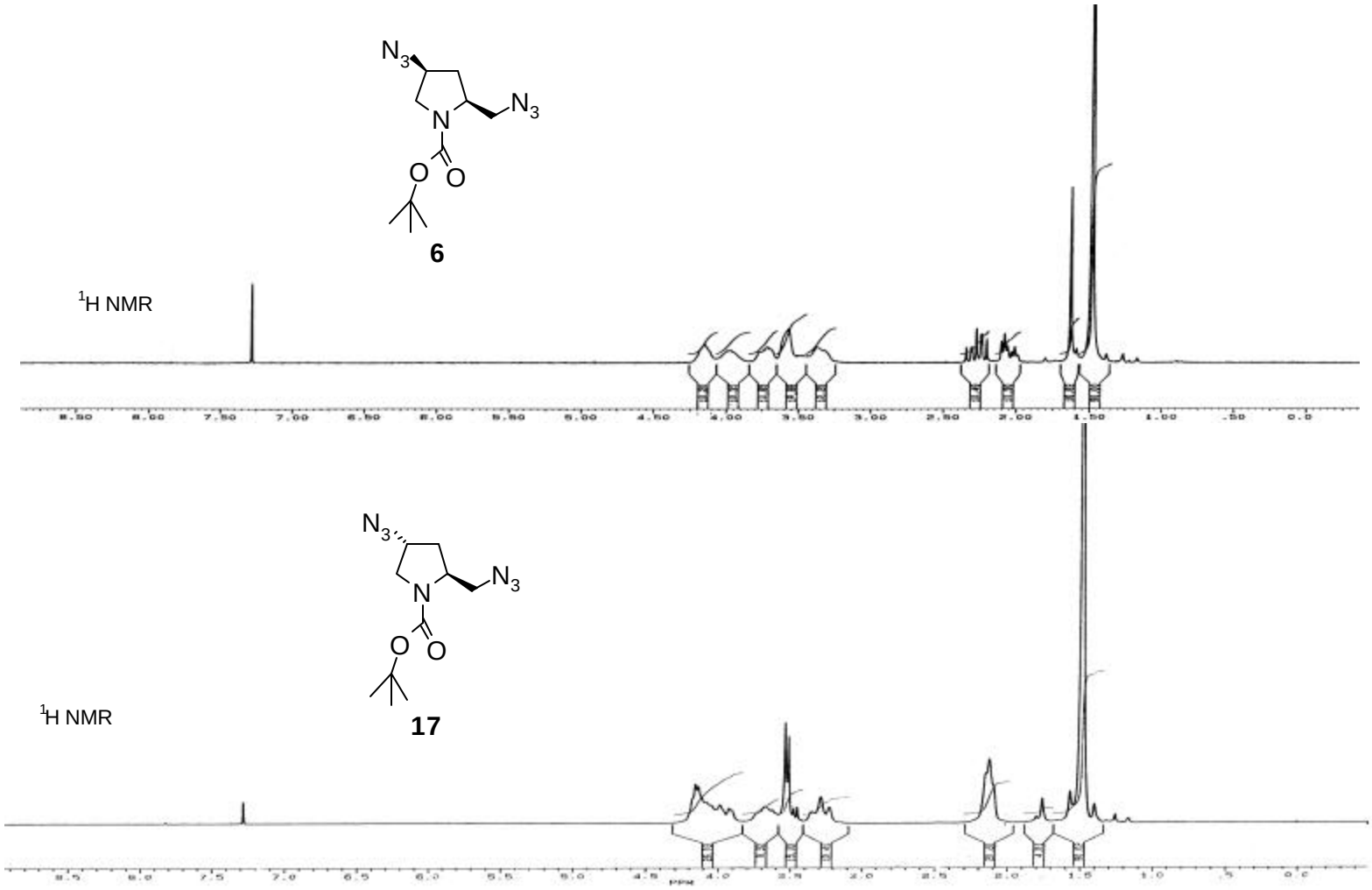
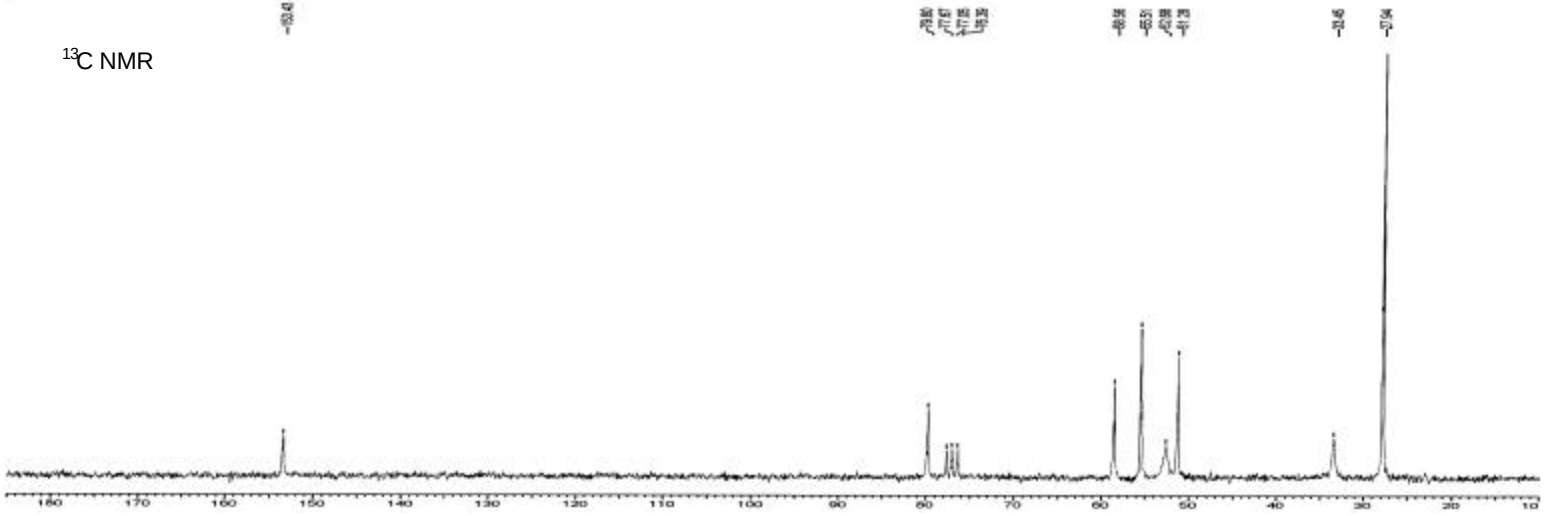
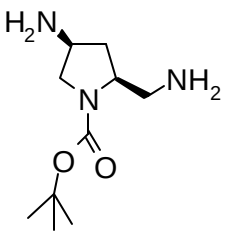

N3
N3
N
O
O
6
1H NMR
N3
N3
N
O
O
17
1H NMR

6

DEPT

^{13}C NMR

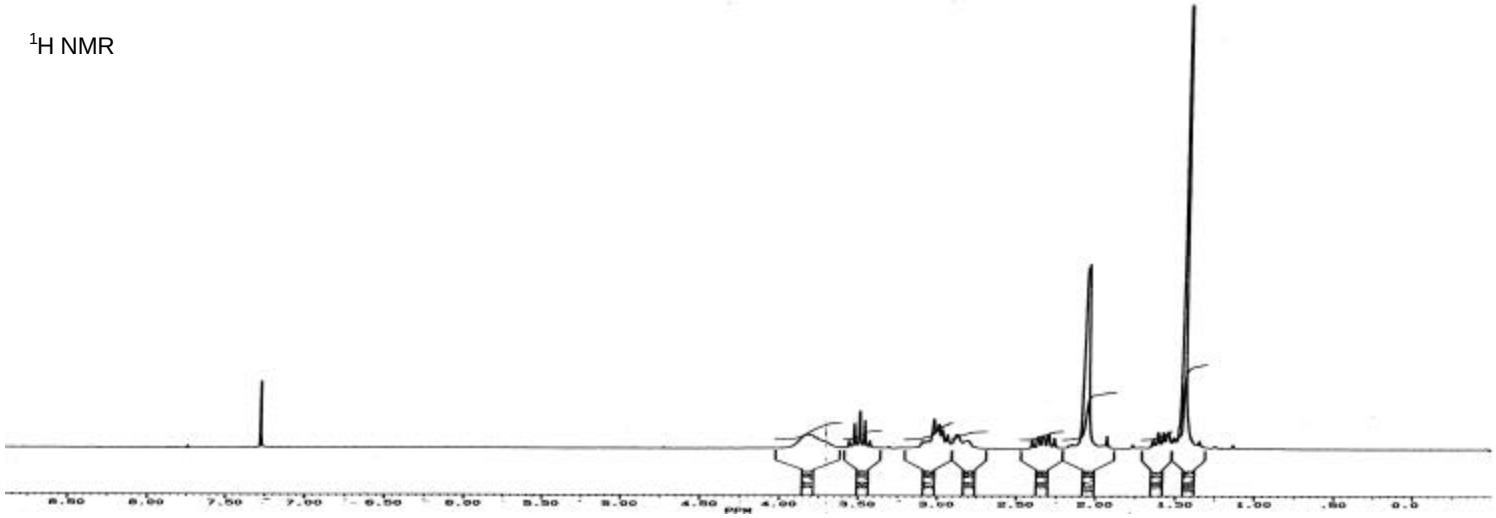




7

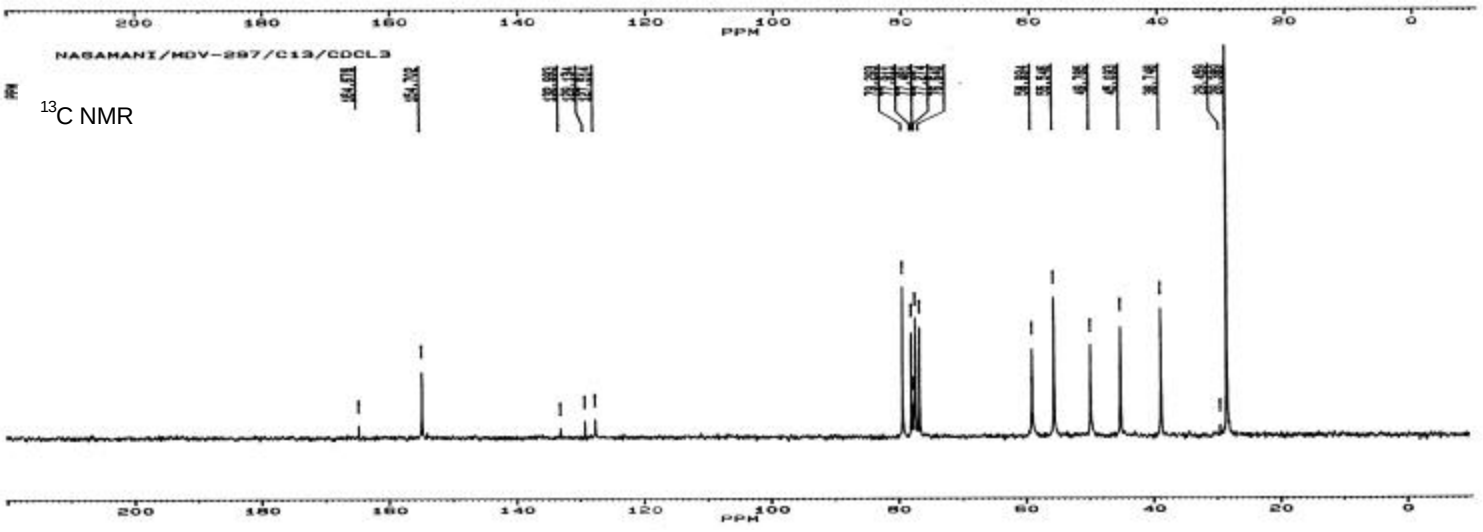
D_2O exchange

^1H NMR

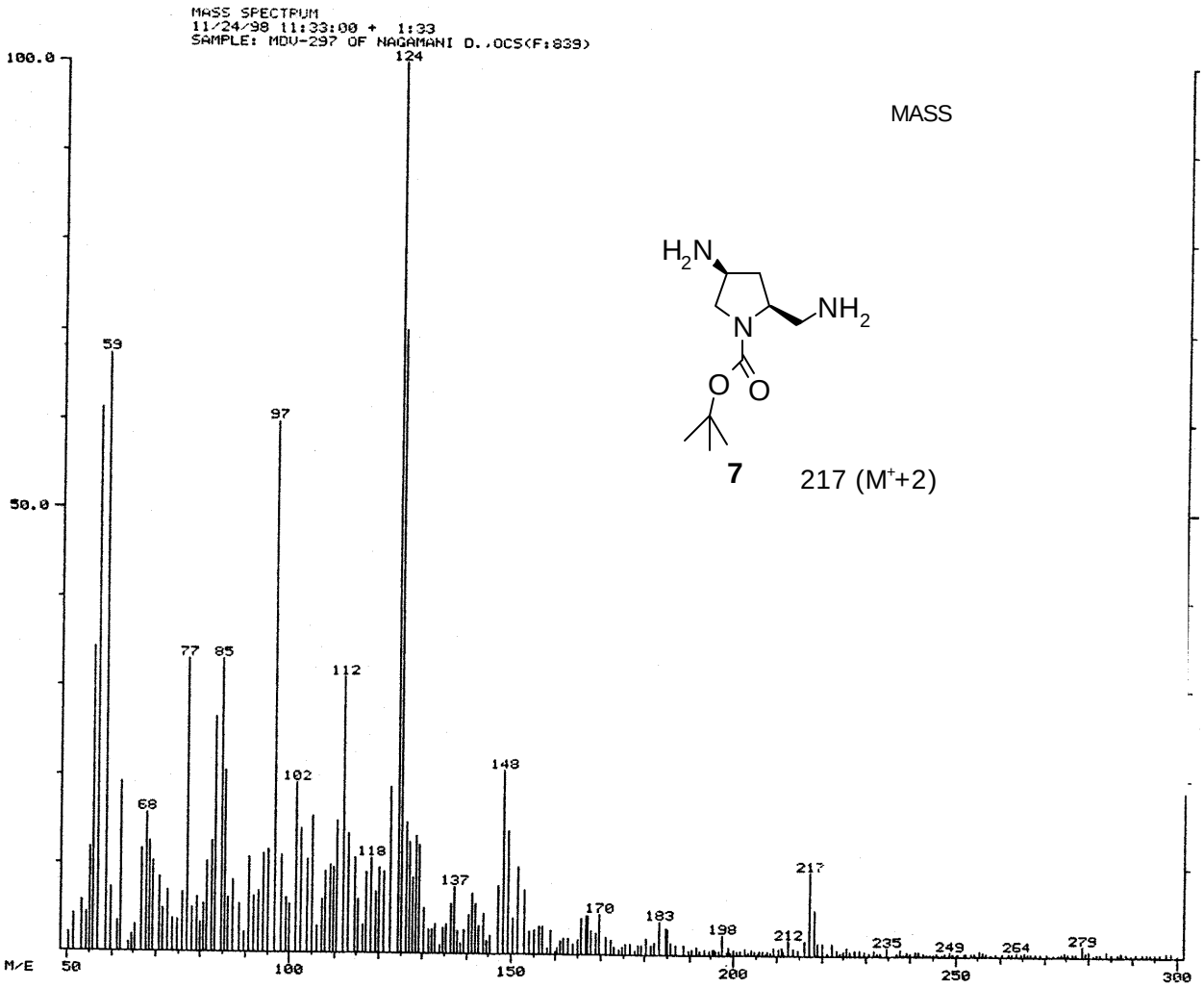


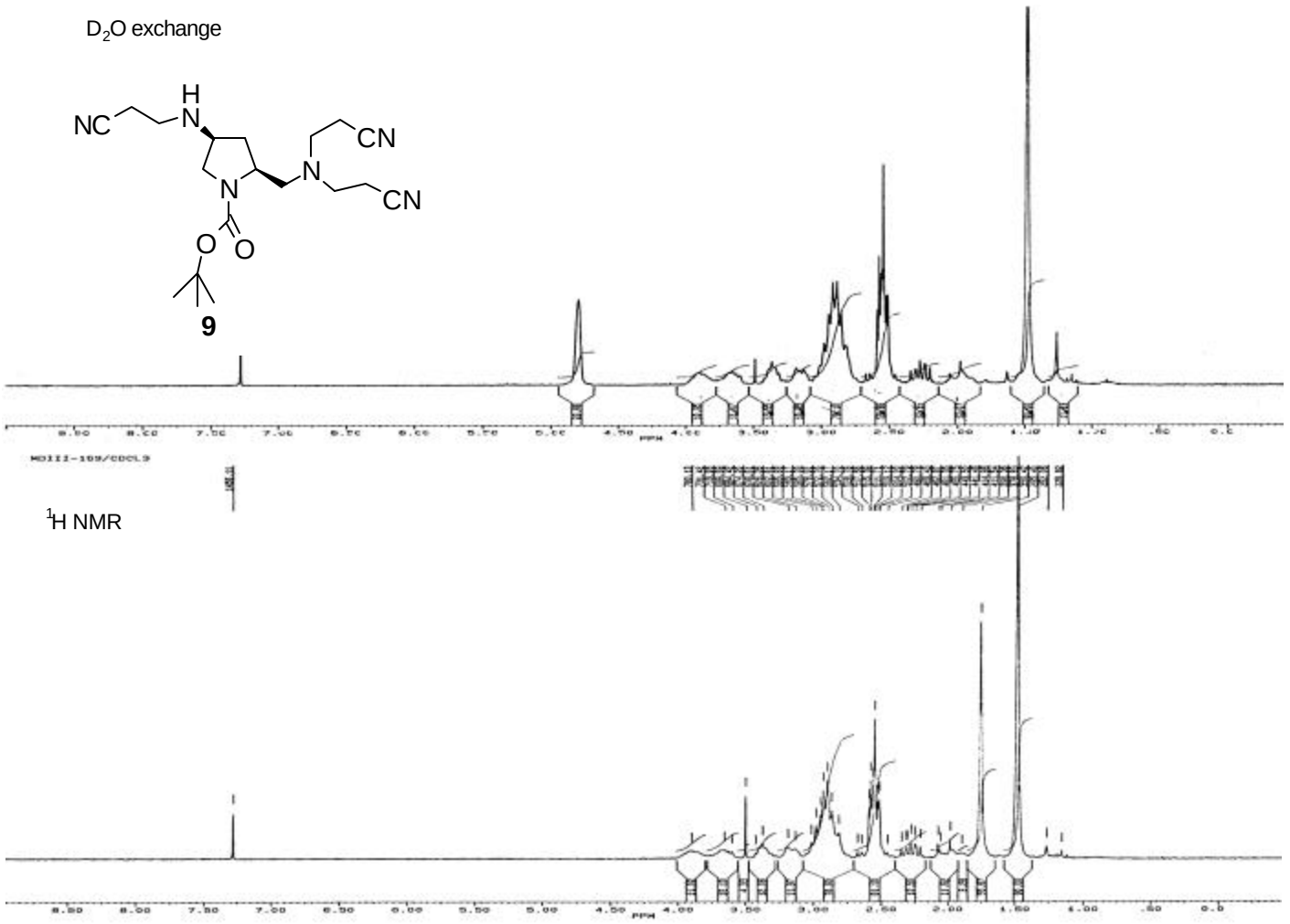
7

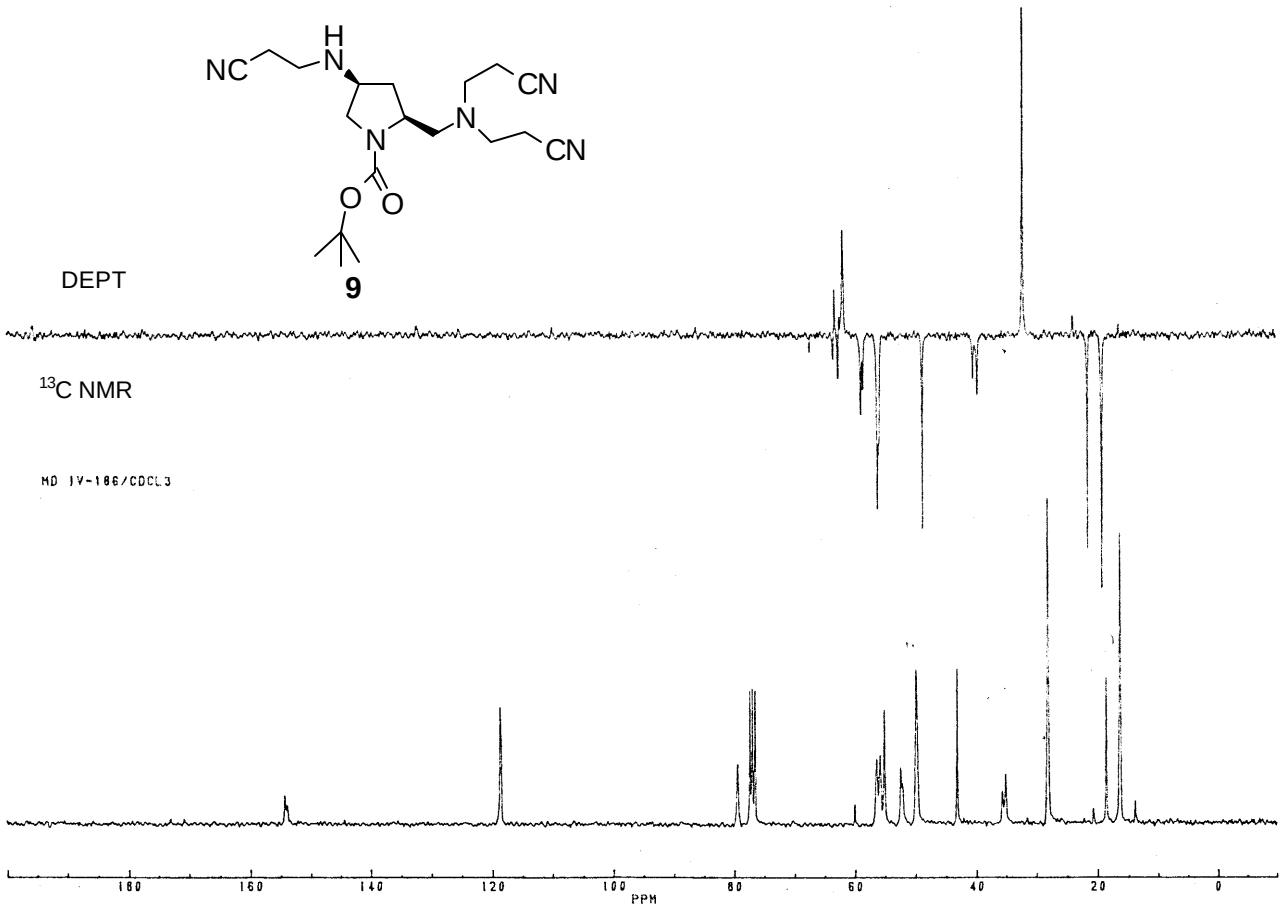
DEPT

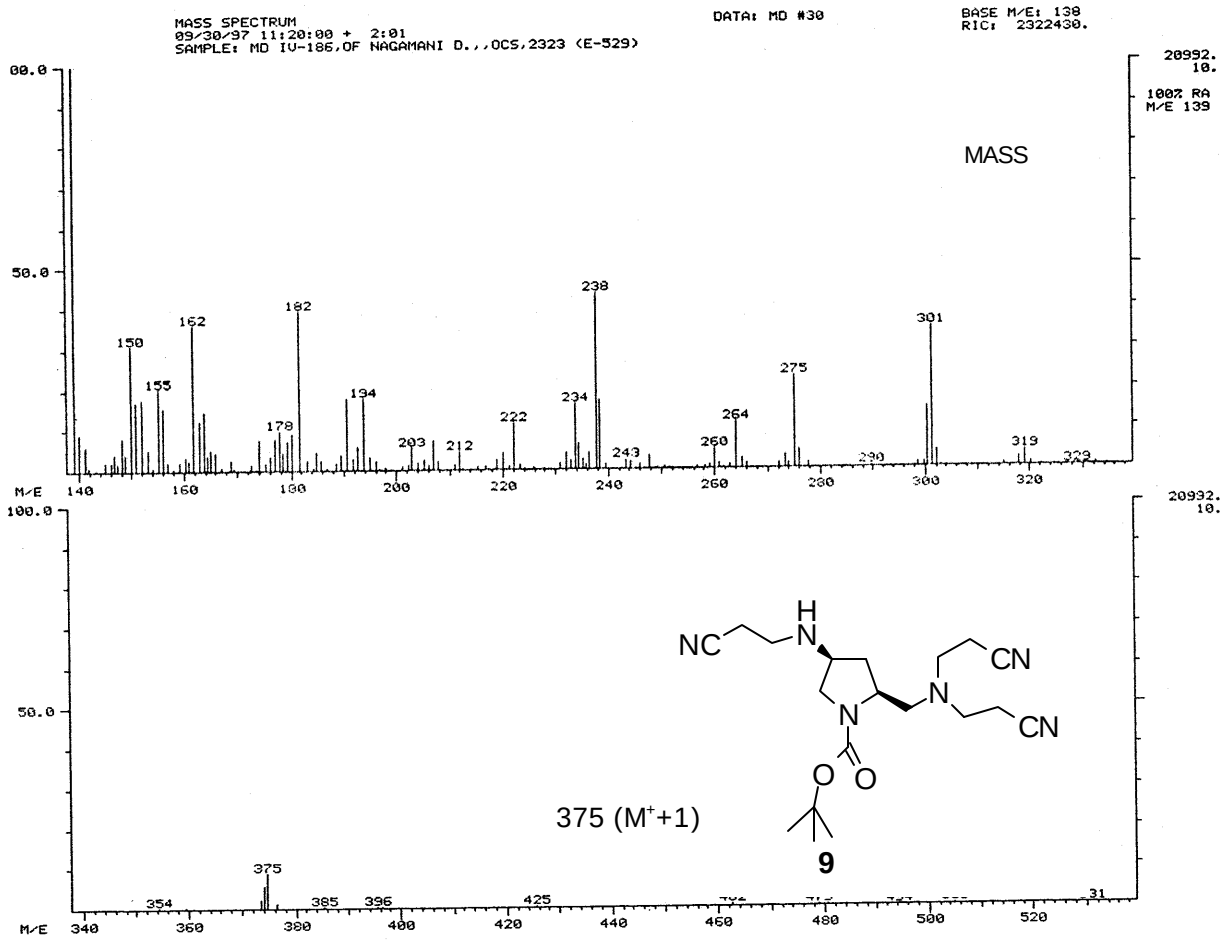


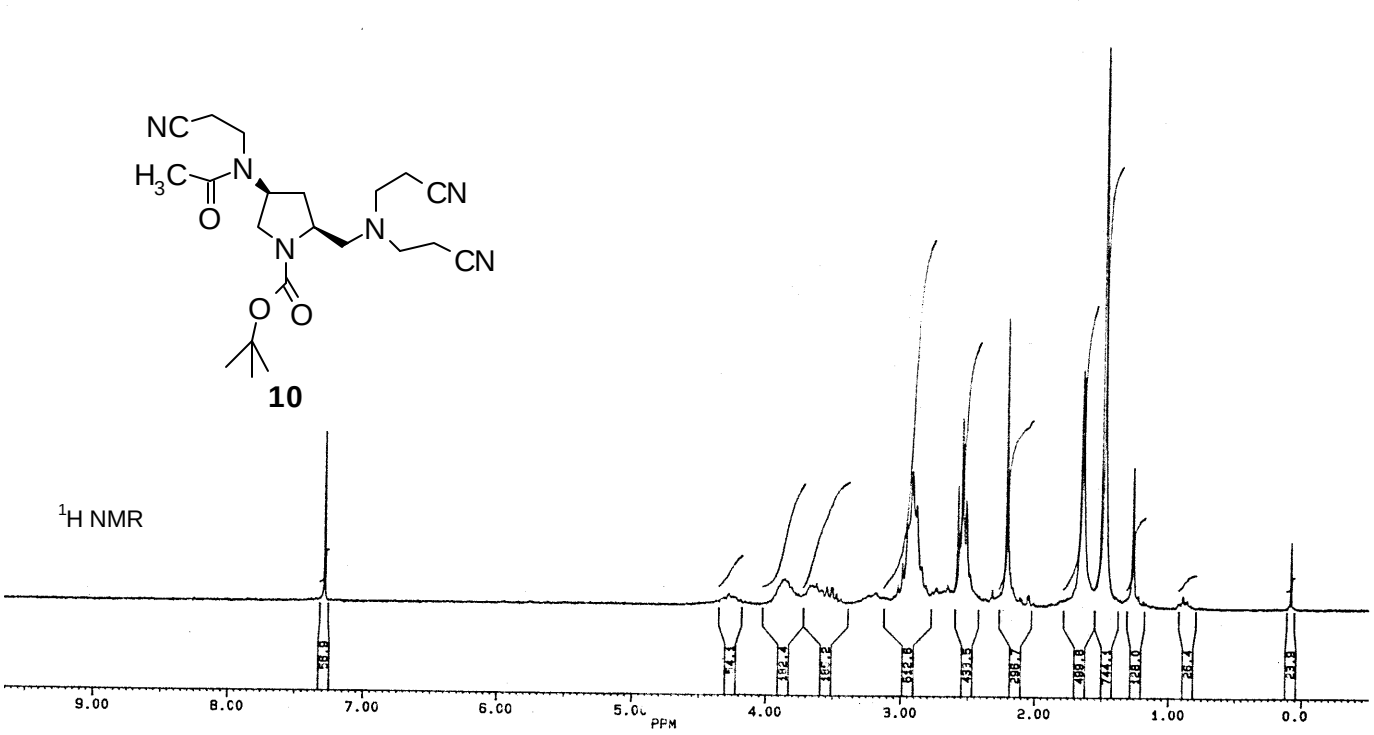
^{13}C NMR

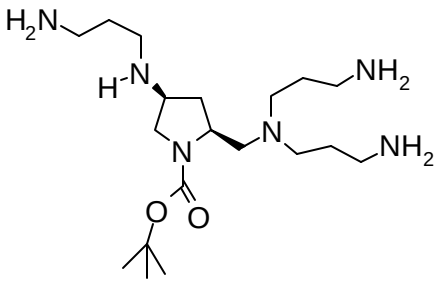

MASS SPECTRUM
11/24/98 11:33:00 + 1:33
SAMPLE: MDV-297 OF NAGAMANI D.,OCS(F:839)
100.0
50.0
M/E
50
100
150
200
250
300
59
77
85
97
102
112
118
124
137
148
170
183
198
212
217
235
249
264
279
MASS
H_2N
NH_2
N
O
O
7
217 (M^++2)


D2O exchange
NC
H
N
CN
N
CN
N
O
O
9
MDIII-169/CDCL3
1H NMR
PPM
PPM


NC
H
N
CN
N
CN
N
O
O
DEPT
9
13C NMR
MD IV-186/CDCL3
180
160
140
120
100
80
60
40
20
0
PPM


MASS SPECTRUM
09/30/97 11:20:00 + 2:01
SAMPLE: MD IV-186,OF NAGAMANI D.,,OCS,2323 (E-529)
DATA: MD #30
BASE M/E: 138
RIC: 2322430.
20992.
10.
100% RA
M/E 139
MASS
M/E
20992.
10.
375 (M+1)
9


NC
H3C
O
N
N
CN
CN
N
O
O
O
10
1H NMR
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.0
PPM

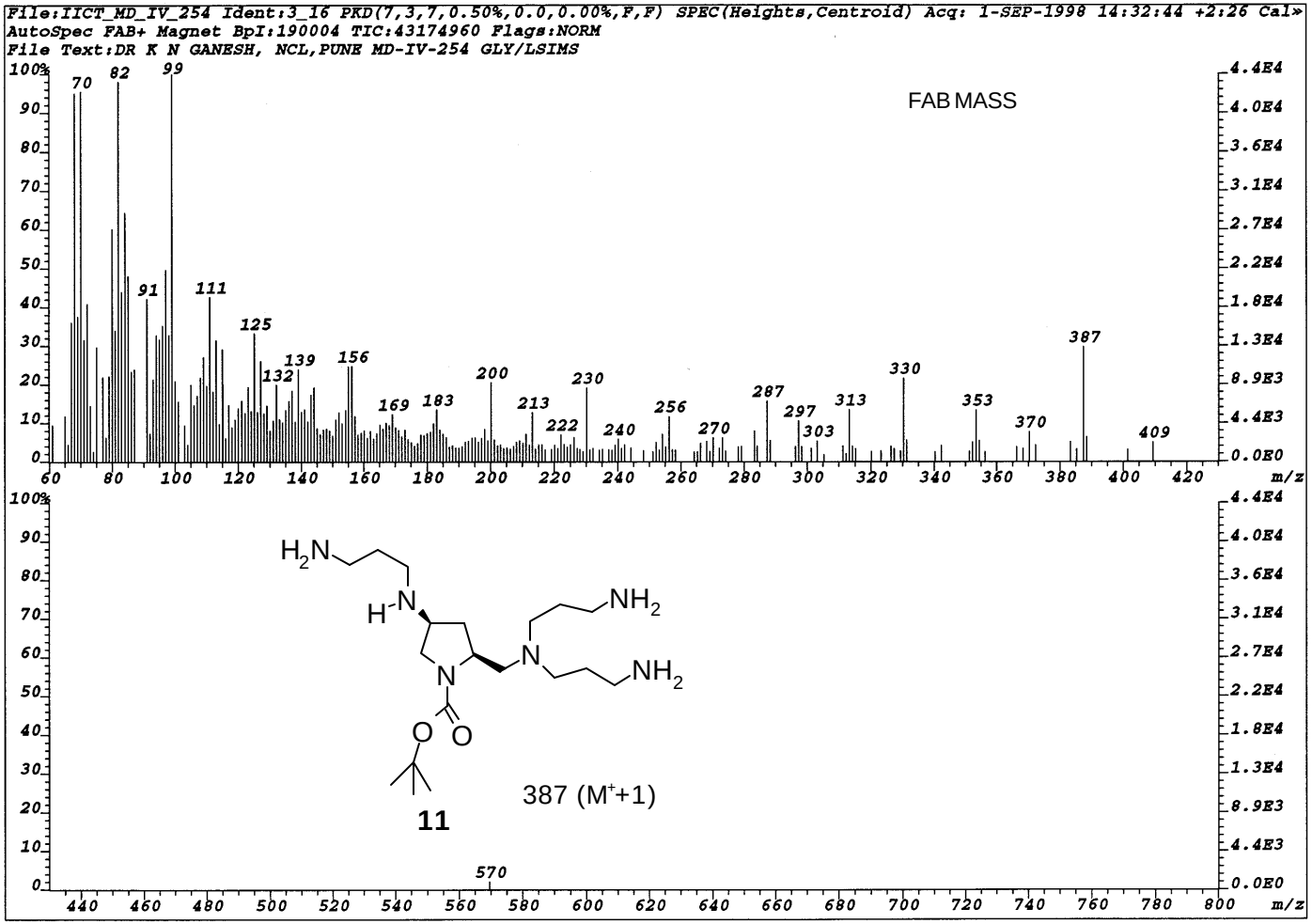


11

^{13}C NMR

FROM: 186.90 ppm TO: 4.60 ppm

Peak	Frecuency	ppm	Intensity
1	13754.37	182.24	1.338
2	11936.73	158.15	2.308
3	6262.01	82.97	1.615
4	4660.77	61.75	0.586
5	4654.69	61.67	0.417
6	4513.45	59.80	1.876
	4484.34	59.41	0.753
	4447.32	58.92	2.933
	4246.53	56.26	0.716
	4233.07	56.08	0.757
	4210.24	55.78	4.478
	4183.90	55.43	1.259
	4159.52	55.11	1.696
	4041.43	53.55	0.675
	4023.18	53.30	0.461
	4015.77	53.21	0.476
	4011.84	53.15	0.447
	4002.71	53.03	0.437
	3974.47	52.66	0.768
	3922.35	51.97	197.038
	3820.85	50.62	0.724
	3707.73	49.12	2.753
23	3681.91	48.78	0.707
24	3242.17	42.96	5.250
25	3223.89	42.71	4.244
26	3023.86	40.06	0.947
27	2646.32	35.06	1.617
28	2440.53	32.34	2.132
29	2332.49	30.90	19.281
30	1994.31	26.42	0.984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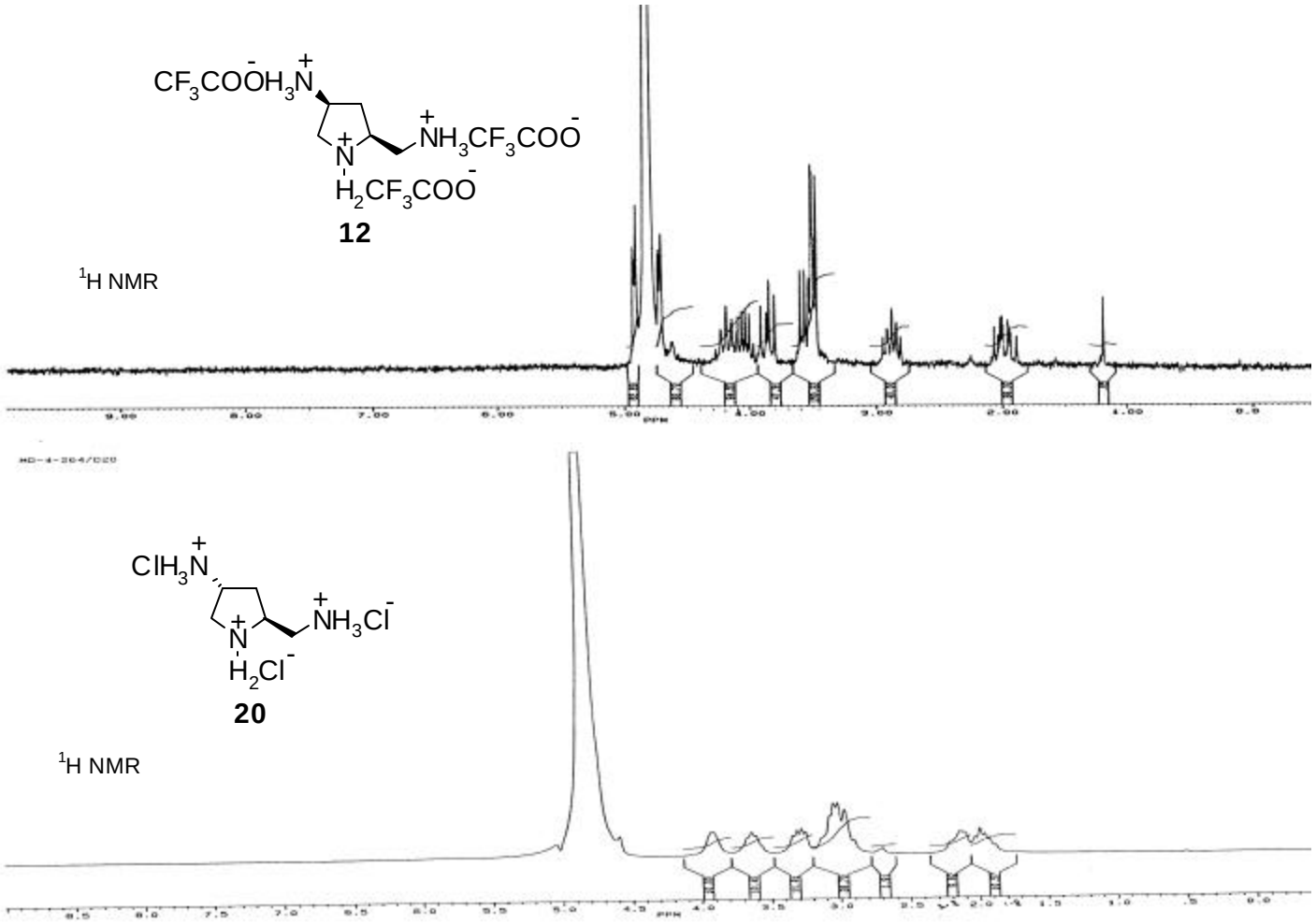
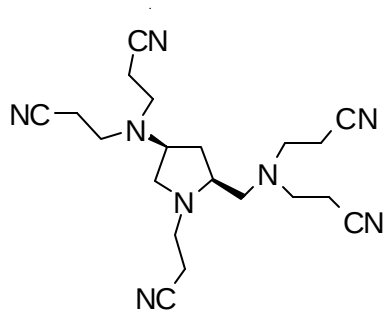
AutoSpec FAB+ Magnet BpI:190004 TIC:43174960 Flags:NORM
File Text:DR K N GANESH, NCL,PUNE MD-IV-254 GLY/LSIMS
FAB MASS
387 (M+1)
11
m/z


CF3COO-H3N+
+NH3CF3COO-
N+
H2CF3COO-
12
1H NMR
ClH3N+
+NH3Cl-
N+
H2Cl-
20
1H NMR


CN
NC
N
CN
N
N
CN
NC

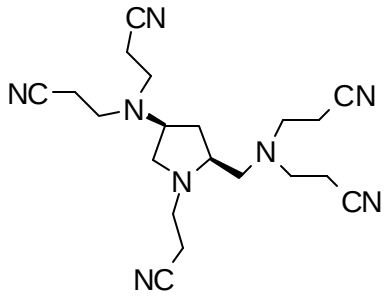
13

^{1}H NMR

131.8
.0
358.8
163.3
1797.1
3410.5
64.0
241.3
282.9
106.5
8.50
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
.50
0.0
PPM

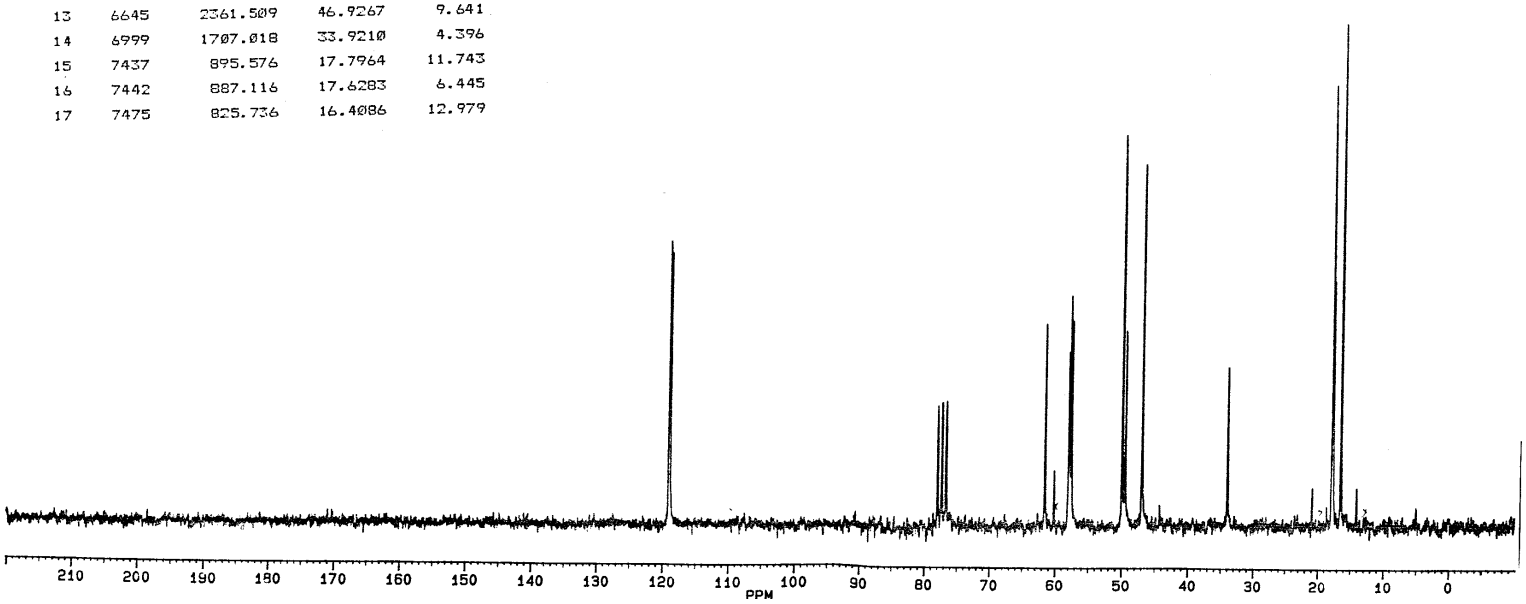
NAGMANI/C13/ MD-IV-190/**SVT

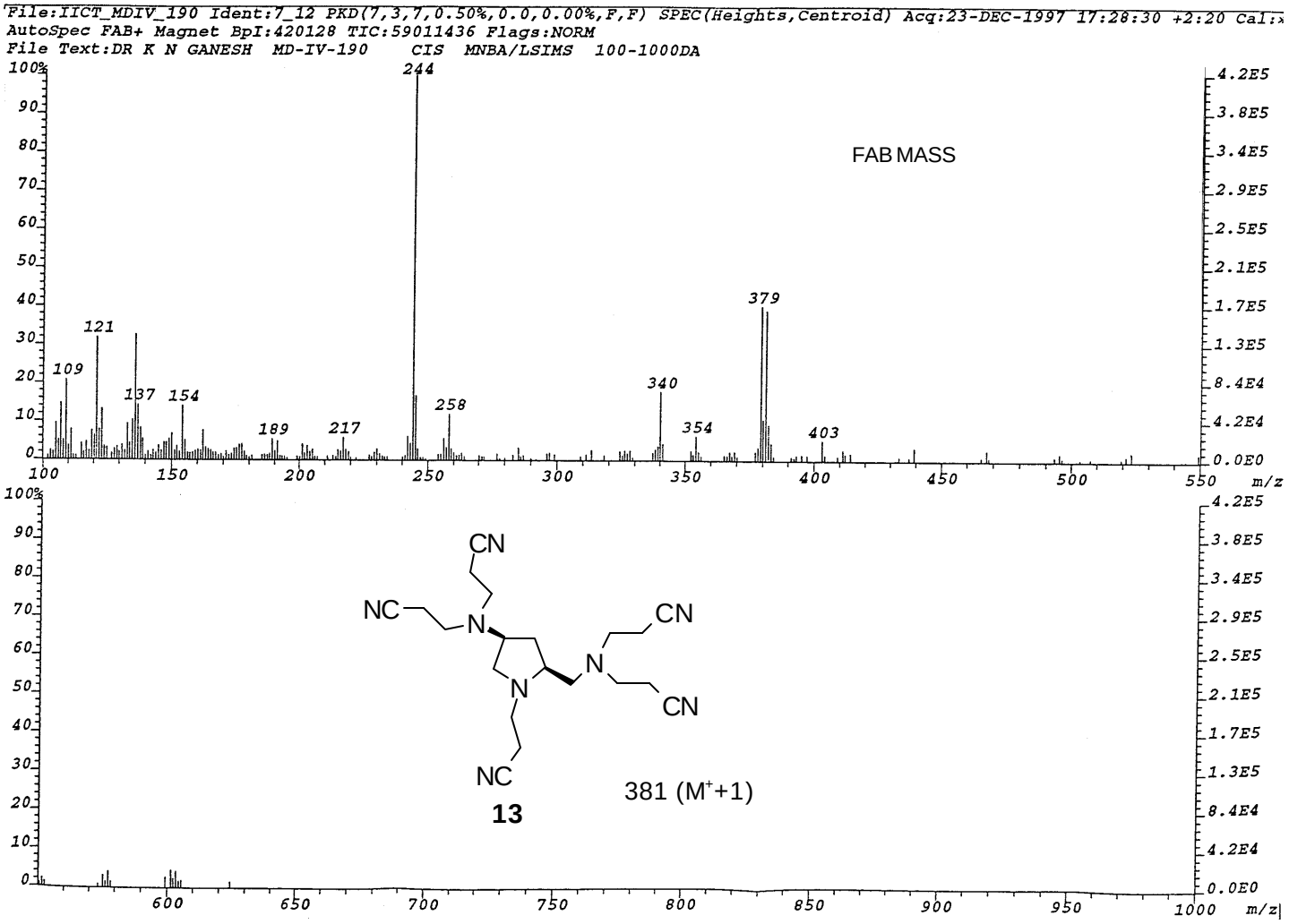
	CURSOR	FREQUENCY	PPM	INTENSITY
1	4682	5990.887	119.0479	7.408
2	4687	5982.627	118.8838	7.009
3	5801	3921.472	77.9255	3.157
4	5819	3889.555	77.2913	3.256
5	5836	3857.051	76.6454	3.246
6	6247	3096.540	61.5329	5.383
7	6286	3024.832	60.1080	1.499
8	6344	2916.715	57.9595	4.850
9	6350	2906.643	57.7594	5.977
10	6356	2895.512	57.5382	5.319
11	6562	2513.893	49.9548	10.401
12	6575	2490.206	49.4841	5.098
13	6645	2361.509	46.9267	9.641
14	6999	1707.018	33.9210	4.396
15	7437	895.576	17.7964	11.743
16	7442	887.116	17.6283	6.445
17	7475	825.736	16.4086	12.979



13

^{13}C NMR

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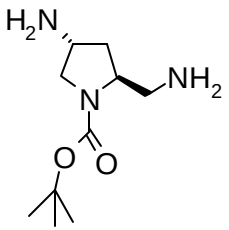
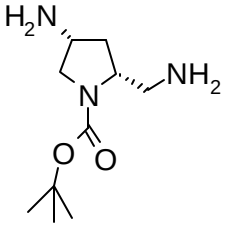
AutoSpec FAB+ Magnet BpI:420128 TIC:59011436 Flags:NORM
File Text:DR K N GANESH MD-IV-190 CIS MNBA/LSIMS 100-1000DA
FAB MASS
244
379
121
109
137
154
189
217
258
340
354
403
m/z
CN
NC
N
CN
N
N
CN
NC
381 (M+1)
13

^{1}H NMR

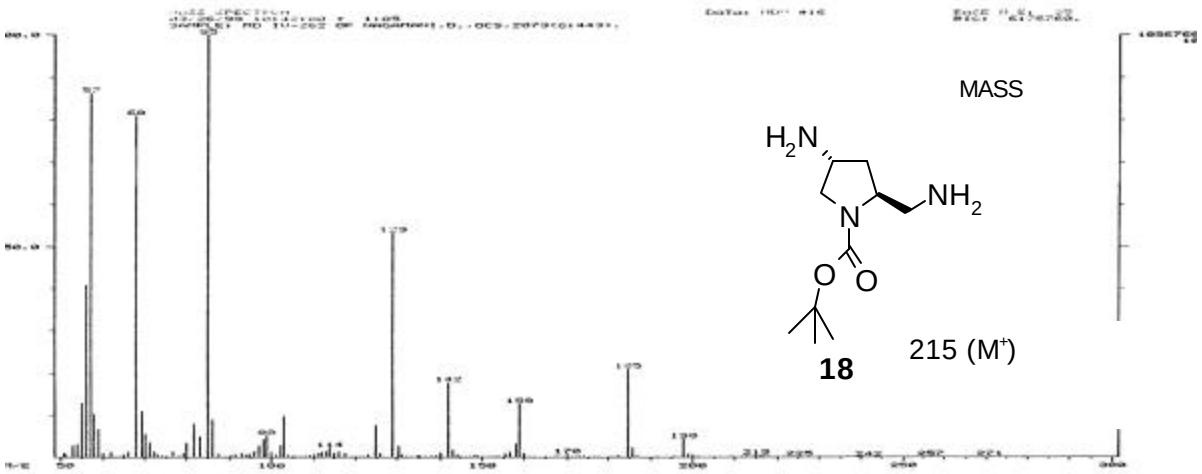


18

^{1}H NMR



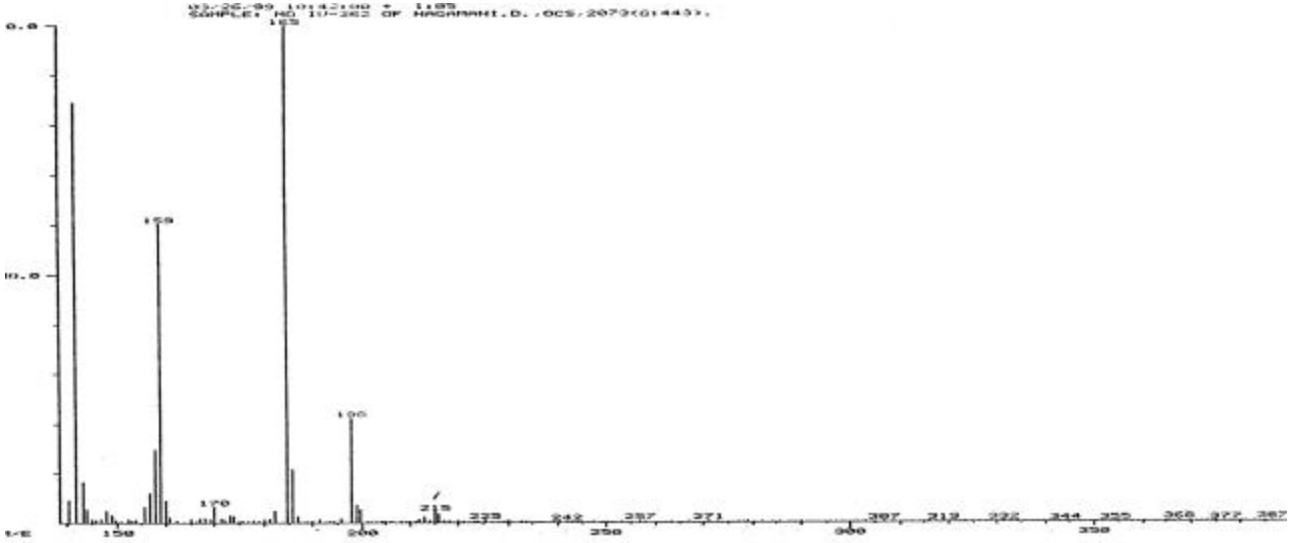
37

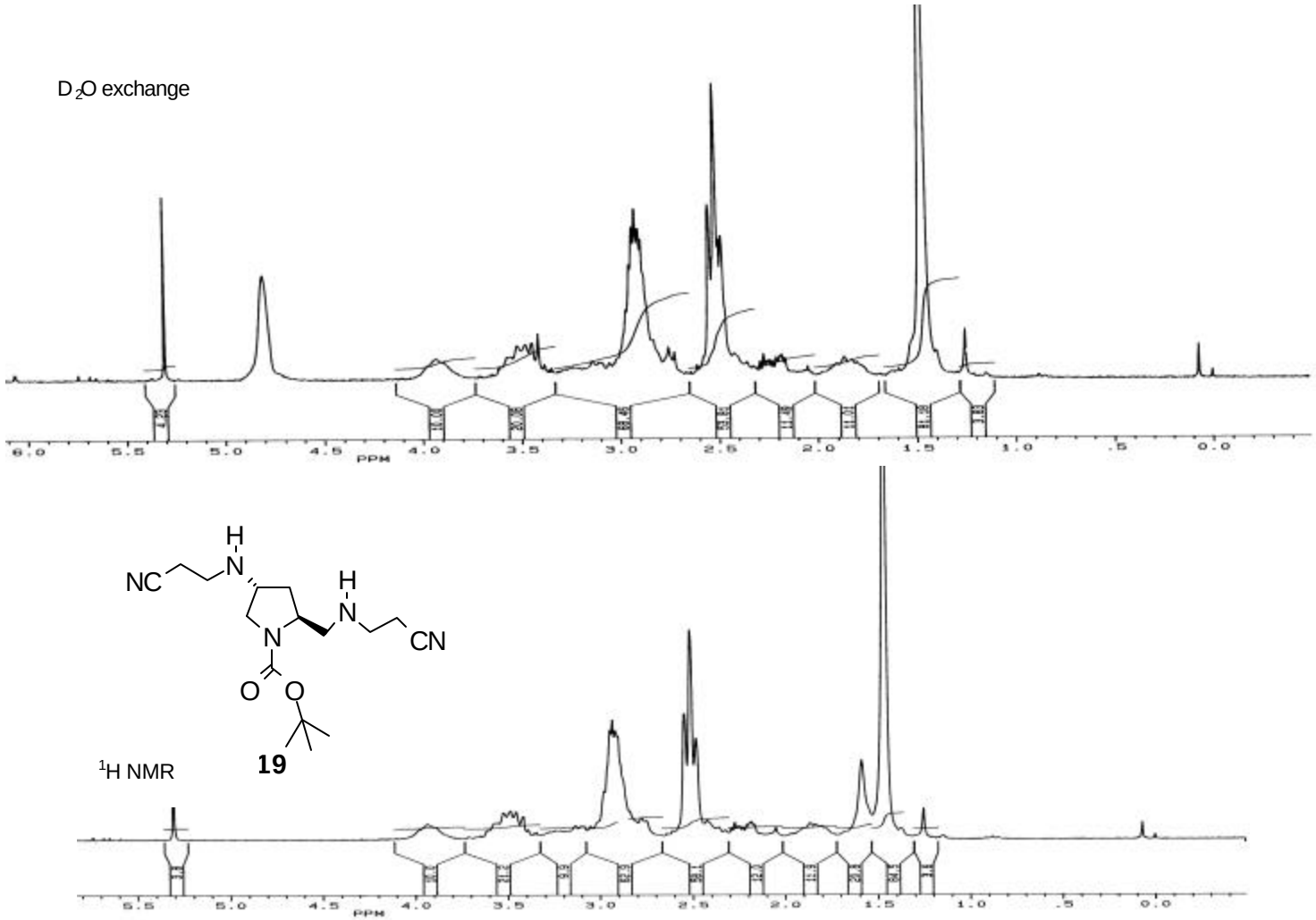


MASS

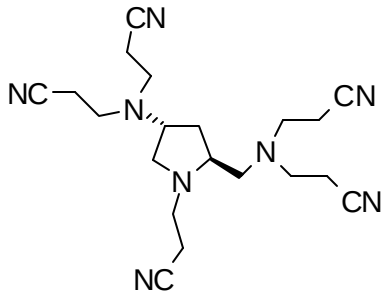
18

215 (M^+)



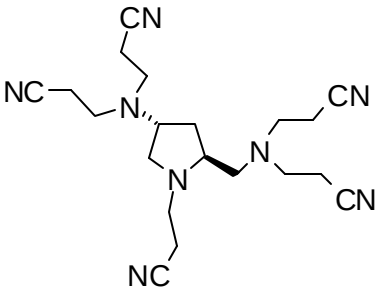

D2O exchange
NC
H
N
H
N
CN
N
O
O
19
1H NMR
PPM
PPM

^{1}H NMR


CN
NC
N
CN
N
N
CN
NC

21

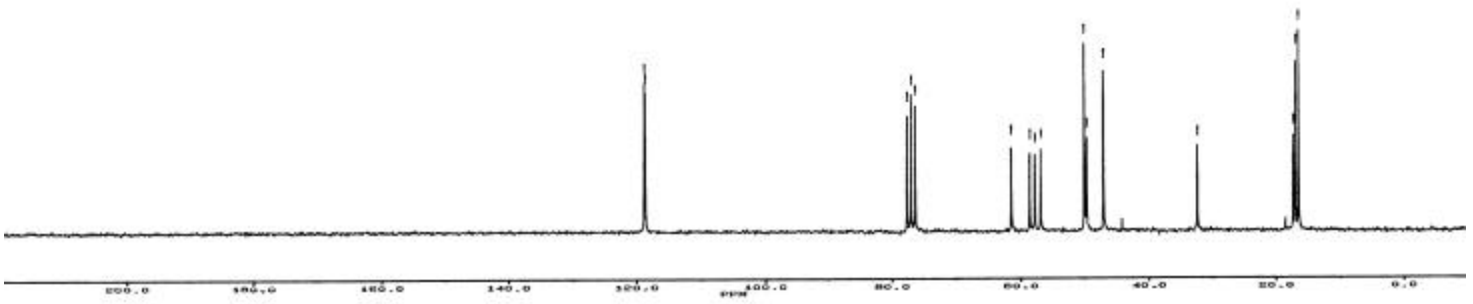
3.22
4.84
20.05
23.67
164.86
235.61
21.75
59.54
12.52
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
PPM

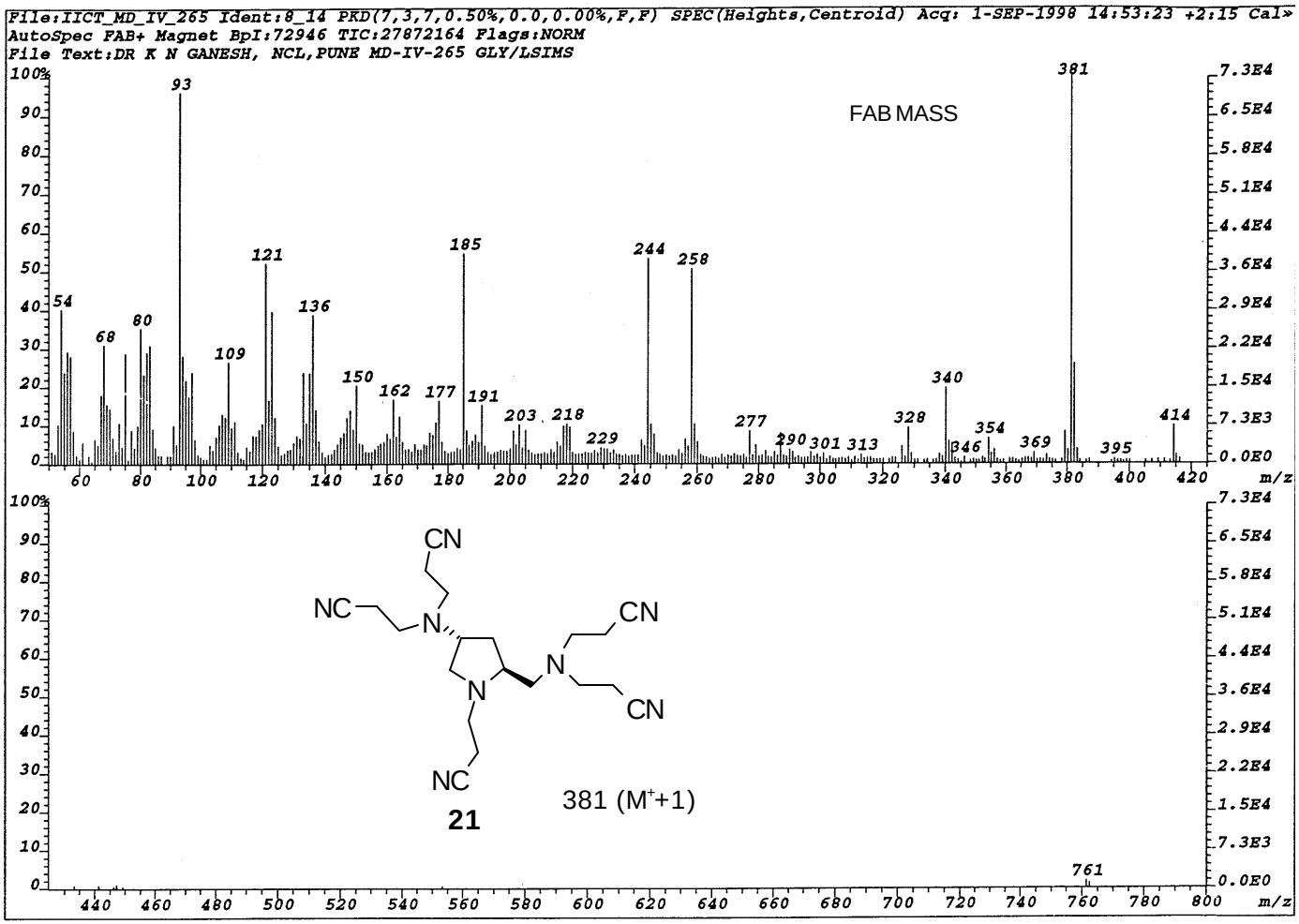


21

DEPT

^{13}C NMR

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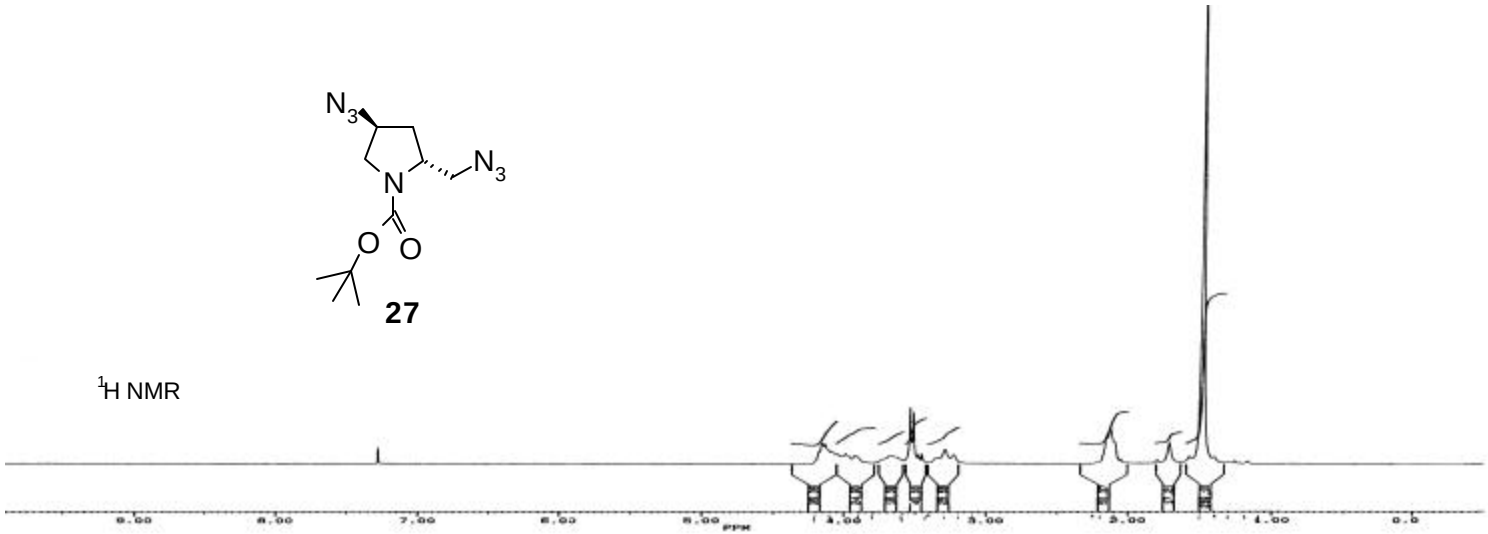
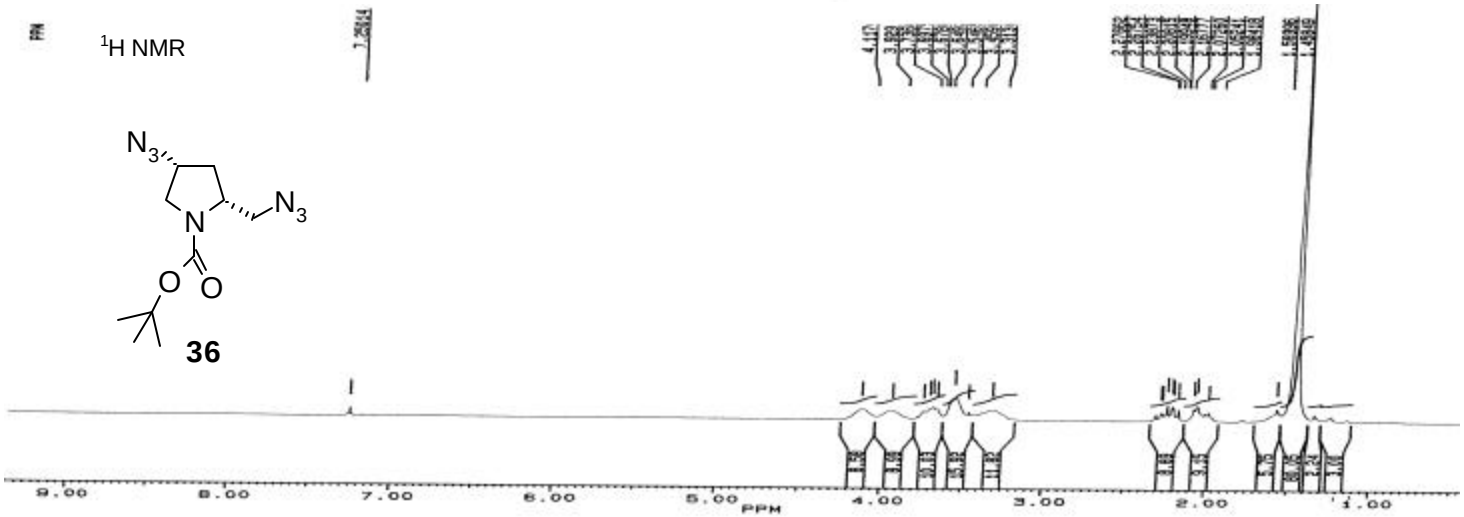
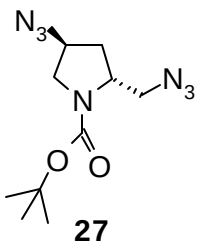
AutoSpec FAB+ Magnet BpI:72946 TIC:27872164 Flags:NORM
File Text:DR K N GANESH, NCL,PUNE MD-IV-265 GLY/LSIMS
FAB MASS
381
93
121
185
244
258
54
68
80
109
136
150
162
177
191
203
218
229
277
290
301
313
328
340
346
354
369
395
414
m/z
CN
NC
N
CN
N
N
CN
NC
381 (M+1)
21
761
m/z


N3
N3
N
O
O
27
1H NMR
9.00
8.00
7.00
6.00
5.00
PPM
4.00
3.00
2.00
1.00
0.0

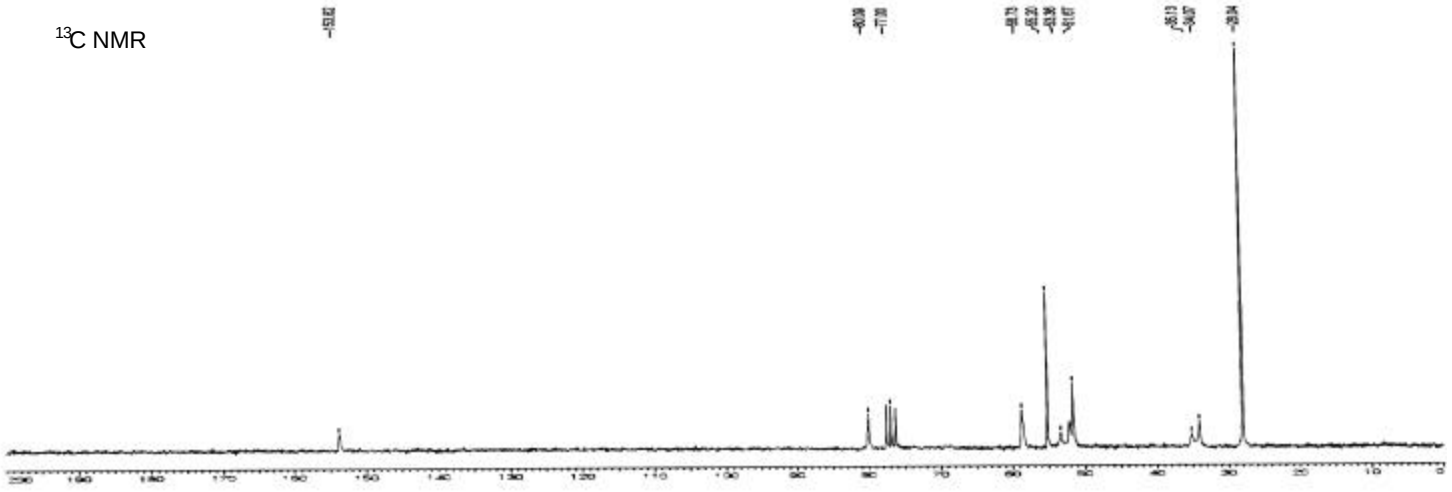

1H NMR
N3
N3
N
O
O
36
9.00
8.00
7.00
6.00
5.00
PPM
4.00
3.00
2.00
1.00

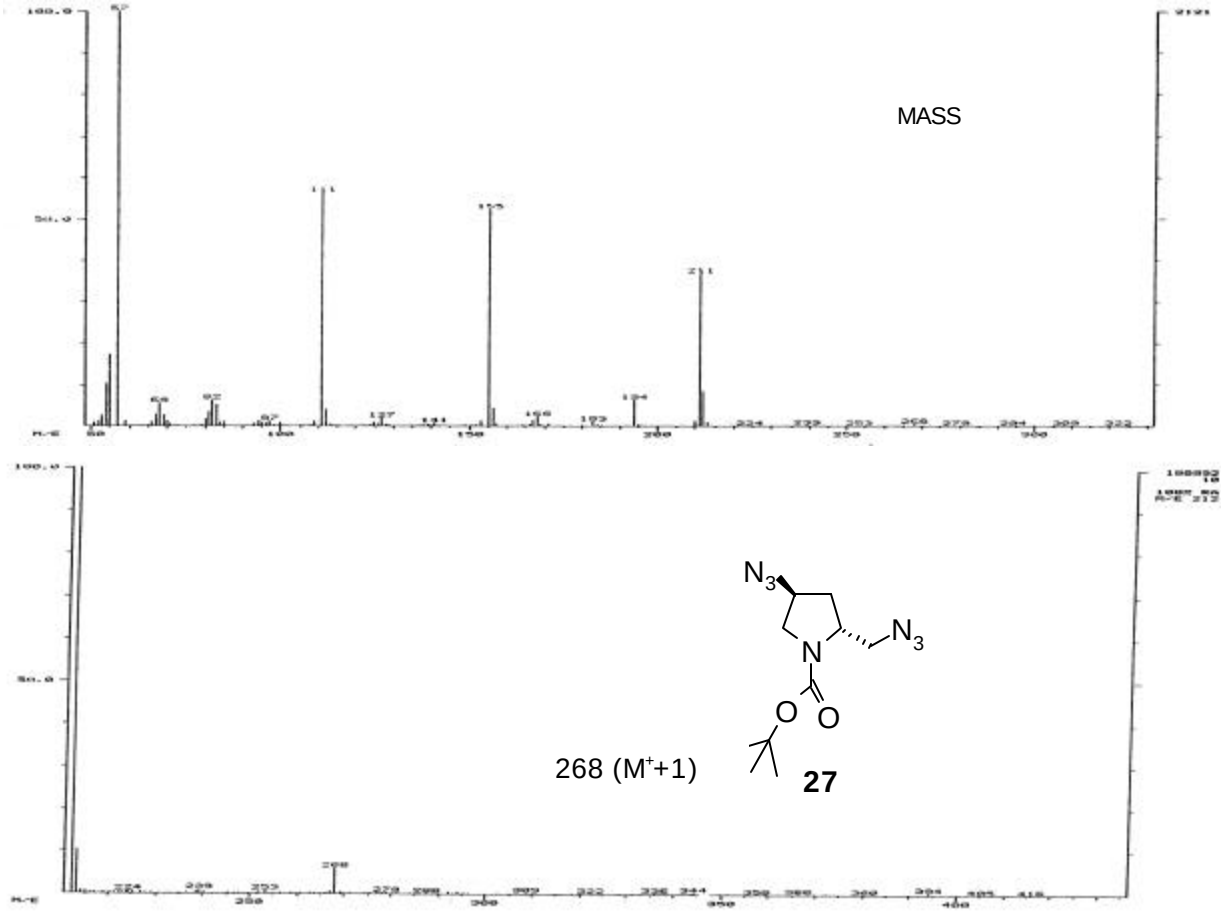


27

DEPT

^{13}C NMR

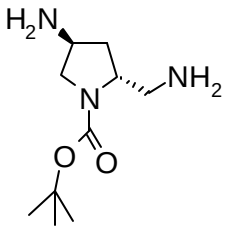




MASS

268 (M^++1)

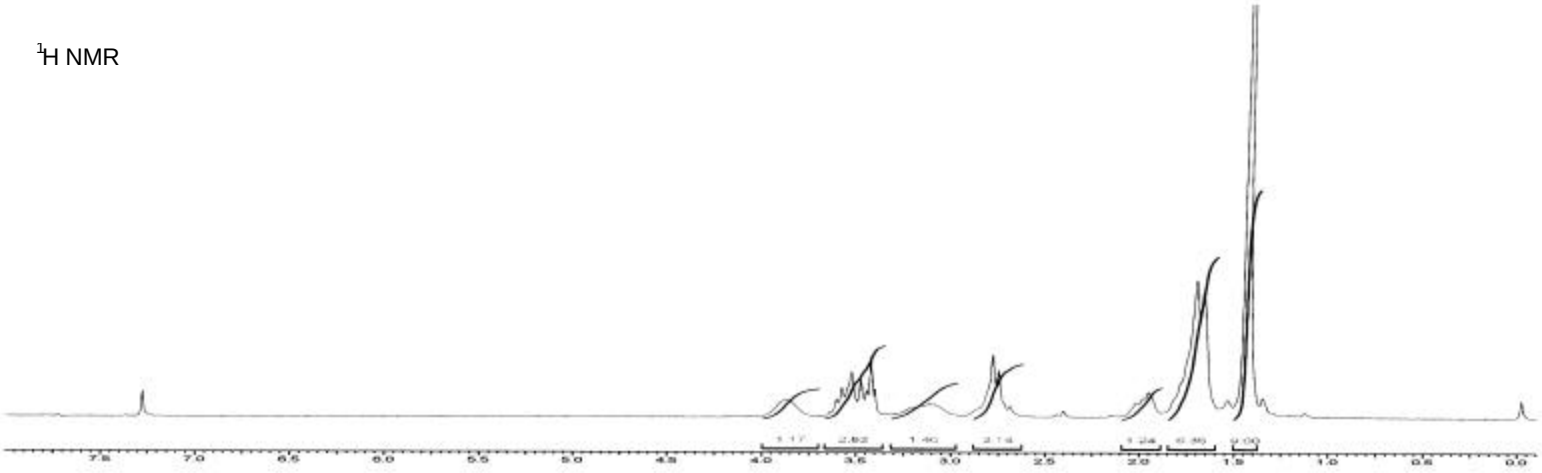
27

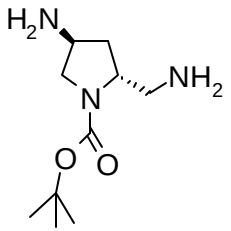


28

D_2O exchange

^1H NMR

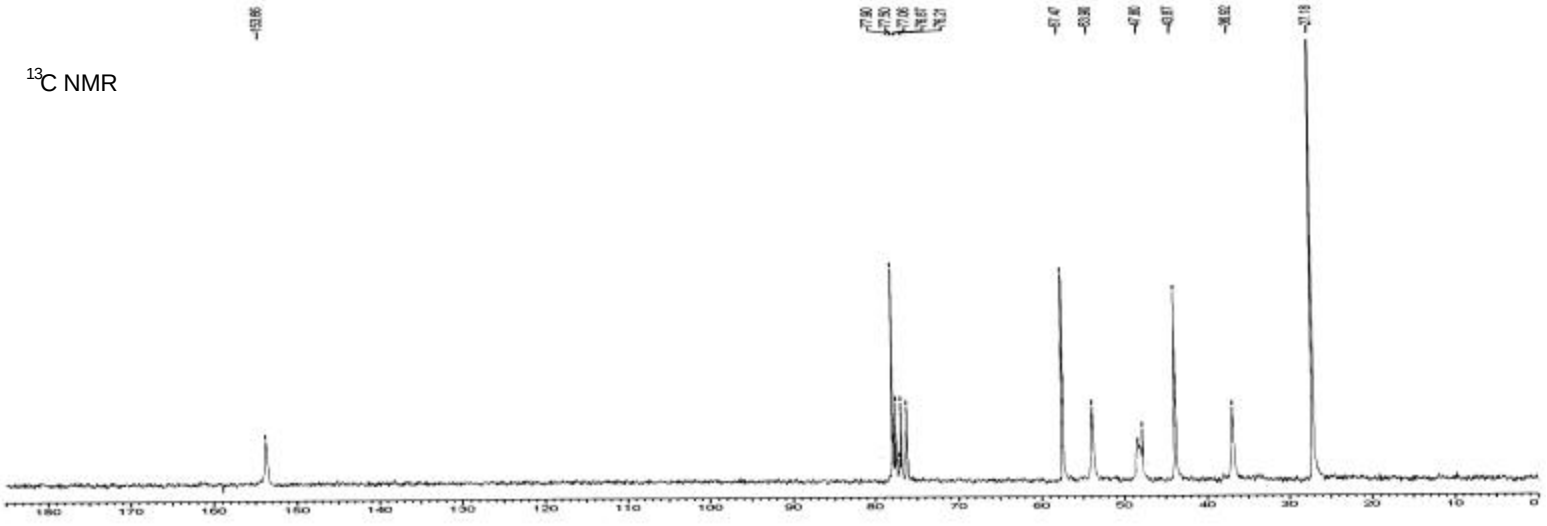




28

DEPT

^{13}C NMR



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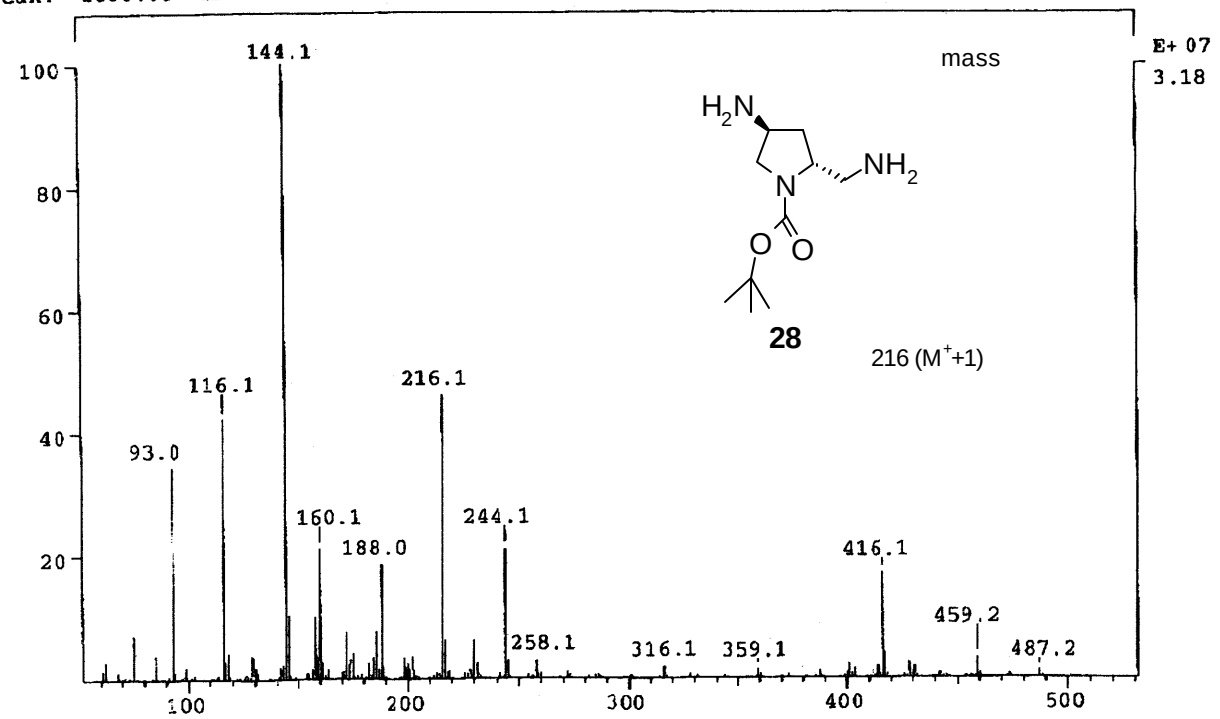
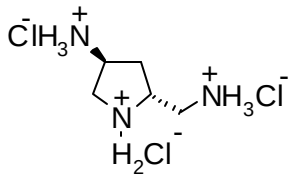
Comm: LR/Isobutane CI Spectrum @ 150eV
Mode: CI +VE +LMR BSCAN (EXP) UP LR Study : Broom(Med.Chem)
Oper: Wenchen Luo Client: K.G.Rajeev Inlet :
Base: 144.1 Inten : 31784704 Masses: 60 > 1000
Norm: 144.1 RIC : 173796681 #peaks: 744
Peak: 1000.00 mmu



28

216 (M^++1)

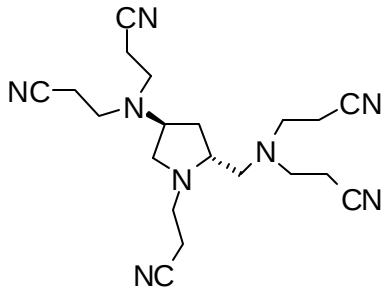
^{1}H NMR



31

20.00
10.58
6.30
10.57
10.76
11.62
8.64
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
PPM
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0

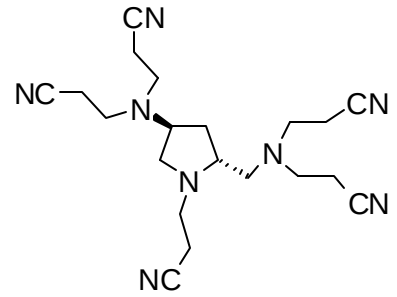
^{1}H NMR


CN
NC
N
CN
N
N
CN
NC

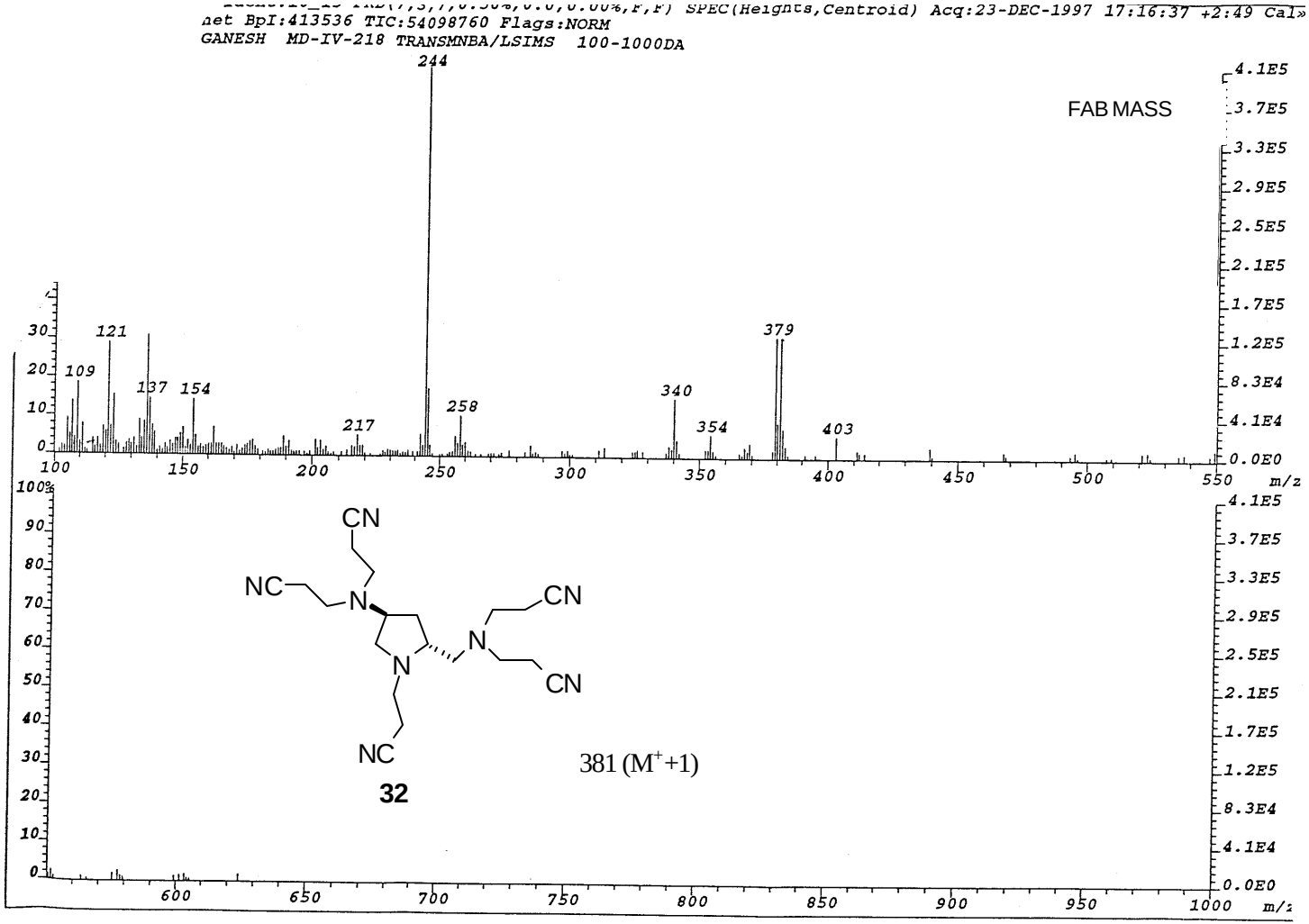
32

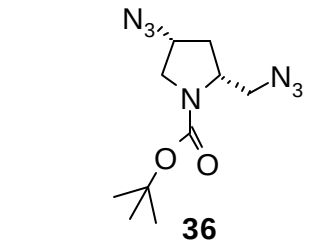
2.5
20.0
40.1
166.3
245.7
37.6
55.2
12.3
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
PPM

NAGAMANI-MD-4-218/C13/CDCL3



32


SPEC(Heights,Centroid) Acq:23-DEC-1997 17:16:37 +2:49 Cal»
Net BpI:413536 TIC:54098760 Flags:NORM
GANESH MD-IV-218 TRANSMNBA/LSIMS 100-1000DA
FAB MASS
244
121
109
137
154
217
258
340
354
379
403
CN
NC
N
CN
N
N
CN
NC
32
381 (M+1)
m/z

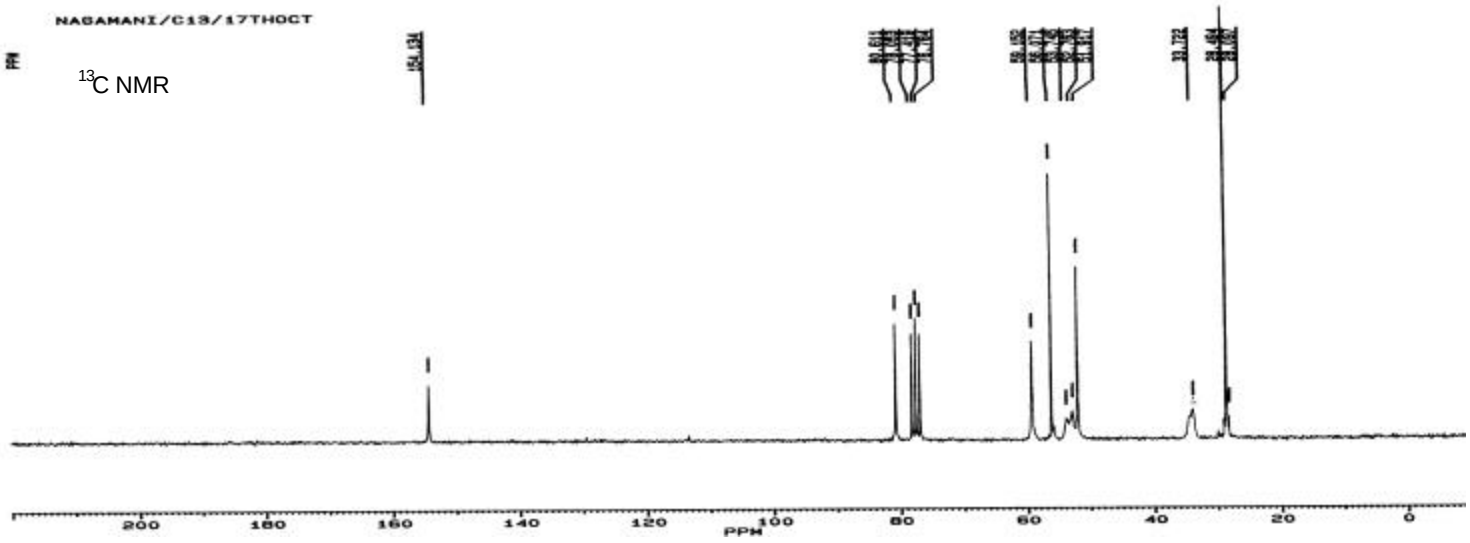


36

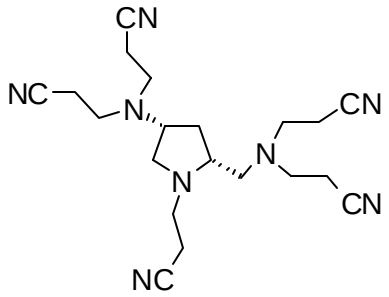
DEPT

NAGAMANI/C13/17THOCT

^{13}C NMR



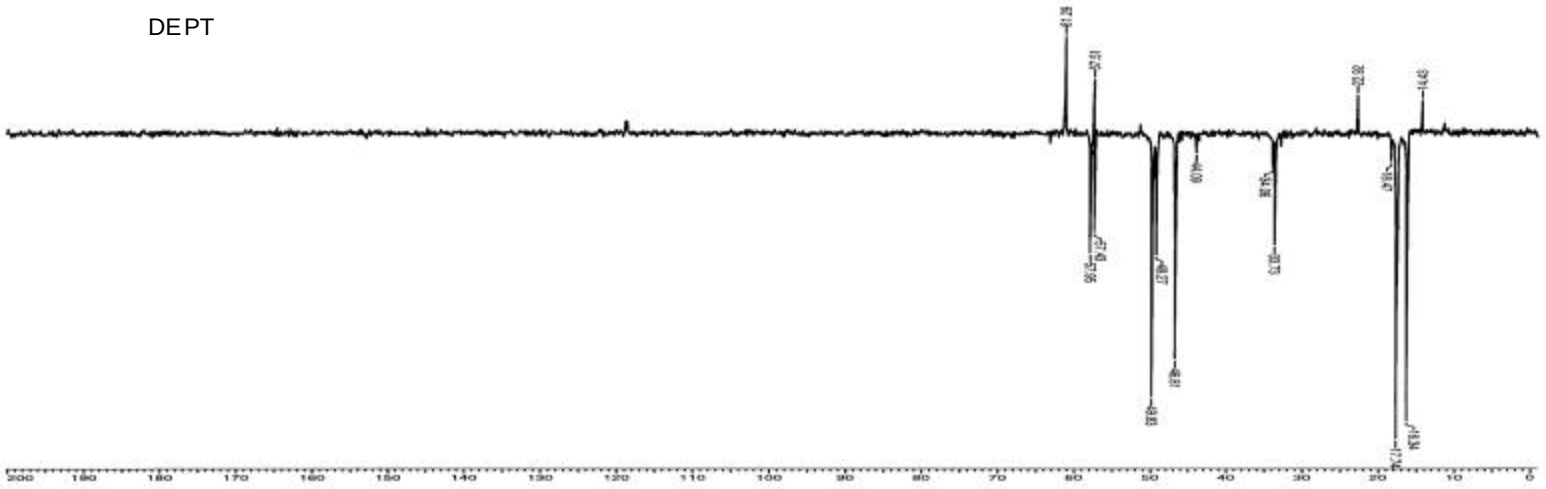
^{1}H NMR


CN
NC
N
CN
N
N
CN
NC

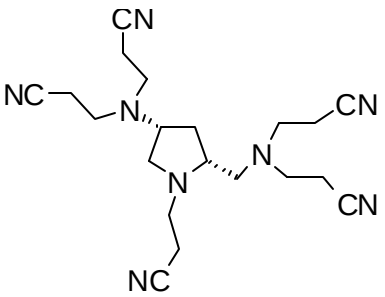
38

0.08
0.37
1.00
1.88
0.34
0.16
0.22
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

DEPT

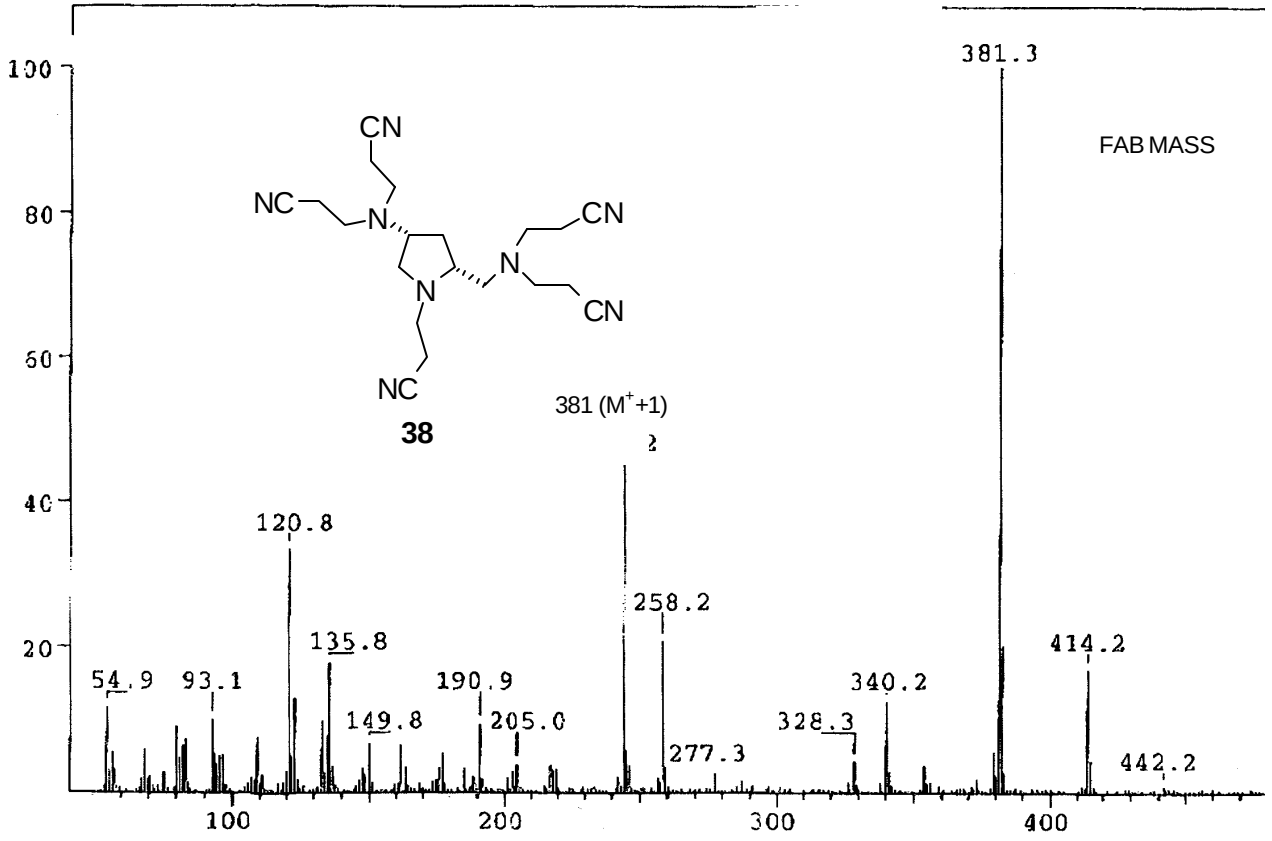


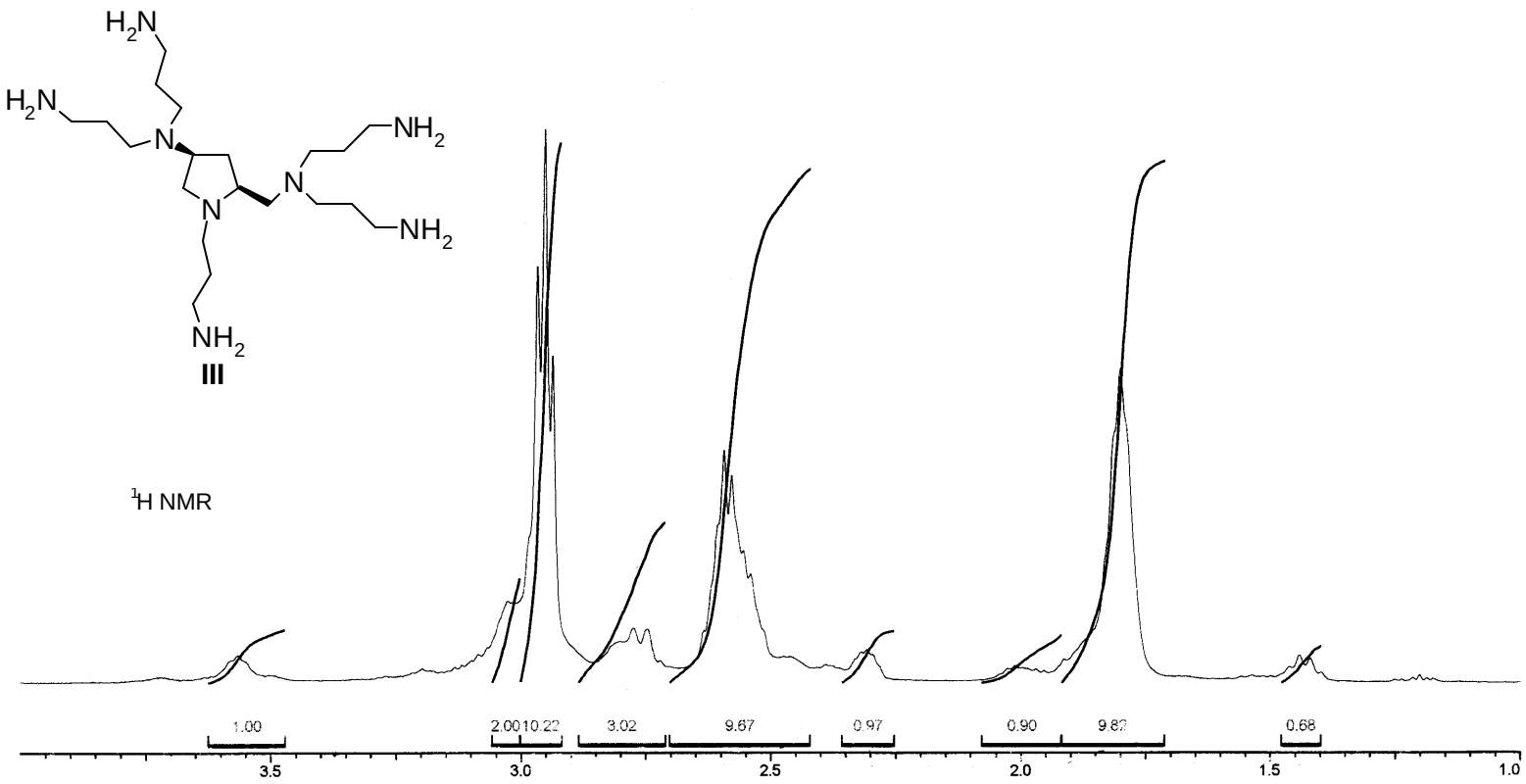
^{13}C NMR

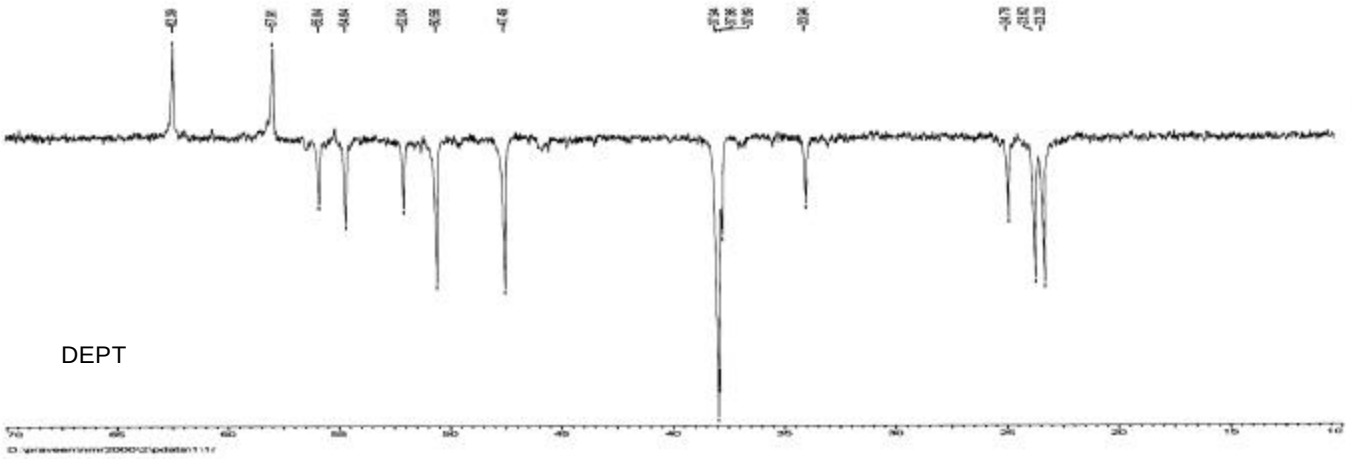


38

SPEC: 99mat1068 10-Nov-99 Elapse: 00:19.1 5
Samp: MDV-316 Start : 14:09:24 13
Comm: LR/FAB(+)/glycercl,MeOH,1%TFA
Mode: FAB +VE +LMR BSCAN (EXP) UP LR Study : Broom(Med.Chem)
Oper: Wenchen Luo Client: K.G.Rajeev Inlet :
Base: 381.3 Inten : 526133 Masses: 10 > 1000
Norm: 381.3 RIC : 3942144 #peaks: 654
Peak: 1000.00 mmu

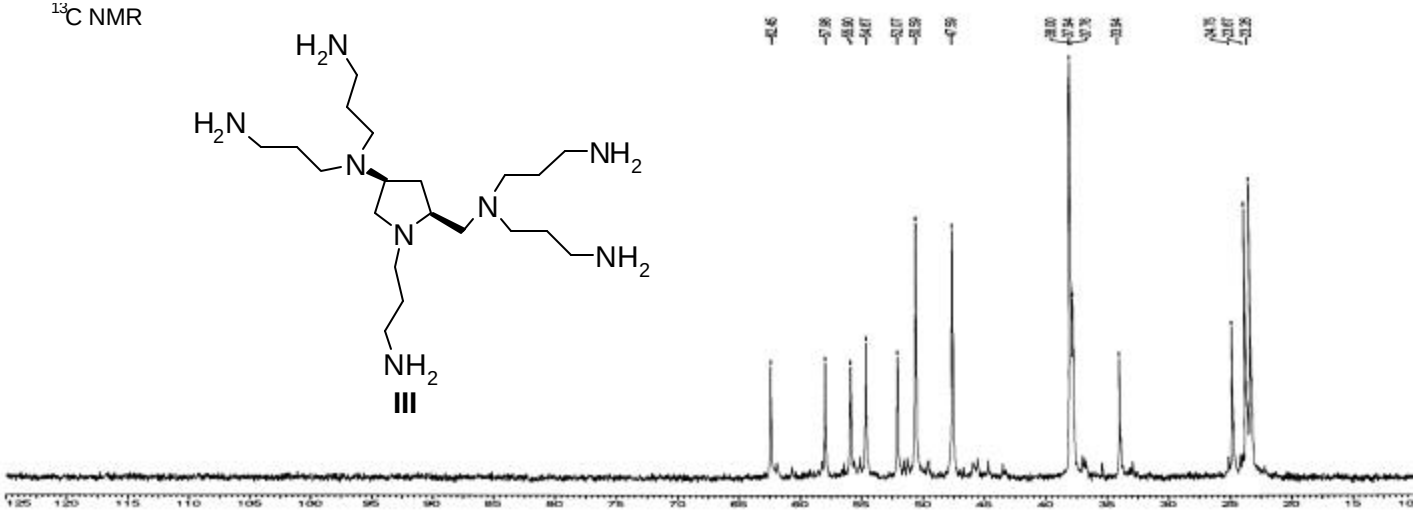



H2N
H2N
N
N
N
NH2
NH2
NH2
III
1H NMR
1.00
2.00
10.22
3.02
9.67
0.97
0.90
9.80
0.68
3.5
3.0
2.5
2.0
1.5
1.0

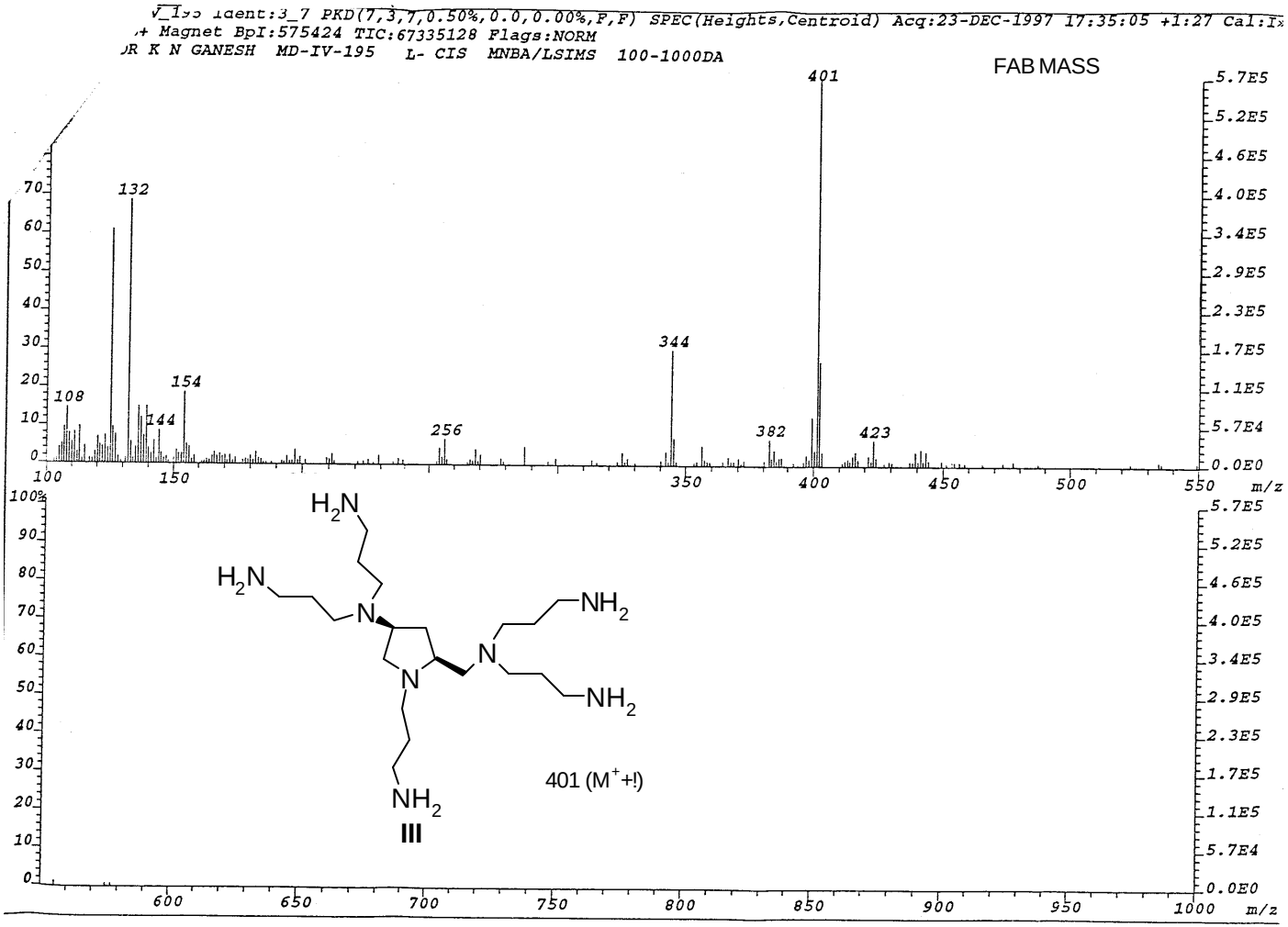


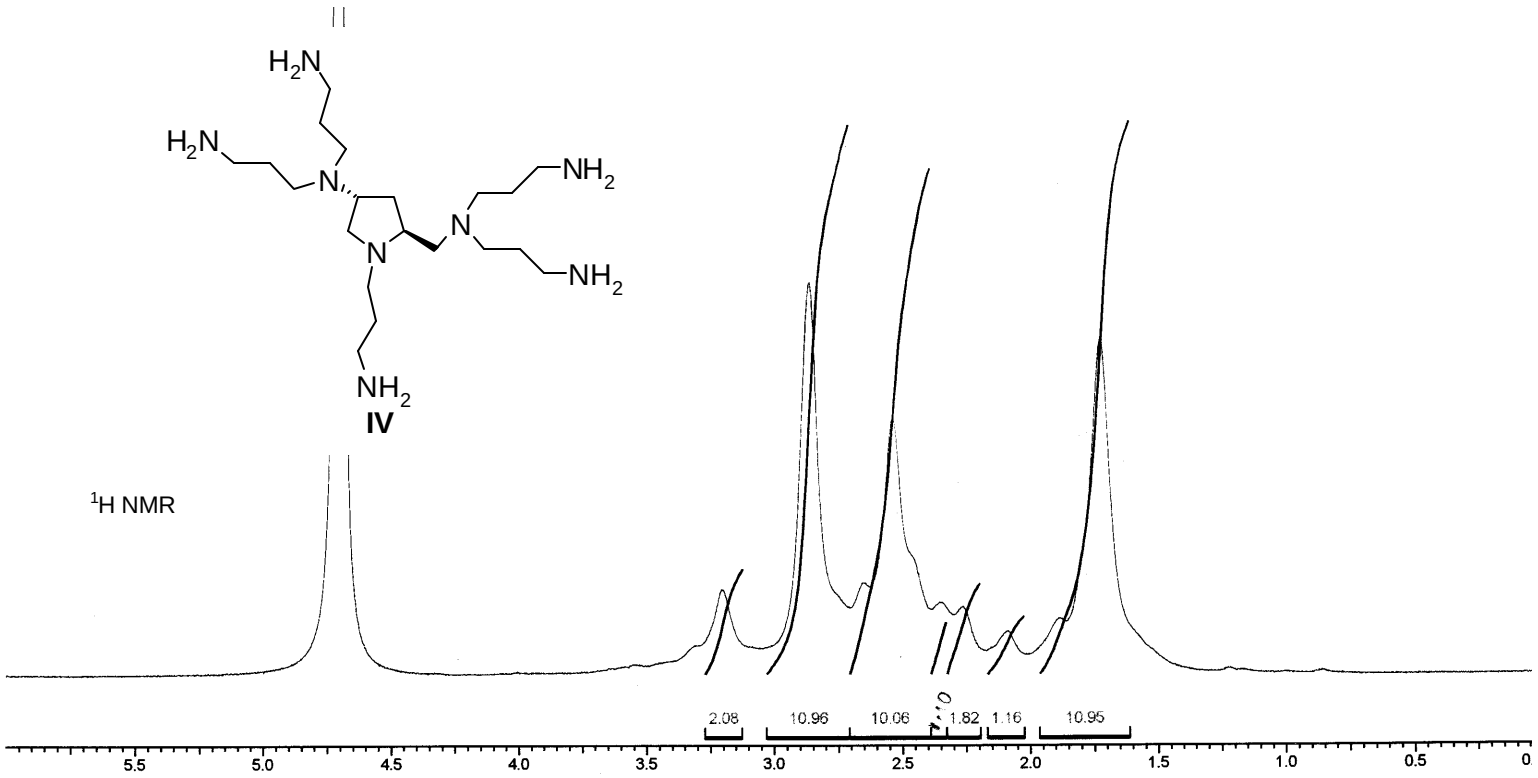
DEPT

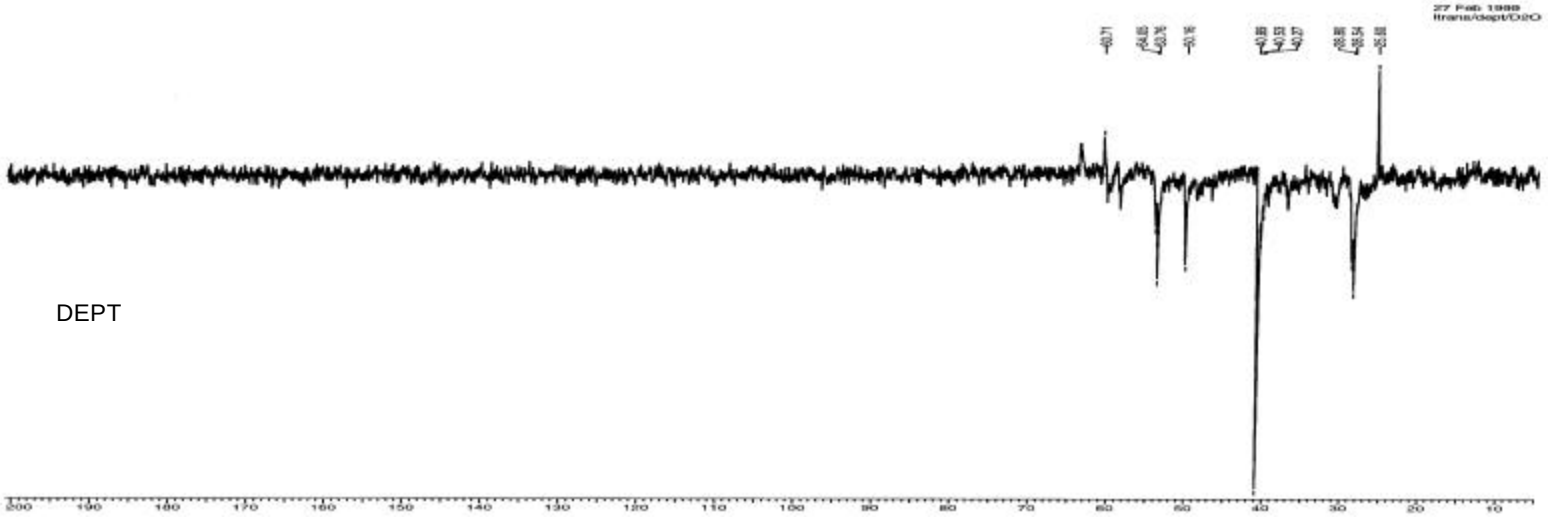
^{13}C NMR

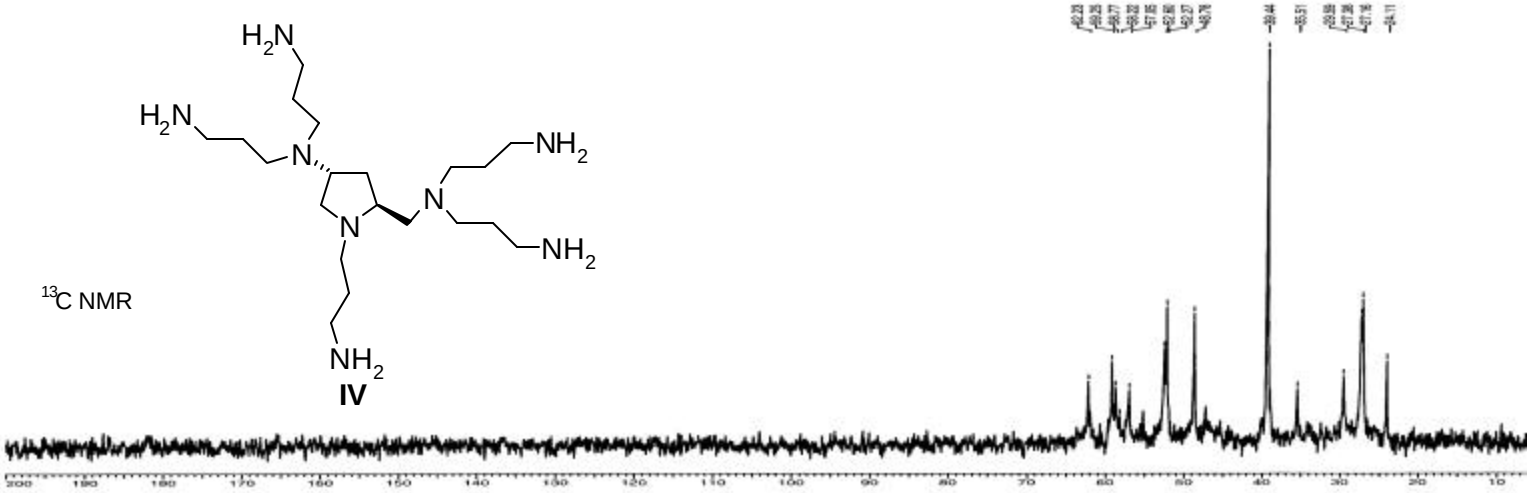


III


FAB MASS
401
132
344
154
108
144
256
382
423
100
150
350
400
450
500
550
m/z
600
650
700
750
800
850
900
950
1000
401 (M+·)
III

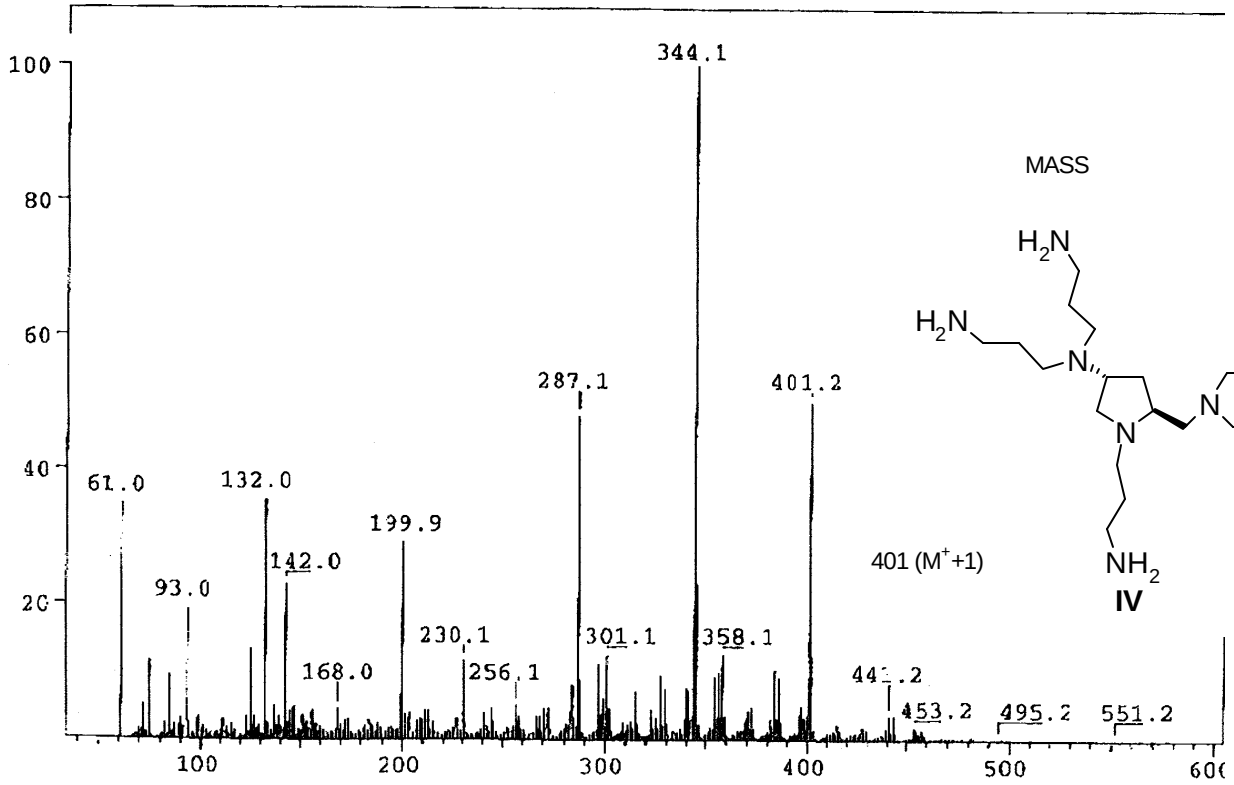

H2N
H2N
N
N
N
NH2
NH2
NH2
IV
1H NMR
2.08
10.96
10.06
1.82
1.16
10.95
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0



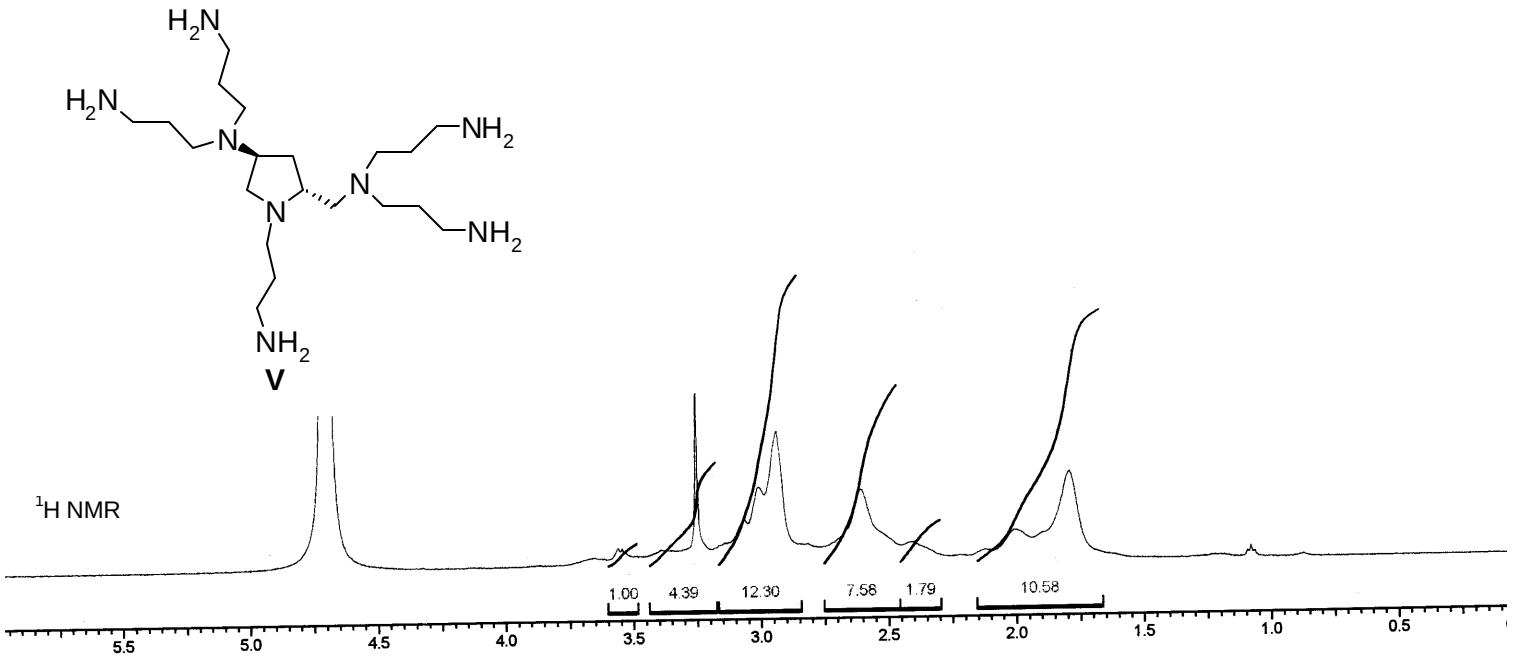


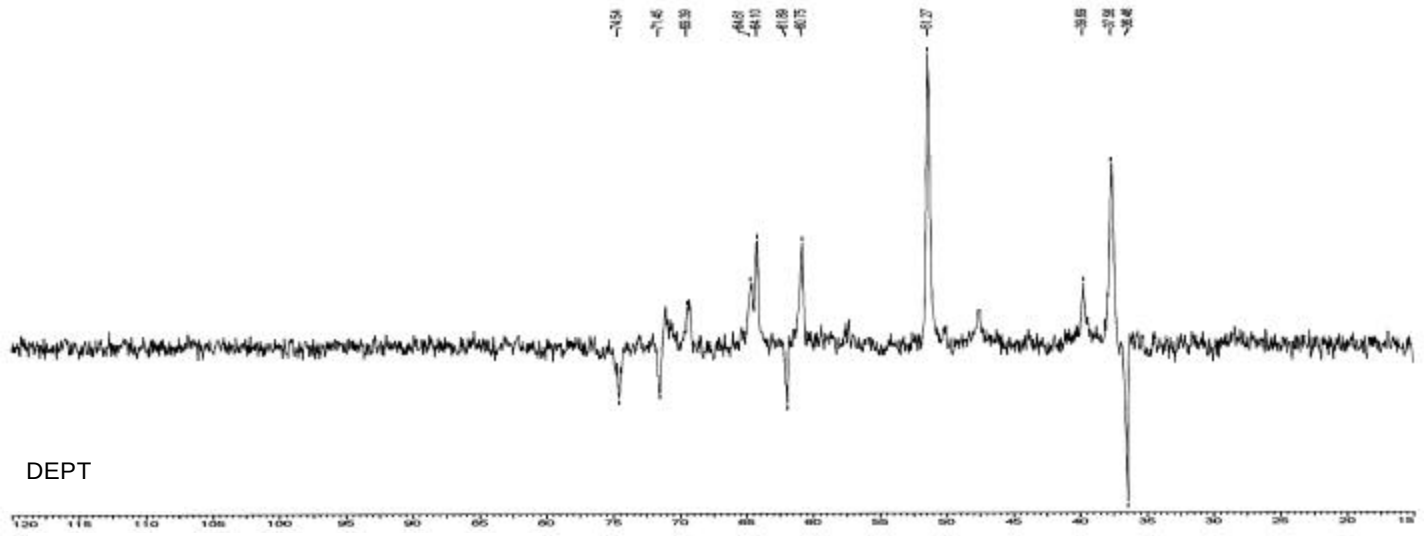
IV

SPEC: 00mat156 28-Feb-00 Elapse: 04:43.2 69
Samp: MDV-267 Start : 15:30:43 98
Comm: LR/CI Spectrum @ 150eV/ isobutane
Mode: CI +VE +LMR BSCAN (EXP) UP LR Study : Med.Chem(Broom)
Oper: Wenchen Luo Client: K.G.Rajeev Inlet :
Base: 344.1 Inten : 3143583 Masses: 60 > 1000
Norm: 344.1 RIC : 35394253 Peaks: 512
Peak: 1000.00 mmu

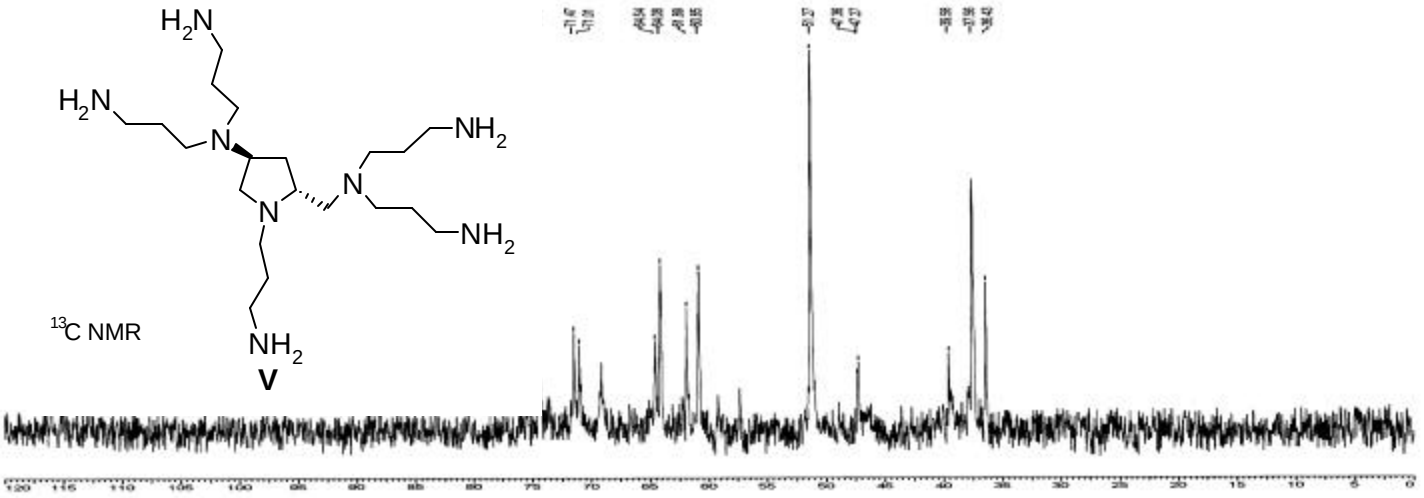


IV


H2N
H2N
N
N
N
NH2
NH2
NH2
V
1H NMR
1.00
4.39
12.30
7.58
1.79
10.58
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5



DEPT

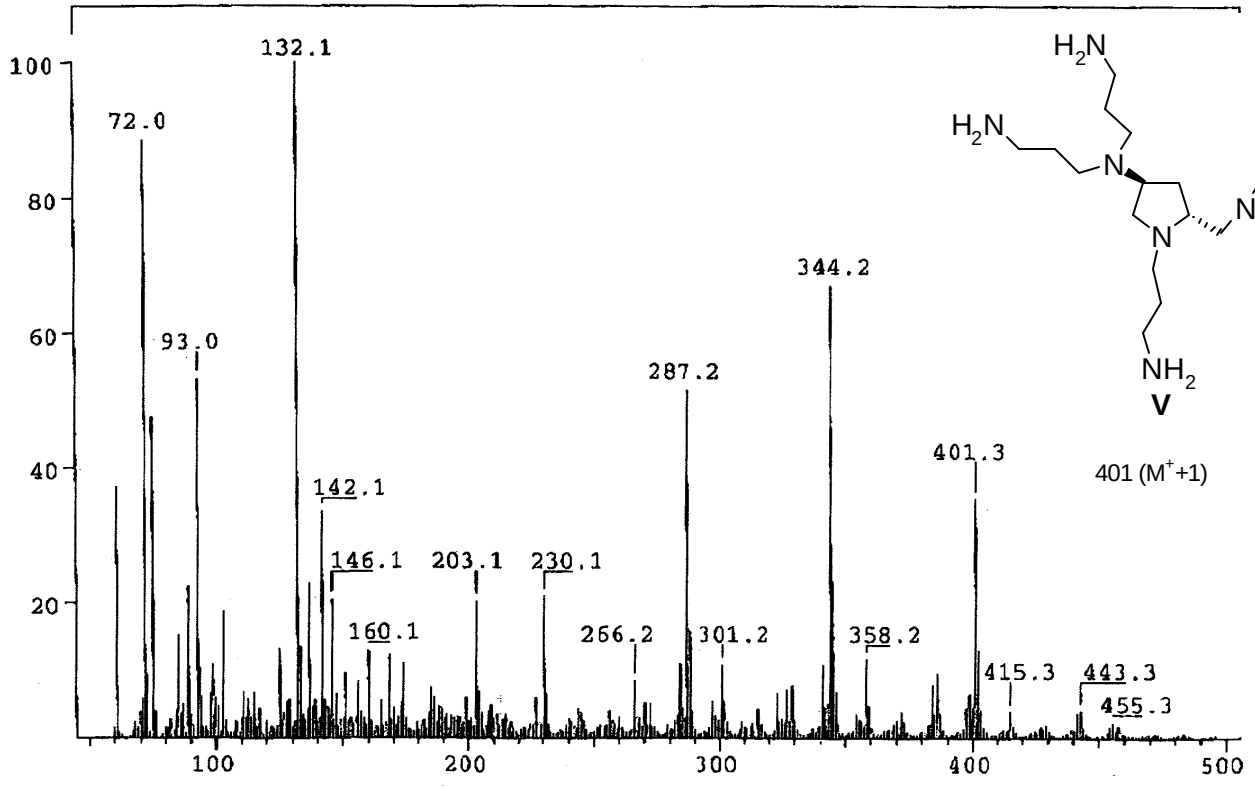


^{13}C NMR

V

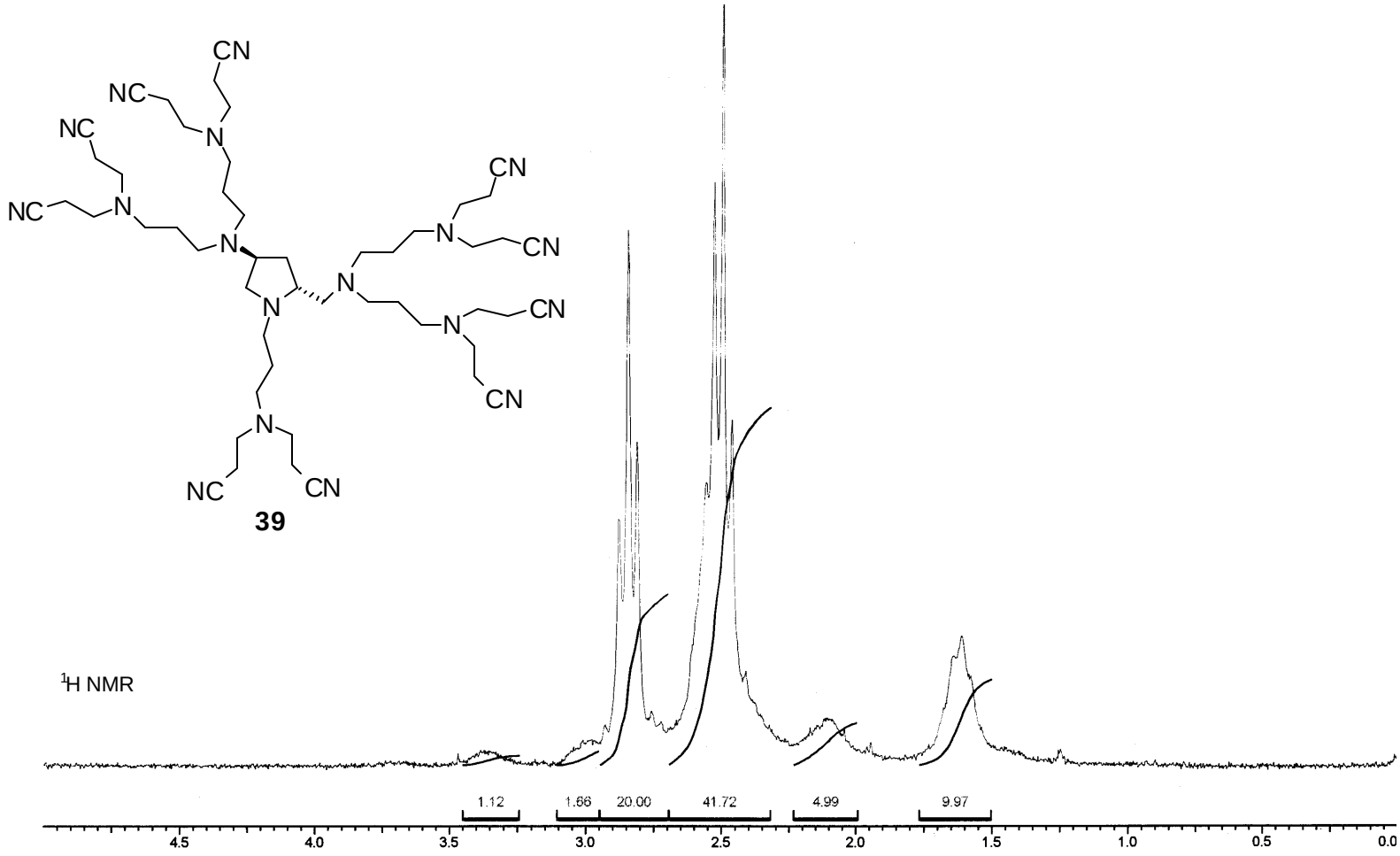
SPEC: 99mat1133 01-Dec-99 Elapse: 08:56.2 130
Samp: MDV-321 Start : 14:43:41 163
Comm: LR/Isobutane CI Spectrum @ 150eV
Mode: CI +VE +LMR ESCAN (EXP) UP LR Study : Broom(Med.Chem)
Oper: Wenchen Luo Client: K.G.Rajeev Inlet :
Base: 132.1 Inten : 6012519 Masses: 60 > 1000
Norm: 132.1 RIC : 102183026 #peaks: 584
Peak: 1000.00 mmu

MASS

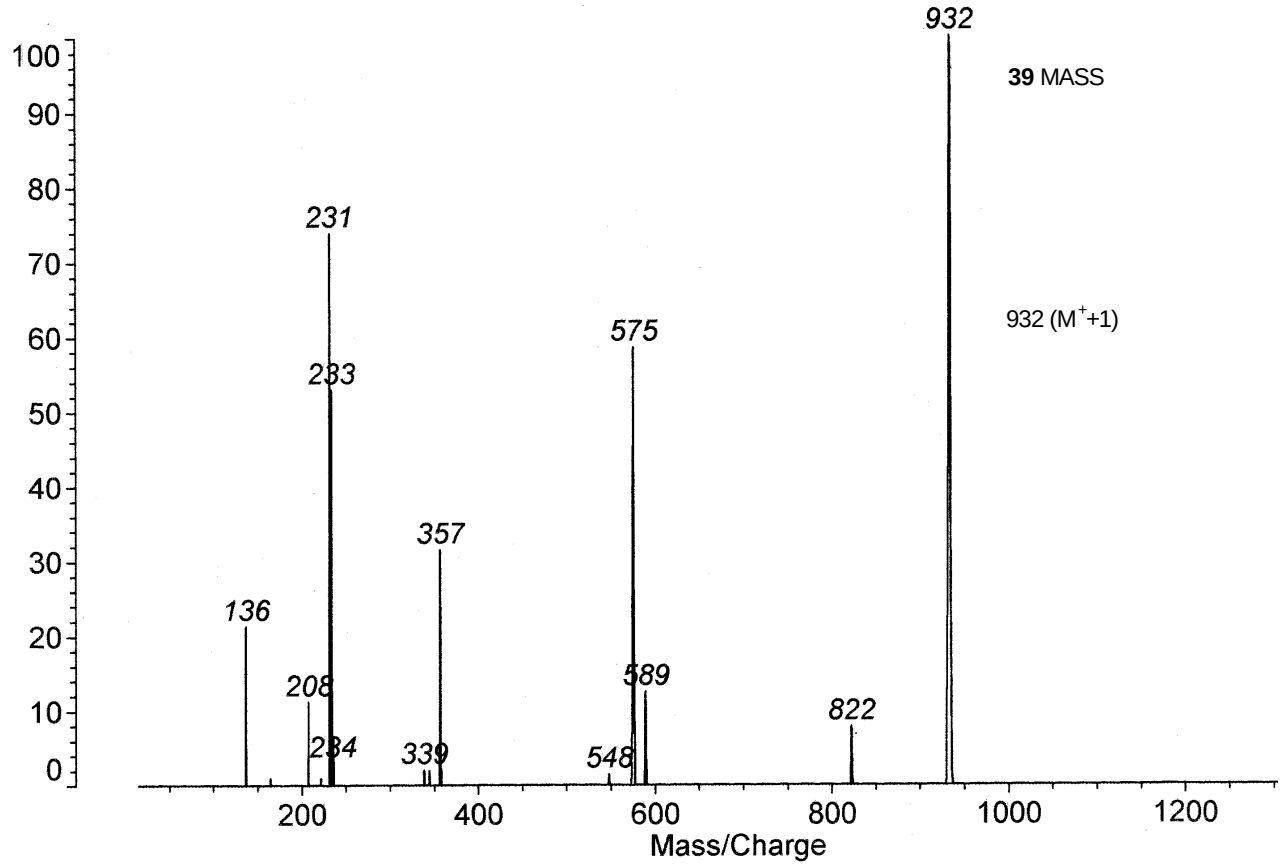


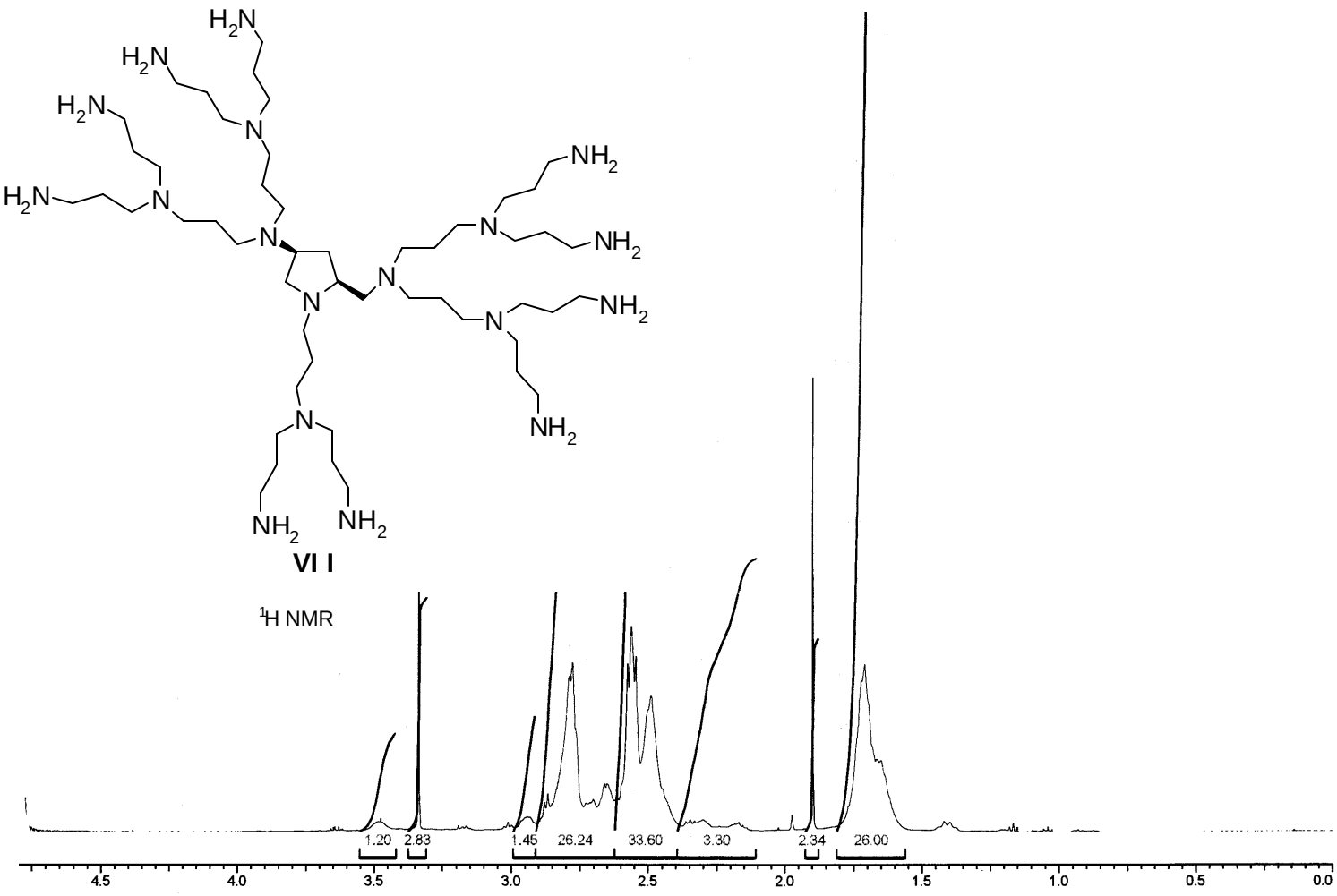
V

401 (M^++1)


CN
NC
NC
NC
N
N
N
N
N
N
N
N
N
CN
CN
CN
CN
CN
CN
NC
CN
39
^{1}H NMR
1.12
1.66
20.00
41.72
4.99
9.97
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

)R K N GANESH
MD-IV-468 MD IV - 468
)ata: MD4680001.15 10 Nov 2000 17:36 Cal: tof 26 Sep 2000 16:21
<ratos PCKompact SEQ V1.2.2: + Linear High, Power: 79, P.Ext. @ 1000 (bin 56)
%Int. 100% = 16 mV[sum= 213 mV] Profiles 1-13 Smooth Av 50




VI I
^{1}H NMR
1.20
2.83
1.45
26.24
33.60
3.30
2.34
26.00
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

CHAPTER 3

BIOPHYSICAL STUDIES OF CHIRAL, BRANCHED PYRROLIDYL POLYAMINES

3.1. INTRODUCTION

The role of polyamines in stabilizing double stranded DNA has been recognized for many years.[1] Spermine is well known to stabilize DNA duplexes and triplexes when added externally[2] and the duplex stabilization by polyamines is governed by several factors like charge, pH and ionic strength of the medium. It was shown that the structural features of polyamines such as number of nitrogens, intervening methylenes etc has a minimal role in duplex DNA stabilization, where as it is important for induction and stabilization of Z-DNA.[3] It was shown that the natural polyamines favor triplex formation at neutral pH due to electrostatic neutralization of high charge density on triplexes.[4] Since the negative charge density of the triplex is higher than that of a duplex, polyamines should bind more tightly to the former and shift the equilibrium in favor of triplex formation. Thus role of polyamines could be important in stabilizing natural triplex formation in purine-rich sequences on chromosomal DNA.[5] Spermine, a linear aliphatic amine has enormous conformational freedom and theoretical calculations have suggested a preferential occurrence of *trans*/gauche conformations at C-N and N-C bonds.[6]

3.1.1. RATIONALE AND OBJECTIVES OF THE PRESENT WORK

In the presently designed and synthesized chiral pyrrolidyl polyamine analogs, (Chapter 2) it was envisaged that the conformational constrain imposed by the five membered pyrrolidine ring and the additional positive charges would influence the interaction of the resulting polycationic molecules with DNA. The relative stereochemistry and the overall hydrophobicity/charge ratio due to the additional aminopropyl chains could be important in their interaction with DNA.

The objectives of this chapter are to systematically study the interaction of the different pyrrolidyl polyamines with duplex and triplex DNA using UV (thermal denaturation studies), CD (analysis of the conformational changes) and fluorescence (ethidium bromide displacement assay) spectroscopic techniques under various pH and salt concentrations. The results should throw more light on structural factors governing the pyrrolidyl polyamine-DNA interactions.

3.2. PRESENT WORK

The pyrrolidyl polyamines used for the biophysical study in this chapter are shown in Figure 2 and the DNA sequences are listed in Table 1.

3.2.1. Design, synthesis and duplex/triplex formation of oligonucleotides

The 5' and 3' ends in duplex (**41:42)** were designed to avoid the concatenation and to resolve the triplex-duplex transition from that of duplex⇔single strand. Since the sequence specificity of the synthesized polyamines was not known, the sequences chosen were polypyrimidine/polypurines that also form triplexes. The length of the duplexes/triplexes was about 18-mers, so that the derived duplexes and triplexes have T_ms in a convenient range. The oligonucleotide sequences (**40-42**) were synthesized on an automated DNA synthesizer (Pharmacia GA plus) by using β-cyanoethyl phosphoramidite chemistry (Scheme 1) on the solid support CPG resin according to standard methods.[7] The synthesis was done according to the manufacturers protocols and efficiency per coupling step was greater than 98% leading to a good overall efficiency. A typical result is shown in Scheme 2 and the synthesis report of ODN **40** is shown in Figure 3. The oligonucleotides were cleaved from the resin and deprotected by ammonia treatment and desalted by gel filtration on NAP columns. The purity of oligonucleotides as checked by C18 reverse phase analytical HPLC and found to be >95%. These were used without further purification.

The duplex (**41:42**) was constituted by hybridization of the constituent 24-mer oligonucleotides in Tris buffer without any salt. In case of the triplex (**40*:41:42**), 18-mer **40** was used as the third strand that hydrogen bonds in Hoogsteen mode.[8]

3.2.1a Triple-Helical Motifs

Polypyrimidine oligonucleotides bind (via Hoogsteen hydrogen bonding) to the Watson-Crick double-stranded DNA of polypurine stretches, resulting in triple-helical structures.[8] This occurs in two modes (a) 'pyrimidine motif' in which AT base pairs are recognized by neutral T, while the base pair GC requires protonated C (C^+) for the triad formation (Figure 1) and the third Hoogsteen strand is parallel to the central purine strand. (b) in the 'purine motif' AT base pairs are recognized by A and the GC base pairs by G for the formation of triad. In this motif no protonation of purine is required and the third strand is antiparallel to the central purine strand. This emphasizes the importance of purine in the central position of the triad in both the motifs triple helix formation is also dependent on several factors such as the length of the third strand, pH and also the ionic concentration. In the present work spermine **I**, chiral and branched pyrrolidyl polyamines **III** – **VI** that are cationic were used to study their role in triplex (pyrimidine motif) stabilization at both the neutral and acidic pH.

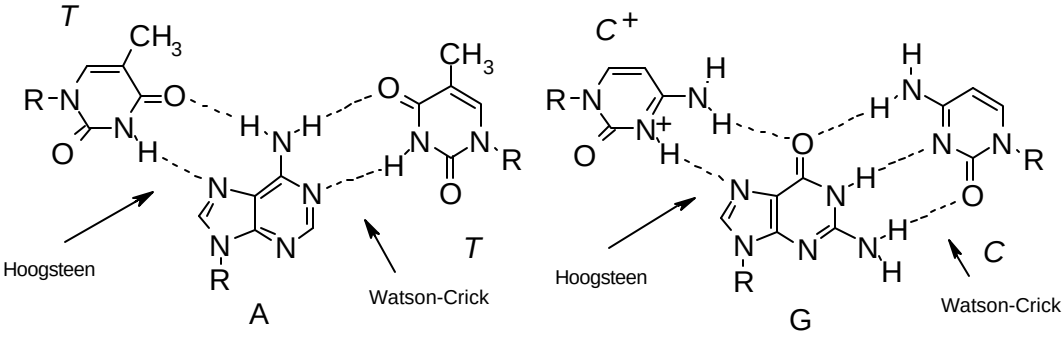


Figure 1. T.A-T and C^+.G-C triplets involving Hoosteen and Watson-Crick base pairing

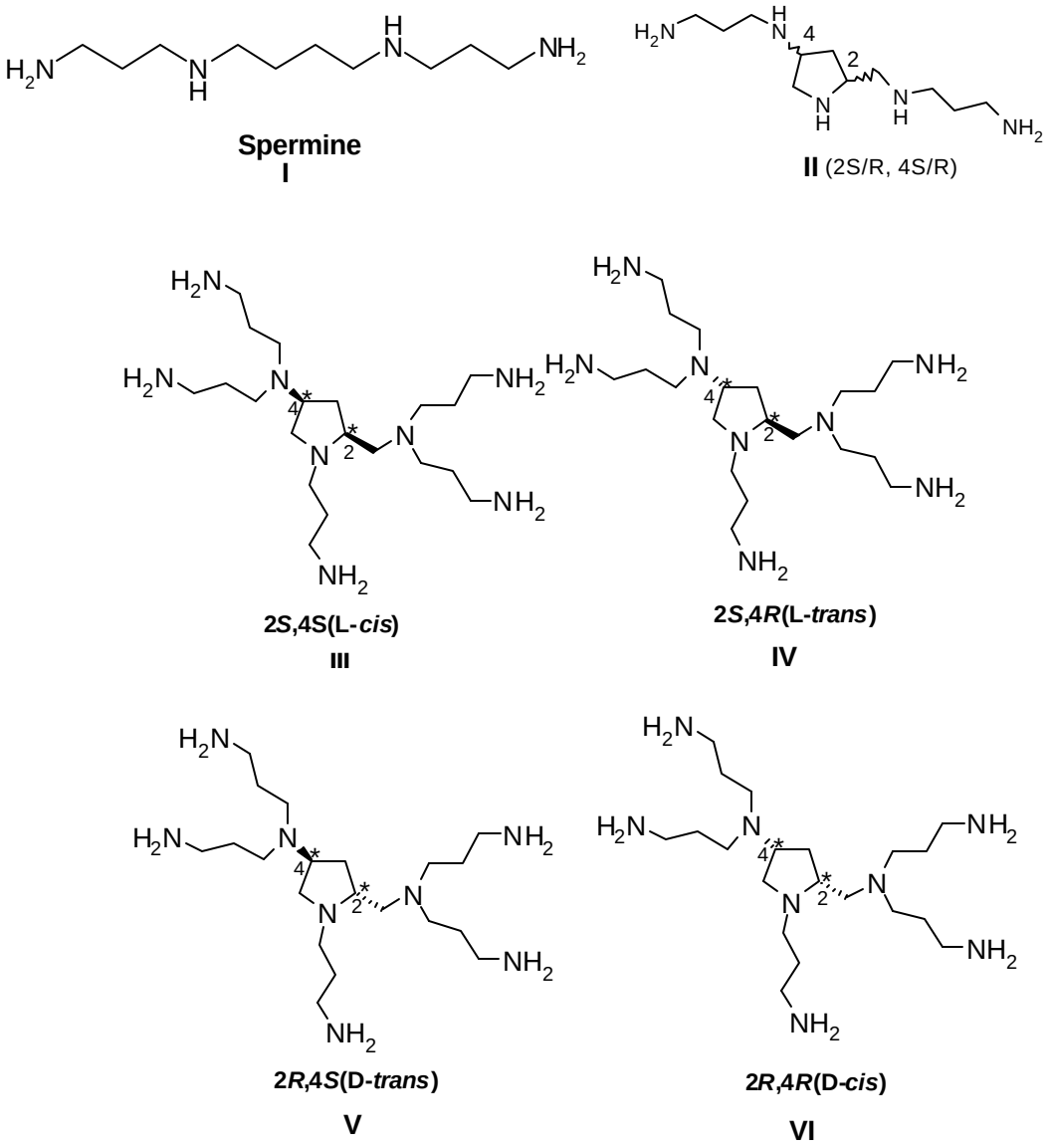
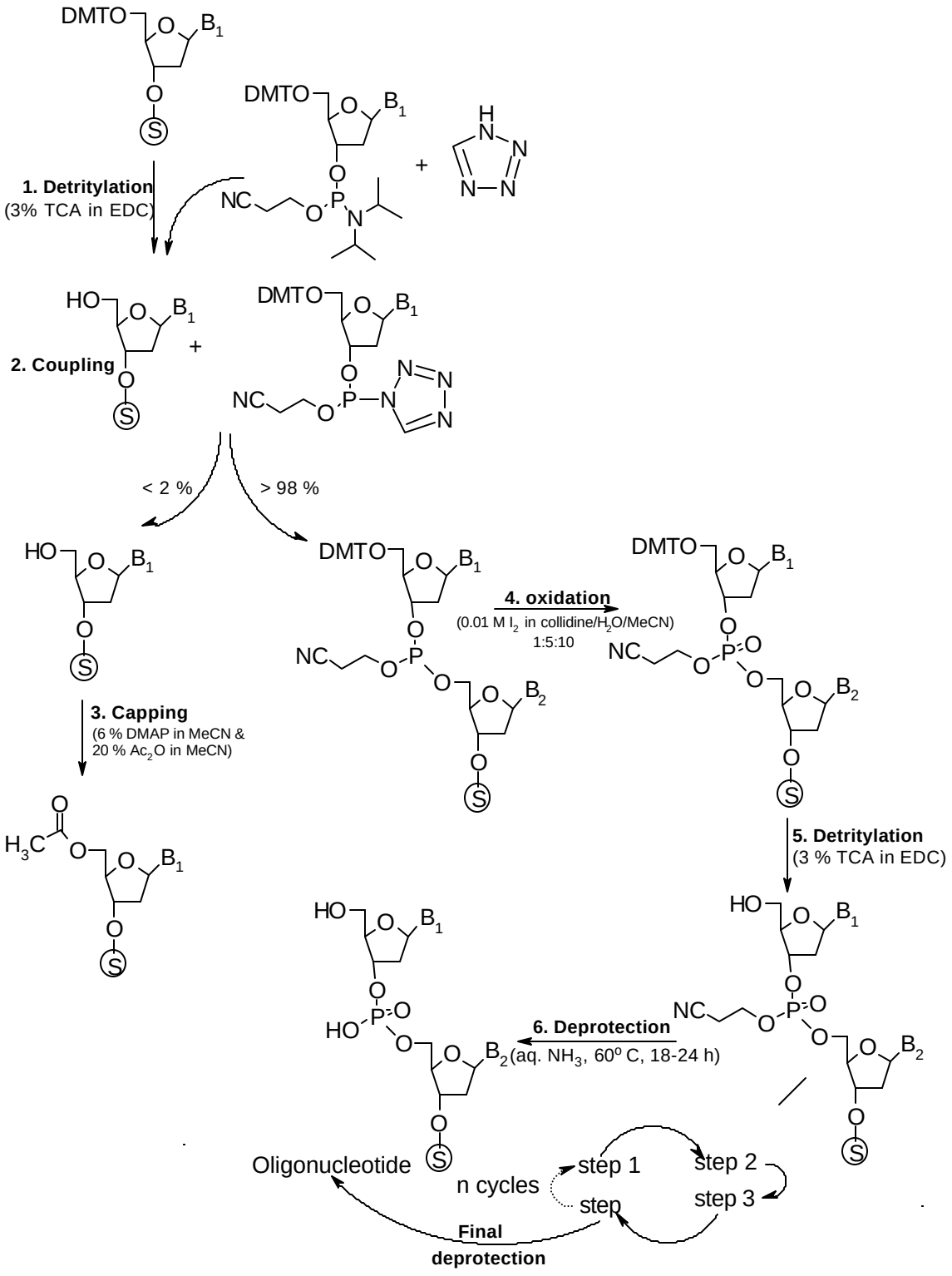


Figure 2. Polyamines

Table 1: DNA oligonucleotide sequences

40*	d T T C T T T T T T C T T T T T T C T
41	d G C C A A G A A A A A A G A A A A A A G A C G C
42	C G G T T C T T T T T T C T T T T T T C T G C G d



Scheme 1. Solid phase synthesis of oligonucletides

Gene Assembler Plus
Date: 02/07/97

Sequence: Mani 1
Synthesis: Mani
Scale: 1.3 micromole
Sequence Lengh: 18
Column: 1
Final Detritylation: Yes
Coupling Efficiency Threshold: 90%

Position	Base	Retention	Duration	Peak ht	Acc Area	Last eff	Ave Eff
		mins	mins		% FS	%	%
18	T	0.62	1.00	3001	583.54	-	-
17	C	0.60	1.00	2962	591.78	-	-
16	T	0.59	1.00	2989	562.44	-	-
15	T	0.59	1.00	3001	564.07	100.3	100.0
14	T	0.60	1.00	3001	561.69	99.6	99.9
13	T	0.59	1.00	3001	550.21	98.0	99.3
12	T	0.59	1.00	3001	548.45	99.7	99.4
11	T	0.59	1.00	3001	549.87	100.3	99.6
10	C	0.55	1.00	3001	541.29	-	99.6
9	T	0.58	1.00	3002	541.12	99.2	99.5
8	T	0.61	1.00	3002	541.66	100.1	99.6
7	T	0.60	1.00	3001	537.93	99.3	99.5
6	T	0.59	1.00	3000	541.26	100.6	99.7
5	T	0.60	1.00	2892	532.89	98.5	99.5
4	T	0.60	1.00	2854	529.09	99.3	99.5
3	C	0.61	1.00	2445	473.22	-	-
2	T	0.61	1.00	2406	450.02	92.2	98.9
1	T	0.60	1.00	2373	485.72	107.9	99.6

Total synthesis yield from start = 93.5%

Figure 3 Solid Phase synthesis report of ODN 40 on Pharmacia GA Plus DNA synthesizer

3.3. SPECTROSCOPIC METHODS IN STUDYING POLYAMINE-DNA INTERACTONS

3.3.1. UV spectroscopy

UV-spectroscopy has been extensively used in studying the thermal stability of nucleic acids[9] by monitoring the absorption at 260 nm as a function of temperature. During this process, as the temperature is increased, structural transitions are observed resulting from the disruption of hydrogen bonds between the base pairs. Duplexes exhibit a single transition into single strands (melting) accompanied by an increase in UV absorption called *hyperchromicity*. According to all or none model,[9] the UV absorbance value at any given temperature is an average of the absorbance of duplex and single strand. A plot of absorbance against temperature gives a sigmoidal

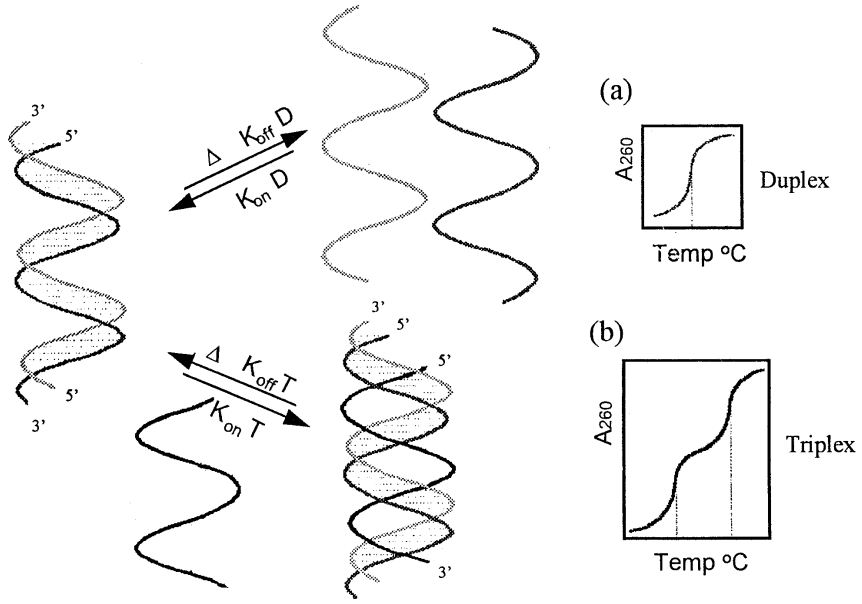


Figure 4. Schematic representation of thermal dissociation of DNA double and triple helices. Dotted lines in the right side boxes correspond to the melting temperature.

curve in case of duplexes and the midpoint of the sigmoidal curve called as the 'melting temperature' (T_m) is the temperature at which the duplex and the single strand exists in equal proportion. In case of triplexes, the first dissociation leads to the duplex (WC duplex) and third strand (Hoogsteen strand), followed by the duplex dissociation to two single strands.[10] The DNA triplex melting shows characteristic double sigmoid transition (Figure 4) and the UV melting temperature for each transition is obtained from the first derivative plots. The lower melting temperature corresponds to triplex ⇔ duplex transition while the second transition gives the T_m of the duplex ⇔ single strand conversion. In the presence of DNA binding ligands, the T_m shifts to a higher or a lower temperature depending on the stabilization or destabilization induced by the ligand. The DNA binding ability of the chiral, branched pyrrolidyl polyamines have been investigated by such temperature dependent absorbance experiments. A detailed analysis of the melting curves also enable evaluation of thermodynamic parameters such as enthalpy, entropy and free energy for the melting transitions.[11]

3.3.2. Circular dichroism (CD) spectroscopy[12]

Circular dichroism is a technique to study chiral molecules that have chromophores. In case of nucleic acids, the sugar units of the backbone provide chirality and the bases attached to sugars are the chromophores. In the CD spectrum of a polynucleotide with stacked bases, the magnitude of CD signals is larger in the 260-280 nm region and significantly higher at 200 nm than that of individual bases. The base stacking in a chiral fashion induces the coupling of CD transitions leading to characteristic patterns. Single stranded DNAs are structurally less well defined than duplex DNAs and their CD signal is smaller. The CD pattern of nucleic acid reflects the polymorphic forms of DNA such as A-, B-, and Z- forms. The CD signature of B-form DNA as seen from longer to shorter wavelength is a positive band centered at 275 nm, a negative band at 240 nm, with a crossover at 258 nm. A-DNA is characterized by a

positive CD band at 260 nm that is larger than the corresponding B-DNA band, a fairly intensive negative band at 210 nm. Naturally occurring RNAs and RNA-DNA hybrids adopt this polymorphic form. The left handed Z-form DNA shows a negative CD band at 290 nm and a positive band at 260 nm.

CD spectra are often the simplest technique to probe DNA conformational changes as a function of ionic strength, solvent, ligand concentration etc. Many DNA binding molecules are themselves achiral. However upon binding to DNA, they acquire induced CD, characteristic of their interaction. CD titration is a convenient method to follow the ligand-DNA association while temperature CD can be used to evaluate the Tm in the same way as UV-T_m.

3.3.3. Fluorescence spectroscopy

DNA has no inherent fluorescence and hence it may seem that this form of spectroscopy is not of much use for studying DNA-ligand interactions when the ligand is also non-fluorescent. However, by employing ethidium bromide as an intermediate probe, fluorescence spectroscopy can be effectively employed to study DNA-ligand interactions. Ethidium bromide (Figure 5) interacts with DNA by intercalation with the base pairs.[13] It is weakly fluorescent, but when intercalated into DNA exhibits a strong fluorescence with emission maxima at 595 nm upon excitation at 475 nm. A competent DNA binding ligand can displace the intercalated ethidium bromide thus leading to a

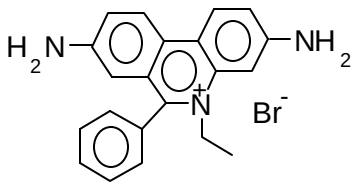


Figure 5

decrease in the fluorescence intensity.[14] An attractive feature of the ethidium binding is that its fluorescence increases 50 fold upon intercalation (with an affinity constant k_a = 10^4 M^{-1}) with DNA making it a very useful probe for monitoring the interactions of DNA with small molecules. When DNA-Ethidium bromide complexes are challenged by the other DNA binding agents (eg. groove binders), it is displaced from the complex leading to a fall in fluorescence intensity. In such experiments, a measure of the decrease in ethiduim bromide fluorescence is useful to compute the relative binding strengths of added ligands to DNA. The displacement of intercalated ethidium bromide by the addition of non-intercalative ligands may happen as a consequence of structural transitions induced by ligands. Thus spectrofluorimetric methods can be used to study the interaction of a range of compounds with DNA.[15]

3.4. RESULTS AND DISCSSION

3.4.1. Studies on the effect of polyamine analogs on DNA duplex stability

Figure 6 shows the UV absorbance-temperature plot of duplex DNA (**41:42**) in

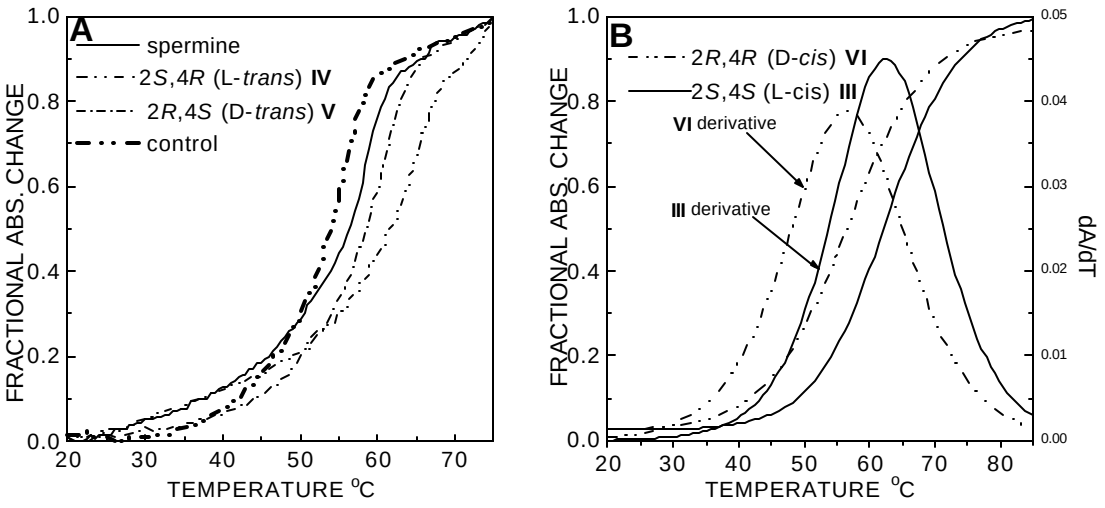


Figure 6. UV melting profile of duplex DNA (**41:42**) in 25 mM TRIS (no added salt) **(A)** with 0.01 mM 2*S*,4*R* (L-*trans*) **III** and 2*R*,4*S* (*D-trans*) **IV** and spermine **I** and control **(B)** in presence of 2*S*,4*S* (*L-cis*) **V** and 2*R*,4*R* (*D-cis*) **VI**

presence of 0.01 mM of spermine **I** and chiral branched pyrrolidyl polyamines (**III** – **VI**) in the temperature range 20-75°C, under identical buffer conditions. It is seen that in all

Table 2. UV-T_m (°C) of Duplex DNA **41:42**

Entry	Compound	T_m	$\triangle T_m$
1	Control	53.8	-
2	Spermine **I**	57.7	+ 3.9
3	2*S*,4*S* (L-*cis*) **III**	65.5	+ 11.7
4	2*S*,4*R* (L-*trans*) **IV**	64.7	+ 10.9
5	2*R*,4*S* (D-*trans*) **V**	62.1	+ 8.3
6	2*R*,4*R* (D-*cis*) **VI**	59.0	+ 5.2

Buffer: 25 mM TRIS (no salt) pH 7.3 T_m values are accurate to (±) 0.5 °C. Experiments were repeated at least thrice and the T_m values were obtained from the peak in the first derivative plots.

cases a sigmoidal plot was observed. The Table 2 shows the UV-T_m values for the DNA duplex (**41:42**) in the absence and presence of spermine **I** and chiral branched pyrrolidyl polyamines **III** – **VI** as determined from UV absorbance-temperature plots. The exact T_ms were derived from the peak maxima in the corresponding first derivative plots. The data in Table 2 indicates that spermine stabilized the duplex (entry 2) by 3.9° over the control (without spermine, entry 1). In comparison, all pyrrolidyl polyamines III-VI exhibited a much higher stabilization of DNA duplex compared to spermine at identical concentrations of the branched pyrrolidyl polyamines. The increased stability of duplex **41:42** followed the order (Table 2), 2*S*,4*S* (L-*cis*) **III**: $\triangle T_m$ 11.7 ° (entry 3) > 2*S*,4*R* (L-*trans*) **IV**: $\triangle T_m$ 10.9° (entry 4) > 2*R*,4*S* (D-*trans*) **V**: $\triangle T_m$ 8.3° (entry 5) > 2*R*,4*R* (D-*cis*) **VI**: $\triangle T_m$ 5.2° (entry 6). These data suggest that the extent

of duplex DNA stabilization seem to depend on the pyrrolidine stereochemistry of the polyamines.

3.4.2. Effect of polyamine concentration

The DNA duplex stability was also measured as a function of increasing concentration of pyrrolidyl polyamines **III**-**VI** in the range 0.01 mM to 1 mM. Figure 7

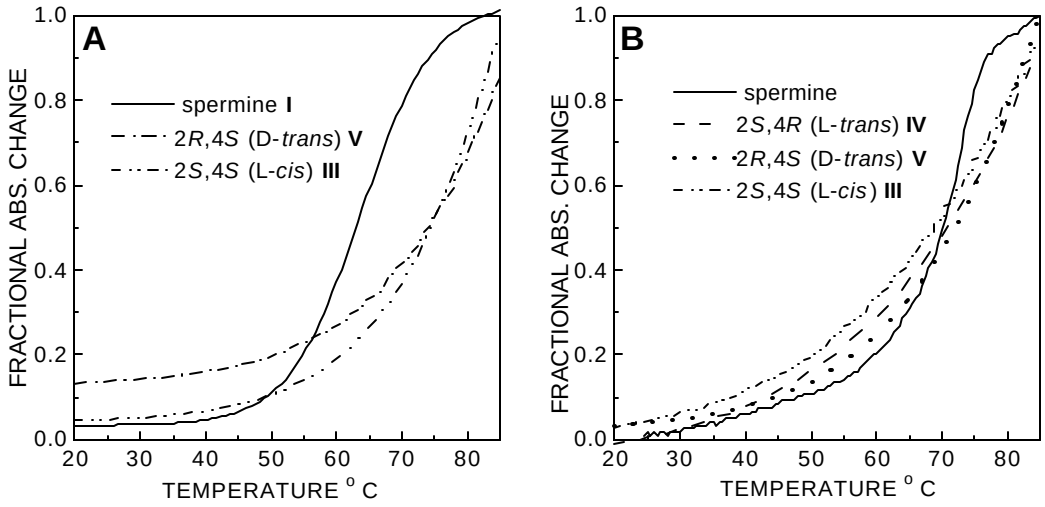


Figure 7. UV melting profile of duplex DNA (**A**) in presence of 0.1 mM of spermine **I**, 2*R*,4*S* (D-*trans*) **V** and 2*S*,4*S* (L-*cis*) **III** and (**B**) in presence of 1 mM of spermine **I**, 2*S*,4*R* (L-*trans*) **IV**, 2*R*,4*S* (D-*trans*) **V** and 2 *S*,4*S* (L-*cis*) **III**

shows the UV melting curve and the data is summarized in Table 3. All T_ms were found to increase with the increasing concentration of the spermine **I** and pyrrolidyl polyamines **III**–**VI**. While for spermine, the ΔT_m in this range was +9.3° at 0.1 mM and +14.7° at 1 mM (entry 2, Table 3), in case of the branched pyrrolidyl polyamines **III**–**VI** the T_m increased with the increase in concentration of the polyamine, with the expected hyperchromicity effects accompanying the melting (Table 3). At concentration 0.1 mM and above no upper plateau was observed in the melting curves even beyond 80° (Figure 4) making it difficult to compute the actual T_m (entries 3, 4, 5 and 6, Table 3). This suggests an incomplete melting due to a very strong electrostatic complexation of

Table 3: UV-T_m (^oC) of DNA duplex **41:42** with different polyamine concentrations

		Polyamine concentration	
Entry	**Compound**	**0.1 mM**	**0.01 mM**
1	Control	53.8	
2	Spermine **I**	67.0 (+9.3)	72.2 (+14.5)
3	2*S*,4*S* (L-*cis*) **III**	Melting incomplete	
4	2*S*,4*R* (L-*trans*) **IV**	Melting incomplete	
5	2*R*,4*S* (D-*trans*) **V**	Melting incomplete	
6	2*R*,4*R* (D-*cis*) **VI**	Melting incomplete	

Buffer: 25 mM Tris, pH 7.3 (no added salt). Spermine **I** and pyrrolidyl polyamines **III – VI** at 0.1 mM and 1 mM. Control is without any polyamines. Values in brackets: $\triangle T_m$ over control. T_m values are accurate to (±) 0.5^o C. The T_m values were obtained from the peaks in the first derivative plots.

the duplex by the pyrrolidyl polyamines (**III**-**VI**). Hence for proper comparison, all experiments were carried out using 0.01mM polyamines and at this concentration, the branched pyrrolidyl polyamines (**III**-**VI**) are remarkably superior to spermine in stabilizing the DNA duplex (as discussed in Section 3.4.1). Further, amongst the four isomers, the (2*S*,4*S*) L-*cis* **III** appeared to be the best in stabilizing the duplex with ΔT_m 11.7^o, followed by (2*S*,4*R*) L-*trans* **IV**, ΔT_m 10.9^o and (2*R*,4*S*) D-*trans* **V,** ΔT_m 7.3^o and least with (2*R*,4*R*) D-*cis* **VI,** ΔT_m 5.2^o (Table 3). These perhaps result from differential electrostatic complexation of DNA by polyamines.

3.4.3. Effect of salt on duplex-polyamine stability

In order to support the electrostatic nature of stabilization effects, studies were carried out by constituting the duplex (**41:42**) with polyamines (0.01 mM) in 25 mM Tris buffer containing 10 mM and 50 mM NaCl and the data is summarized in Table 4. In

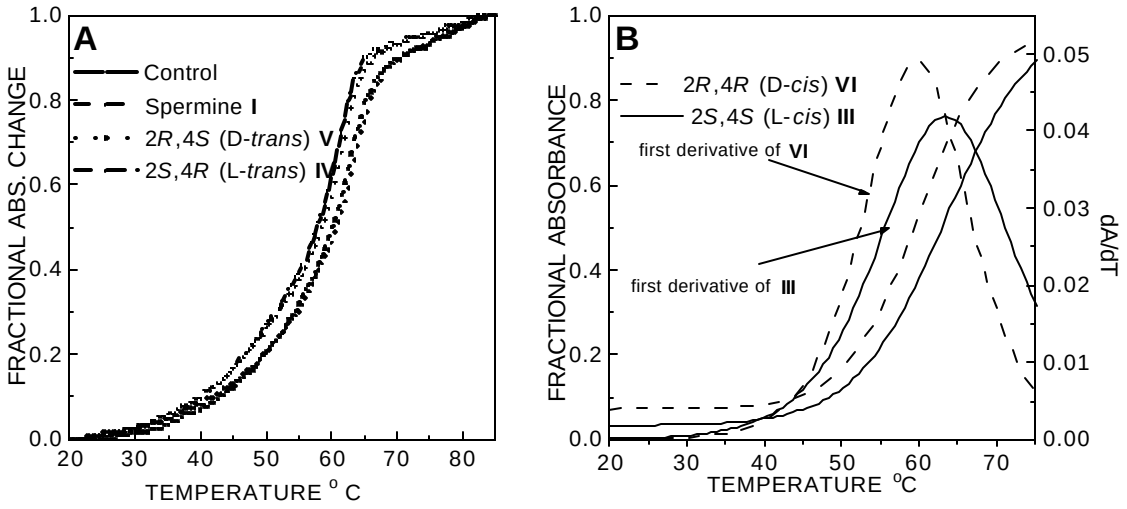


Figure 8. UV melting profiles of duplex DNA (**A**) control, 0.01 mM spermine **I**, (2*R*,4*S*) D-*trans* **V** and (2*S*,4*R*) L-*trans* **IV**. (**B**) (2*R*,4*R*) D-*cis* **VI** and (2*S*,4*S*) L-*cis* **III** along with first derivatives.

Table 4. UV-T_m (^oC) of Duplex DNA with polyamines at different salt concentrations

Entry	Compounds	Salt effects		
		No salt	10 mM	50 mM
1	Control	53.8	55.1 (1.3)	61.0 (7.2)
2	Spermine **I**	57.7	57.9 (0.2)	61.4 (3.7)
3	(2*S*,4*S*) L-*cis* **III**	65.5	65.9 (0.4)	65.7 (0.2)
4	(2*S*,4*R*) L-*trans* **IV**	64.7	63.2 (-1.5)	63.2 (-1.5)
5	(2*R*,4*S*) D-*trans* **V**	62.1	60.0 (-2.1)	62.7 (-0.6)
6	(2*R*,4*R*) D-*cis* **VI**	59.0	59.2 (0.2)	62.4 (3.4)

Buffer: 25 mM TRIS with 10 mM and 50 mM NaCl pH 7.3. Values in brackets: ΔT_m over no salt Tm . values are accurate to (±) 0.5 ^oC. Experiments were repeated at least thrice and the T_m values were obtained from the peaks in the first derivative plots.

the presence of externally added NaCl the T_m of control duplex increased by 1.3^o at 10

mM NaCl (entry 1) and by 7.2^{o} in presence of 50 mM NaCl. For spermine, the increase was less (ΔT_m 0.2^{o} and 3.7^{o} respectively, entry 2) compared to control. Such a salt effect was much less pronounced (<3^{o}) for the DNA duplex containing the polyamines **III**–**VI**, under identical salt concentrations. This negligible effect of ionic strength suggests that the observed DNA duplex stability with branched polyamines predominately originate from the electrostatic interactions. In the presence of 50 mM NaCl, these relative differences were reduced and the (2*S*,4*S*) L-*cis* isomer **III** (entry 3) was still superior to other isomers in duplex stabilization. The overall results indicate that the chiral, branched polyamines presented in this study stabilize DNA duplex by electrostatic interactions and the degree of stabilization is dependent on their stereochemistry.

3.4.4. Fluorescence spectroscopy: Ethidium bromide displacement assay

To examine whether the differential stabilization observed in DNA duplex by pyrrolidyl polyamines **III - VI** are due to their altered binding affinities to the DNA duplex, the displacement of ethidium bromide upon binding of polyamines was followed by fluorescence assay. The experiment was performed by incremental addition of DNA duplex (**41:42**) to ethidium bromide solution and recording the fluorescence emission intensity at 595 nm till the saturation point is attained. The resulting ethidium bromide-DNA complex was individually titrated with aq. solutions of pyrrolidyl polyamines (**III–VI**) and the fluorescence intensity changes recorded (Figure 9). The drop in fluorescence is much slower for spermine compared to the pyrrolidyl polyamines, which exhibited a drastic decrease. The data on C_{50} values obtained as the concentration of polyamine required to quench 50% of maximum fluorescence is summarized in Table 5 and it indicates that spermine **I** (entry 1) displaced intercalated ethidium bromide inefficiently compared to the pyrrolidyl polyamines: spermine **I** (16.4 μM), (2*S*,4*S*) L-*cis* **III** (0.96 μM), (2*S*,4*R*) L-*trans* **IV** (1.1 μM), (2*R*,4*S*) D-*trans* **V** (2.9 μM) and (2*R*,4*R*) D-*cis*) **VI**

(2.55 μM). Thus within the experimental limitations, the (2*S*,4*S*) L-*cis* polyamine **III**

Table 5. C_{50} values of Spermine **I** and pyrrolidyl polyamines **III - VI**

Entry	Compound	C_{50} values (mM)
1	Spermine **I**	16.4
2	2*S*,4*S* (L-*cis*) **III**	0.96
3	2*S*,4*R* (L-*trans*) **IV**	1.1
4	2*R*,4*S* (D-*trans*) **V**	2.55
5	2*R*,5*R* (D-*cis*) **VI**	2.9

Buffer: 10 mM Tris + 1 mM EDTA pH 7.4, 20°

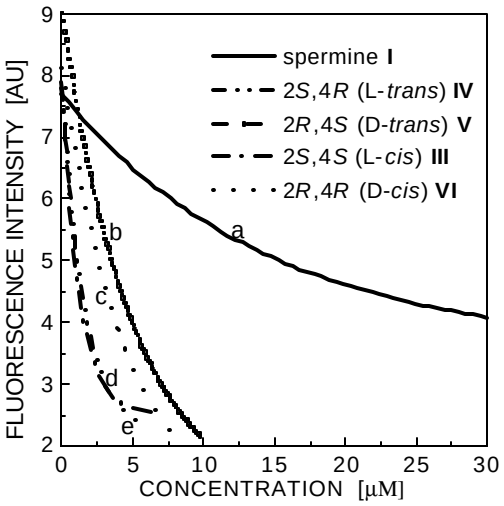


Figure 9. Ethidium bromide displacement fluorescence assay

binds to duplex DNA strongest followed by the polyamines **IV**, **V** and **VI**. This order roughly reflects that seen from the UV melting temperatures and all polyamines bound duplex at least 8-15 times as strong as spermine under identical conditions.

3.5. STUDIES ON THE EFFECT OF POLYAMINE ANALOGS ON DNA TRIPLEX STABILITY

In order to examine the abilities of pyrrolidyl polyamines **III**–**VI** to stabilize the triplex DNA (**40*41:42**) under physiological conditions the effects of the branched pyrrolidyl polyamines on triplex structure were studied by measuring their melting temperatures by thermal UV absorbance method. The DNA triplexes were constituted

Table 6. UV-melting temperature T_m of triplex DNA-polyamine complexes

Entry	Compound	T_{m1}	T_{m2}
1	Control	-	53.6
2	Spermine	-	58.2
3	2*S*,4*S* (L- *cis*) **III**	41.1	64.1
4	2*S*,4*R* (L- *trans*) **IV**	42.7	62.6
5	2*R*,4*S* (D - *trans*) **V**	36.8	60.2
6	2*R*,4*R* (D - *cis*) **VI**	35.1	60.1

Buffer: 25 mM TRIS (no added salt) pH 7.3, Experiments were repeated atleast thrice and the values are accurate to (+) 0.5C.

from mixing equimolar amounts of oligonucleotides **40**, **41** and **42** in the presence and absence of 0.01 mM polyamines **III**-**VI**. Figure 10 shows the UV-Tm profile and the appearance of double sigmoidal transition is indicative of successful triplex formation and Tm data for triplexes is shown in Table 6. In buffer at pH 7.3, no triplex formation. was seen in the control samples with or without 0.01 mM spermine (entry 1) while triplexes were observed in case of all polyamines (entry, 3-7). Among the different polyamines, 2*R*. series (entry 3, 4) exhibited better effects than the 2*R* stereomers (entry 5, 6) in triplex stabilization. Such a salt effect was much less pronounced ($<3^{o}$) for the DNA duplex The slight differential effect of polyamines on stability of underlying

duplex in triplex is seen in the values of $T_{m2,}$ which is higher for **III** than **IV**, **V** and **VI**. Comparison addition of 0.01 mM of pyrrolidyl polyamines facilitated the triplex formation (entries 3, 4, 5 and 6). Among the different stereomers, 2*S* polyamines (entry 3, 4) were marginally better than of entries 3 and 4 shows a reversal of stability order for **III** and **IV** in stabilizing duplex and triplex indicating that the factors responsible for stabilization effects may have a stereochemical basis.

3.5.1. Effect of salt on triplex stability in presence of polyamines

The stability of triplex (**40*41:42**) was studied in presence of different concentrations of sodium chloride. The presence of even 10 mM NaCl induced triplex

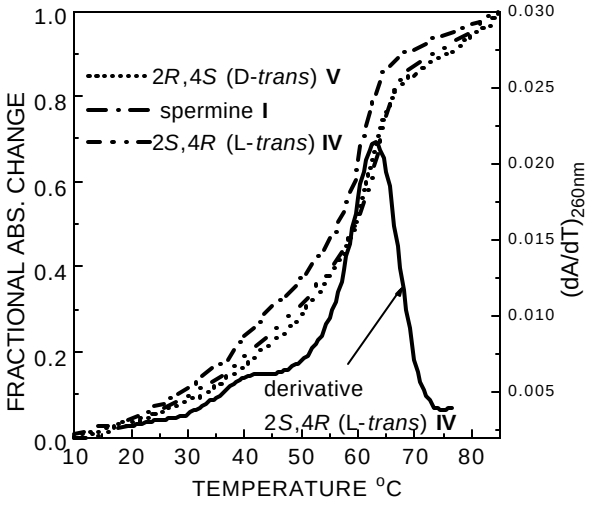


Figure 10. UV melting profile of Triplex (**40*:41:42**) in 25 mM Tris + 50 mM NaCl at pH 7.3 with 0.01 mM spermine **I**, pyrrolidyl polyamines (2*R*,4S) D-*trans* **V** and (2*S*,4*R*) L-*trans* **IV**

formation in both control and the triplex containing spermine (0.01 mM). The latter with T_m 35.2° (entry 2, Table 7) gave a slightly higher melting (ΔT_m=+1.4°) than the control T_m= 33.8°. Increasing the salt concentration to 50 mM (Figure 10), further increased the triplex T_ms to ≈ 40.7° (not shown). In comparison, 0.01 mM of pyrrolidyl polyamines **III**-

VI facilitated triplex formation even without any salts and addition of 10 mM salt decreased the triplex Tm slightly. The T_m of triplexes showed significant dependence on the stereochemistry of the polyamines. Among the different stereoisomers 2*S*,4*R* (L-

Table 7. UV-T_m of Triplex (**40*41:42**) in presence of polyamines and 10 mM NaCl

Entry	Compound	T_{m1}	T_{m2}
1	Control	33.8	55.5
2	Spermine **I**	35.2	57.7
3	2*S*,4*S* (L-*cis*) **III**	41.3	64.8
4	2*S*,4*R* (L-*trans*) **IV**	38.7	61.6
5	2*R*,4*S* (D-*trans*) **V**	36.6	60.9
6	2*R*,4*R* (D-*cis*) **VI**	34.8	59.3

Buffer: 25 mM Tris + 10 mM NaCl pH 7.3. Experiments were repeated at least thrice and the values are accurate to (±) 0.5°C.

trans) **IV** was the most effective triplex stabilizing agent followed by the other stereoisomers (2*S*,4*S*) L-*cis* **III** > (2*R*,4*R*) D-*cis* **VI** > (2*R*,4*S*) D-*trans* **V**.

When the buffer contained 10 mM NaCl, triplex formation was facilitated in case of control as well as with 0.01 mM spermine. In presence of 50 mM NaCl, T_m was almost similar in all the cases. This indicated an effective competition from salt at higher concentration that nulls the effect of polyamines that are present in much lower concentration (0.01 mM). As expected, the T_m of control significantly increased in the presence of salt.

3.5.2. Effect of pH on DNA triplex-polyamine complexes

For DNA sequences containing CG bases, the triplex stability is known to be a function of pH with a maximum around pH 5.5 at which C is protonated at N3.[10] Hence the triplex melting experiments were also done at pH 5.5 in sodium acetate buffer

containing 10 mM NaCl (Table 8). Under these conditions, the triplexes were seen in all cases, except with *cis* polyamines **V** and **VI** (Figure 11) where aggregation induced by

Table 8. pH dependent UV-Tm data for DNA triplex –polyamine complexes

Entry	Compounds	Tm			
		PH 7.3		PH 5.5	
		10 mM	100 mM	10 mM	100 mM
1	Control	33.8	44.9	35.7	44.2
2	Spermine **I**	35.2	44.5	37.0	44.2
3	2*S*,4*S* (L-*cis*) **III**	41.3	44.9	---	44.6
4	2*S*,4*R* (L-trans) **IV**	38.7	44.2	40.7	44.5
5	2*R*,4*S* (D-*trans*) **V**	36.6	44.9	39.2	44.5
6	2*R*,4*R* (D-*cis*) **VI**	34.8	44.9	---	43.0

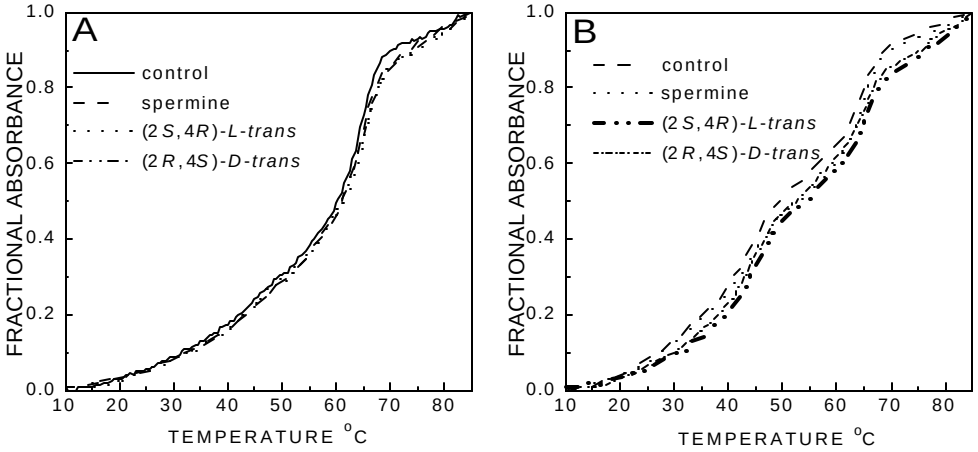


Figure 11. UV melting curves of DNA-triplex polyamine complexes with 100 mM NaCl. **A**, pH 7.3. **B**, pH 5.8

strong binding[16] precipitated DNA out of solution. The (2*S*,4*R*) L-*trans* polyamine **III** exhibited a 5° increase in T_m (entry 3) over that of control as compared to 3.5° by (2*R*,4*S*) D-*trans* polyamine **IV** (entry 4) and 1.3° by spermine (entry 2) over the control

T_m. In the presence of higher NaCl concentration (100 mM), the triplexes showed an increase in T_m but the relative differences among the different triplexes in the presence of polyamines disappeared similar to that seen at pH 7.3. This again suggested the weak effect of polyamines in the presence of a large excess of competing salt.

3.5.3. Effect of stereochemistry on polyamine binding to DNA: Molecular modeling studies

In order to examine the possible variations in the surface charge distribution as a function of the pyrrolidine core stereochemistry, preliminary molecular modeling of all

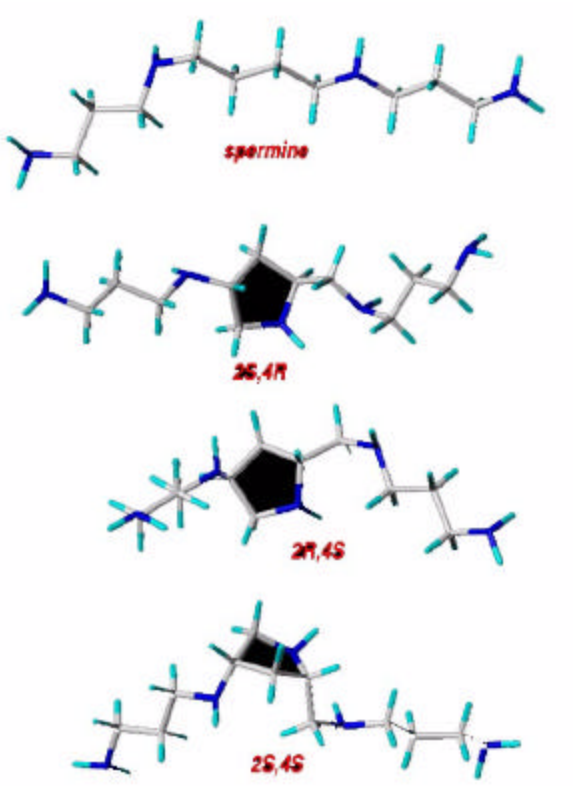


Figure 12. Energy minimised structures for spermine analogs

the stereoisomers was done, using Tripos SYBYL software. Figures 12-14 show the minimized structures for spermine **I** (Figure 12), pyrrolidyl polyamines **II-VI** (Figure 13) and the dendramine **VII** (Figure 14). In Figure 11, it is seen that conformational constrain on spermine results in shrinking of its backbone the extent of which depends on the stereochemistry. While the (2*S*,4*R*) *trans* amine **II**, shows an extended structure, the (2S,4S) *cis* amine **II** adopts a bent-V type.

The polyamine **II** corresponding to other stereochemistries show a crescent

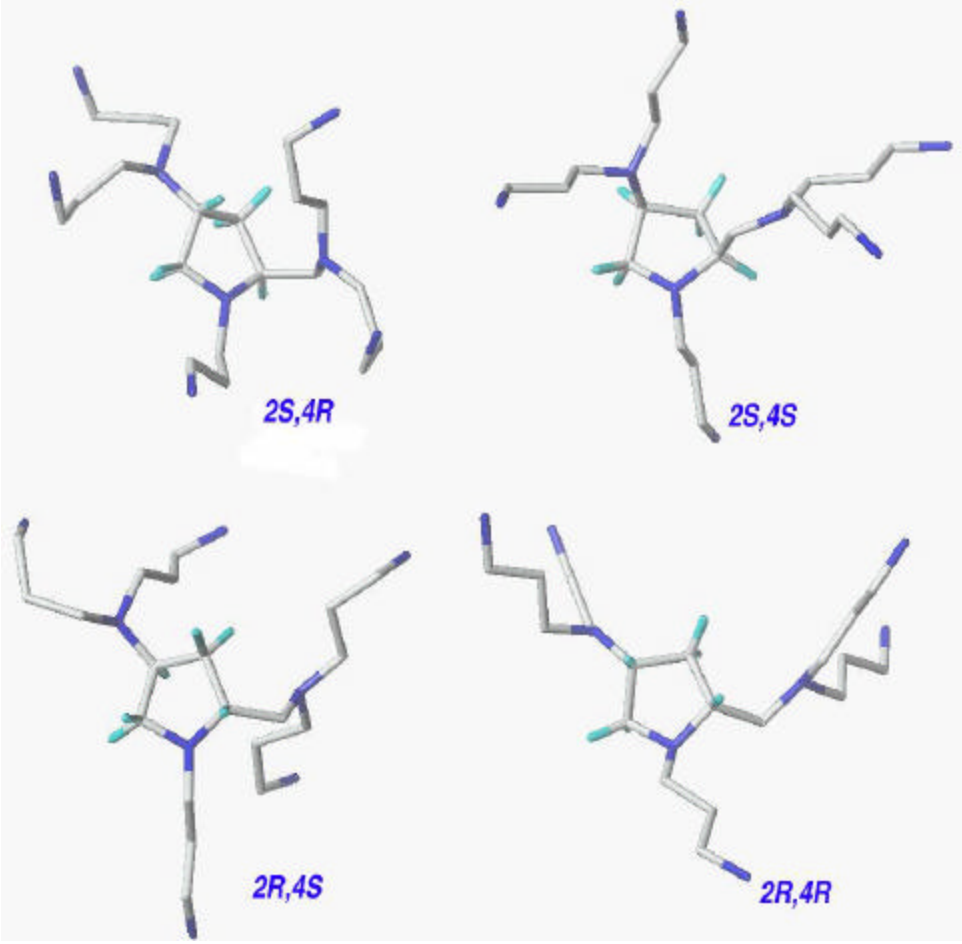


Figure 13. Energy minimized structures for polyamines **III-VI**

structure with conformational features in between the above. Thus the intramolecular distance between the cationic amino groups vary considerably among these, resulting in differences in their overall shape and hence probably their interactions with DNA duplexes and triplexes.

The structures of pyrrolidyl polyamines in Figure 13 suggest that the stereochemistry of the internal core considerably influences the spatial distribution of the terminal amino groups on the branches. Viewed from the same direction of the molecules, the *cis* isomers (2*S*,4*S*)-L-*cis* and (2*R*,4*R*)-D-*cis* seem to have their branches more dispersed than those of the analogous *trans* (2*S*,4*R*)-*L*-*trans* and (2*R*,4*S*)-*D*-*trans* isomers that are more compact. The distance between the terminal

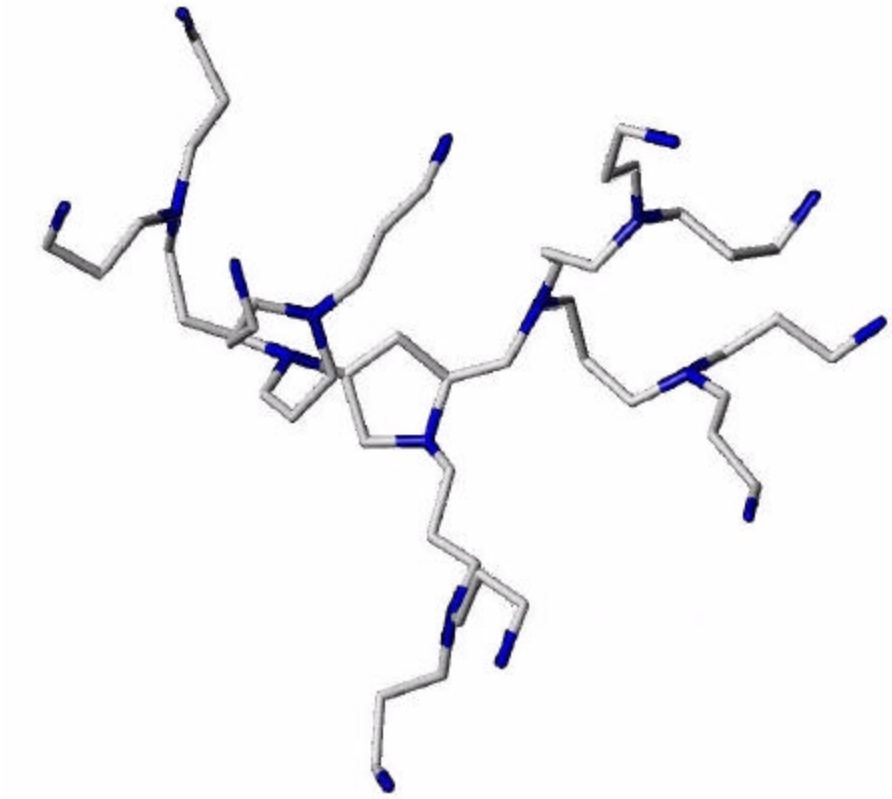
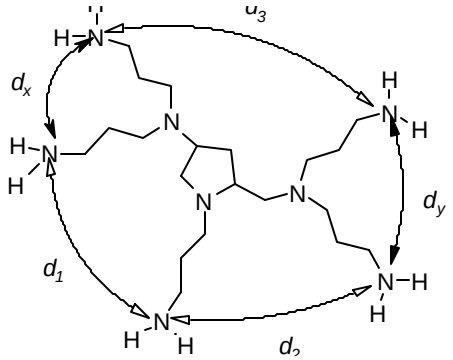


Figure 14. Energy minimised structure for second generation dendramine **VII**

amino groups (Figure 15) on the same branch in all the isomers varied in the range 5.5 to7.7Å. In contrast, the distances between the amino groups located on the adjacent branches in *cis* isomers were in the range of 8.5 to 11.7Å, significantly different from a shorter range 5.2Å to 7.7Å seen in *trans* isomers. Though it is difficult to correlate these values directly with the DNA geometry to explain the differential binding of the isomers, it is clear that the isomeric polyamines do have differential distribution of surface cationic amino functions, determined by their stereochemistry at the interior pyrrolidine core. It is difficult at this stage to reason why the (2*S*, 4S)-*L*-*cis* isomer is different from



Compound	d_1	d_2	d_3	d_x
(2*S*,4*S*)-*cis*	10.84	10.81	8.59	6.86
(2*R*,4*R*)-*cis*	11.67	11.01	11.34	7.10
(2*R*,4*S*)-*trans*	11.55	6.30	5.90	5.89
(2*S*,4*R*)-*trans*	6.74	5.19	8.50	5.58

Figure 15. Intramolecular distances among the amine groups

the (2*R*,4*R*)-*D*-*cis* isomer. Nevertheless, preliminary molecular modeling studies point to a significant variation in the topological variations in the surface charges. Figure 14

shows the minimized structure for the second generation (2*S*,4*S*) dendramine **VIII**, which reiterates the operating theme of this work that conformational restriction and chirality at the center leads to varied topologies and thus the presently synthesized pyrrolidyl polyamines are good stereoisomeric scaffolds. Very recently such stereo scaffolds based on a trisubstituted cyclopentane ring has provided a good library for design of new drugs that are potent inactivators of γ-aminobutyric acid aminotransferase..[17]

3.5.4. CD Spectral analysis of DNA-polyamine complexes

The electrostatic binding of a polyamine may condense DNA leading to a change in its conformation.[18] Normally this happens only at high concentrations of salts or other counterions. To examine whether the present polyamines indulge in such conformational changes, CD spectra were recorded for the DNA duplex **41:42** and triplex **40*41:42** (Figure 16). The CD spectrum of DNA duplex **41:42** exhibits a B-form

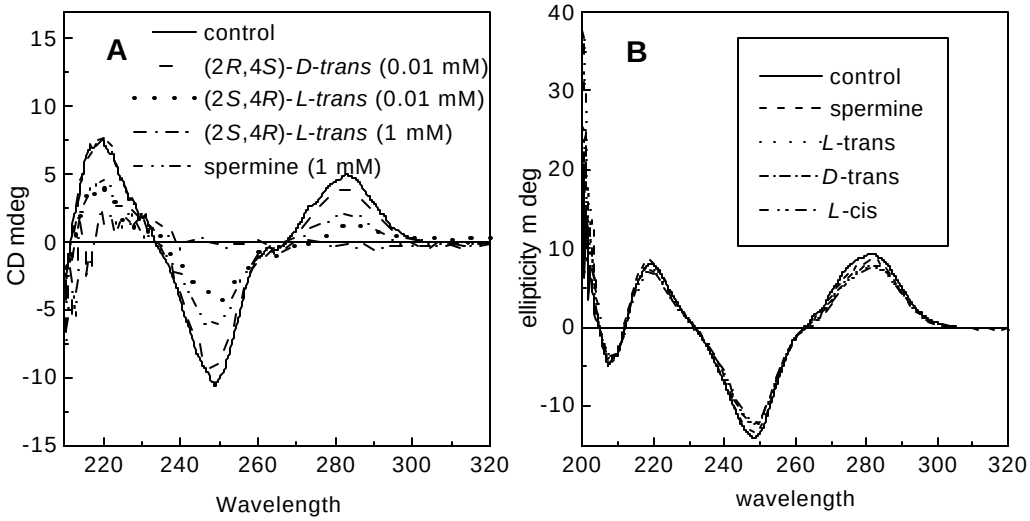


Figure 16. CD spectra of DNA duplex (**A**) and triplex (**B**)

pattern with a positive band at higher wavelength (270-280 nm) and a negative band at lower wavelength (240-250 nm). The addition of spermine or other polyamines to DNA duplex lead to a decrease in intensity of both positive and negative bands, without any significant shifts in their wavelength maxima (Figure 16A). The chiral polyamines lack any absorbing chromophore and hence they do not contribute significantly in the region 240-280 nm. However, upon binding to DNA they may contribute to the overall CD depending on their chirality, leading to intensity changes. In contrast, no significant intensity changes were noticed for DNA triplexes in presence of polyamines (Figure 16B), though UV data clearly indicated a stabilization of triplexes under these conditions. Any condensed form of DNA should show characteristic CD bands[18] that were absent in this case indicating that under these conditions, no condensation of DNA has occurred. The CD spectral results thus rule out any major changes in the base stacking or polymorphic form of B-DNA duplex and triplexes in presence of polyamines.

3.6. DISCUSSION

Spermine, a linear polyamine is well known to stabilize DNA duplexes and triplexes.[19] At a molecular level, the terminal ammonium cations interact electrostatically with the anionic phosphate on DNA backbone. The internal amino moieties may also be involved in specific hydrogen bonding with the nucleobases in the major groove, with a cross groove binding for AT base pairs and down groove binding for GC base pairs[20] (Figure 2, Chapter 1). In triplexes, where the negative charge density is higher, stabilization by cationic polyamines assumes a major role.[21] Our earlier studies[22] involved substitution of terminal primary amino groups of spermine by guanidine functions that led to selective enhancement in DNA triplex stability. In a second approach,[23] the enormous conformational freedom in linear polyamines was restricted by the introduction of a pyrrolidine ring and such analogs improved the

stability of DNA triplexes, perhaps due to a conformational pre-organization effect. The present approach constitutes a combination of above in which additional aminopropyl side chains are introduced as branches on the pyrrolidine nucleus to act as sources of spatially distributed positive charges.

The UV melting data clearly indicated that the branched pyrrolidine polyamines are much superior to linear spermine and the earlier reported pyrrolidine analogs[23] in stabilizing the DNA duplexes. Even at as low a concentration of 0.01 mM of the target polyamines **III-VI** and in the absence of salts, considerable gain in T_m is seen while in the presence of salts, the enhancing effect evens out. Thus the branched polyamines exert their stabilizing effect predominantly via electrostatic interactions of their polycationic charges with anionic phosphates of DNA and at higher NaCl concentrations, the Na^+ ions competitively displace them, leading to the disappearance of the relative stabilizing effects as compared to the control. Such is the stabilizing effect of the branched polyamines, that above 0.1 mM concentrations, the duplex does not melt completely even beyond 80^{o}C. This is remarkably higher than that observed with their previous congeners.

DNA triplex formation also occurs with these polyamines at as low concentrations as 0.01 mM, significantly even at physiological pH and in the absence of any salts, under which conditions no triplex formation could be seen either with or without spermine in the control sample. In the presence of 10 mM NaCl at pH of 5.5, triplexes were seen in all cases, but the magnitude of the Tm differences between spermine and the polyamines (**III**) / (**IV**) are not as large as that seen with duplexes. At slightly higher salt concentrations (100 mM), no differences existed in their Tm, since the polycationic ligands are competitively displaced by Na^+ ions. These results indicate that the branched pyrrolidyl polyamines, in the absence or at very low salt concentrations act as a powerful source of cations and bring about enormous stability in DNA duplexes and triplexes.

In addition to electrostatic stabilization, it is noteworthy that two additional interesting features are specifically seen in the interaction of the four chiral, branched polyamines with DNA duplex and triplex. First, the different chiral polyamines exhibit slight differences in their binding strength to DNA duplex (seen both in their T_m as well as fluorescence C_{50} values), as a function of the relative stereochemistry of the side chains, with the *cis* polyamine **V** being most strongly bound. This suggests that the stereochemical disposition of the side chains on pyrrolidine nucleus determine the spatial location of terminal amino functions thereby governing the topological distribution of positive charges. This seems to be an important factor in modulating their duplex binding, which varies among the different diastereomers. The spatial arrangement of charges for DNA interaction is perhaps optimal with the *cis*-polyamine **V**. Secondly, the topology of polyamine-DNA interactions in triplexes differ considerably from that in duplexes and thus, among the four stereoisomeric polyamines, while the *cis*-analog **V** is the most efficient for duplex stabilization, the (2*S*,4*R*)–*trans* isomer **III** was better in triplex stabilization. The CD results suggested no major departure of DNA conformation from B-form, in presence of polyamines.

3.7. CONCLUSIONS

The biophysical studies discussed in the above sections using the chiral, pyrrolidyl polyamines discussed in Chapter 2 indicates the high affinity of these compounds to DNA duplexes and triplexes. Moreover, these compounds showed a better stabilization compared to the linear, natural polyamine spermine and were superior to the previous analogs, as seen by their remarkable stabilization properties at concentrations as low as 0.01mM. The stabilization is mediated essentially through the higher charge density on these compounds and electrostatic interactions that were evident from the salt dependent experiments. Stereochemistry of the inner core was also found to have an important role in the interaction of these compounds with DNA,

which perhaps determines the topological disposition of the positive charges of the terminal amino groups located on the surface. These observations were confirmed by the preliminary molecular modeling experiments. This kind of nucleic acid binding agents form first generation dendrimers and are novel since the chiral core determines the arrangement of the growing branches leading to chiral dendrimers.[24] and these may be good mimics of aminoglycoside antibiotics[25] and useful for potential applications, for eg. in material chemistry and chiral separations.[26] Polycationic compounds that bind DNA efficiently may also have utility as DNA transfection agents.[27] Further studies are needed to study the effect of these novel polyamine analogs on the possible structural transitions of DNA.

3.8. EXPERIMENTAL

3.8.1. Oligonucleotide synthesis

All oligonucleotides were synthesized on 1.3 μM scale on a Pharmacia GA plus DNA synthesizer using β-cyanoethylphosphoramidite chemistry, The synthesis cycle consists of four steps. (I) Deprotection of the Dimethoxy trityl group using 3% dichloroacetic acid in EDC, washed with EDC followed by washing with acetonitrile to get water free conditions for the coupling. (ii) formation of the 3'-5' internucleoside linkage with the phosphorous containing group of the 5'-O-(4,4'-dimethoxytrityl)deoxyribonucleoside-3'-O-{(diisopropylamino)-β-cyanoethylphosphoramidite] monomers (0.1 M in CH_3CN) using tetrazole (0.5 M in CH_3CN) as coupling reagent, followed by acetonitrile wash to remove the unreacted amidites and the tetrazole. (iii) capping of the unreacted 5' hydroxyl group with acetic anhydride (20% in CH_3CN) in presence of DMAP followed by acetonitrile wash. (iv) oxidation of the phosphite triester to the phosphate using iodine (0.1 M in CH_3CN) and collidine. After the completion of the synthesis the oligonucleotides were cleaved and deprotected from the support by treating with aqueous ammonia at 55°C for 16 hours.[28]

All the oligonucleotides were purified by gel filtration (Sephadex G25) and their purity checked on reverse phase HPLC C18 column using buffer system A: 5% CH3CN in 0.1 M triethyl ammoniumacetate (TEAA) and B: 30% CH3CN in 0.1 M TEAA using a gradient A to B of 1.5%/min at a flow rate of 1 mL./min and the oligomers showed more than 90% purity and were used as such without further purification.

3.8.2. UV experiments

UV-Tm of duplex and triplex by melting experiments were carried out in Tris (25 mM) buffer at pH 7.3 containing 10,50 or 100 mM NaCl. Triplex melting experiments at pH 5.5 were carried out in sodium acetate (50 mM) buffer containing 10 or 100 mM NaCl. Appropriate ODNs, each at a strand concentration of 1 μmol based on UV absorbance at 260 nm calculated using molar extinction coefficients (A = 15.4, C = 7.3, G = 11.7, T = 8.8 cm^2/mmol) were mixed in the buffer containing known amount of polyamines, heated to 85°C for 3 minutes and cooled to room temperature, followed by overnight storage at 4°C.

All the UV melting experiments (A_{260} at various temperatures) were recorded on a Perkin-Elmer λ15 UV-Vis Spectrophotometer equipped with a water jacketed 5-cell holder and a Julabo temperature programmer with a heating rate of 0.5° C/min. over the range 5-85° C. Purging argon/nitrogen gas through the solutions for 2-3 minutes prior to the experiments degassed the samples. Dry nitrogen gas was flushed in the spectrophotometer chamber to prevent moisture condensation at temperature below 15°C.

Each melting experiment was repeated at least thrice. The normalized absorbance at 260 nm was plotted as a function of temperature. The T_m was determined from midpoint of the first transition in the plots of fractional absorbance change vs temperature and was further confirmed by the differential (dA/dT vs T) curves. The Tm values were accurate to ± 0.5° C over the reported values.

For the salt dependent experiments, the samples were annealed in buffers containing appropriate concentration of the salt. In the pH dependent experiments DNA triplexes were constituted in a sodium acetate buffer (pH 5.8) containing appropriate concentration of salt.

3.8.3. CD Studies

CD spectra were recorded on a JASCO J-715 spectropolarimeter. CD spectra of a single strand, duplex and triplexes were recorded at different polyamine concentrations in Tris buffer (25 mM). All the CD spectra were recorded at 20^{o}C and an accumulation of 4 scans from 320 nm to 195nm using 1 cm cell. A resolution of 0.1 nm, band width 1.0 nm, sensitivity 2 mdeg, response 2 sec and a scan speed of 50 nm/min.

3.8.4. Fluorescence Experiments

Fluorescence spectra were recorded on Perkin-Elmer LS-50B luminescence spectrometer. Ethidium bromide displacement assay was performed as reported in literature.[29] Typically, to 1 μM of EtBr solution, DNA duplex (20 μM stock) was added incrementally (2 μL) until the rise in fluorescence of the probe attained saturation (λ_{ex} = 475 nm, λ_{em} = 610 nm). To this complex small aliquots (2 μL) of spermine/polyamine solution (0.01 mM stock) were added till the drop in intensity reached a constant value. C_{50} value is the concentration of the drug, which caused a 50% drop in fluorescence intensity in Ethidium bromide-DNA complex.

3.8.5.Molecular Modeling

Preliminary molecular modeling was done on Silicon Graphics Computer using Tripos SYBYL software version 6.1 and a conjugate gradient with a minimum energy change of 0.01 K Cal/mol, and 1000 iterations.

3.9. REFERENCES

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SECTION B

CONSTRAINED PEPTIDE NUCLEIC ACID ANALOGS

CHAPTER 4

INTRODUCTION

PNA ANALOGS AND DERIVATIVES

4.1. INTRODUCTION

Self-recognition by nucleic acids through complementary base pairing is one of the fundamental processes in biological systems.[1] DNA is the basic genetic material, consisting of two complementary strands held together by Watson-Crick hydrogen bonds through the donor-acceptor sites of the four nucleobases A, T, G and C. (Figure 1)

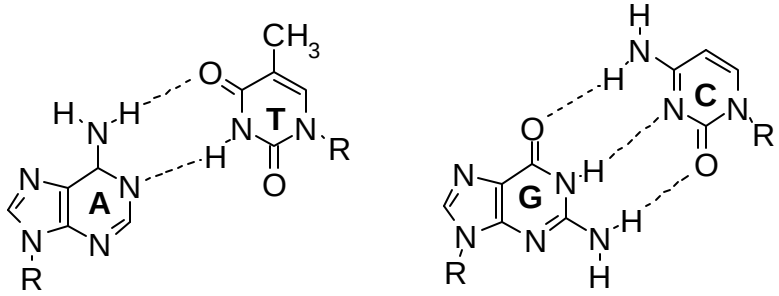

Figure 1. Base pair recognition by Watson-Crick Hydrogen bonding

Since many of the biological functions of DNA take place via molecular recognition, attempting chemical modifications[2] of DNA towards practical therapeutic and diagnostic applications has been a desirable goal for chemists. The concept of `antisense oligonucleotides' as potential therapeutic agents[3] introduced by Zamechnik and Stephenson[4] has aroused much interest in search of potent DNA mimics.

Antisense oligonucleotides (Figure 2) recognize a complementary region on target *m*-RNA through Watson-Crick base pairing and form a duplex (RNA-DNA hybrid) that is not processed by the protein synthesis machinery and hence would retard the expression of the corresponding protein. When target proteins are disease related, this will have therapeutic value. The prime requisites for oligonucleotides to be effective as antisense oligonucletides are (a) they should have high specificity to the RNA template, the sense

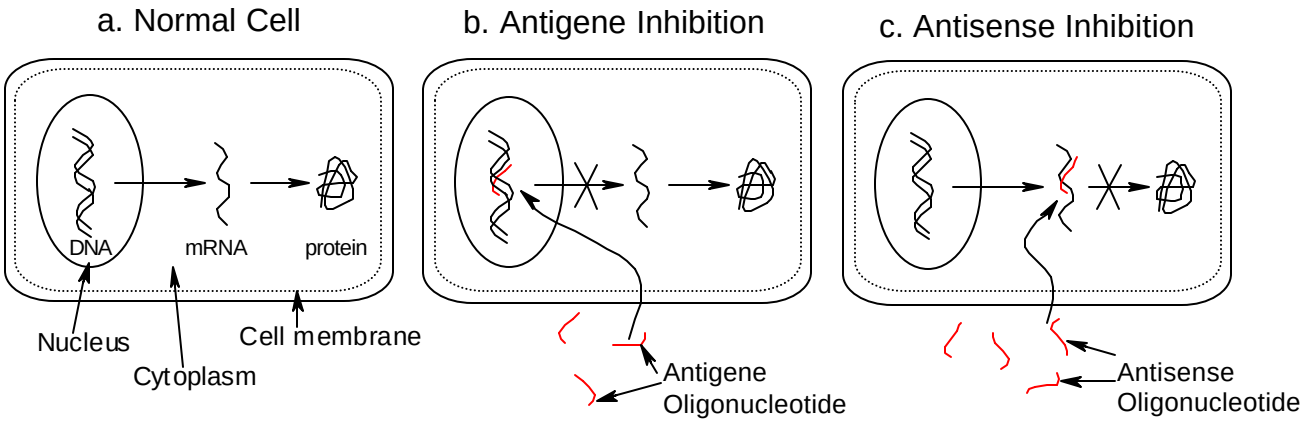

Figure 2. Mechanism of action of Antisense and Antigene Oligonucleotides

strand, (b) improved cellular uptake and (c) resistance to cellular enzymes eg. nucleases and proteases.

In another approach, the `antigene strategy', (Figure 2) interference with gene expression can be accomplished by binding of oligonucleotides to duplex DNA through Hoogsteen hydrogen bonds (Figure 3) leading to the formation of a triple helix.[5] Thus the double stranded DNA itself can act as a target for the third strand oligonucleotides or

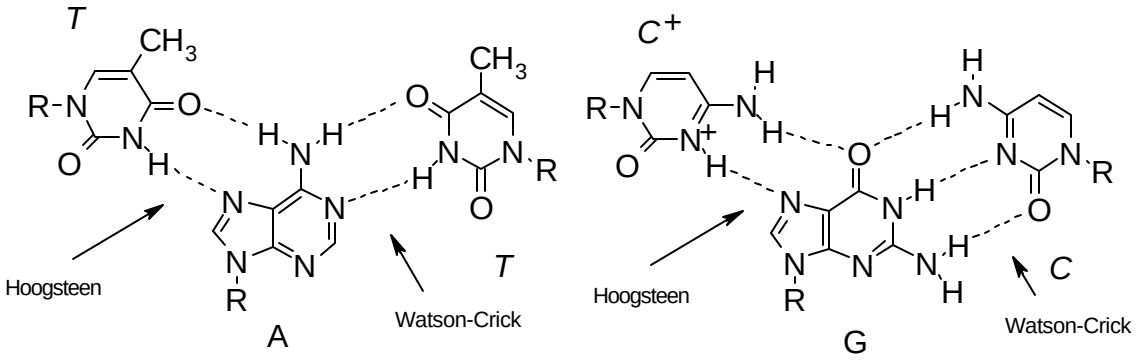

Figure 3. T.A-T and C^{+}.G-C triplets involving Hoosteen and Watson-Crick base pairing

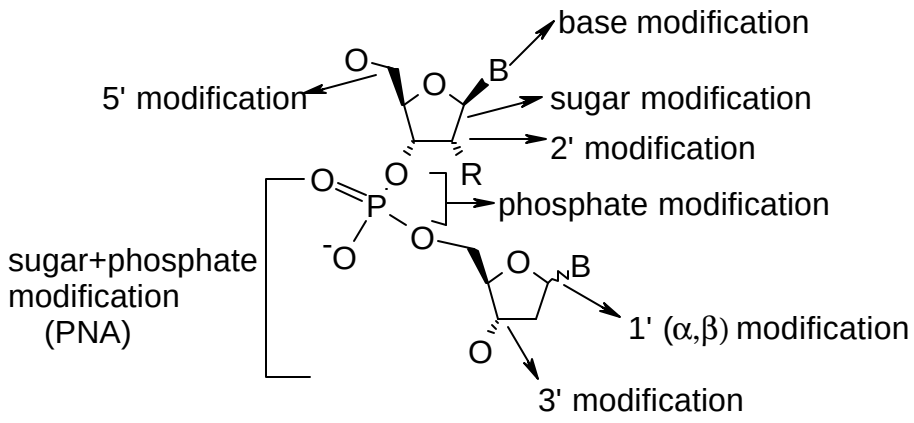

Figure 4. Position and type of Oligonucleotide Modification

analogs and the limitation for triplex formation is that it is possible only at homopurine stretches of DNA..[5,6] Natural oligonucleotides have been shown to exhibit both antisense and antigene properties in vitro.[7] However, a serious drawback that limits the use of oligonucleotides as therapeutic agents is that they are rapidly degraded by nucleases *in vivo*.[7a] This has lead to several chemical modifications[8] of the oligonucleotide structure to impart them the resistance towards cellular enzymes.[3,] The various possible sites of modification in a nucleotide are shown in Figure 4.

In the first generation 'antisense oligonucleotides' the phosphodiester backbone has been replaced by phosphorothioates **1a**, phosphorodithioates **1b**, methyl phosphonates **1c**, hydroxymethyl phosphonates **2**, phosphotriesters **3**, and phosphoramidates **4** as shown in Figure 5.

The backbone modifications displayed a greatly improved resistance towards nucleases[9] and a therapeutic agent based on phosphorothioates already has been recently approved as a drug by USFDA.[10] The chemical modifications also modulate the binding ability of analogs to complementary sequences. To increase the nuclease resistance and

binding affinity, several modifications have been introduced. In most of these modifications, four atom chain W, X, Y and Z in DNA phosphodiester backbone of **5** has

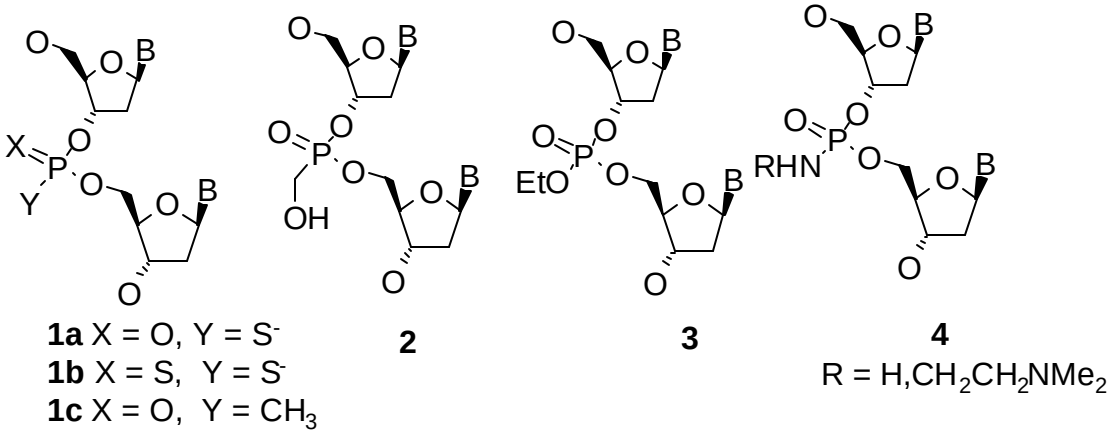

Figure 5. Phosphate Modifications

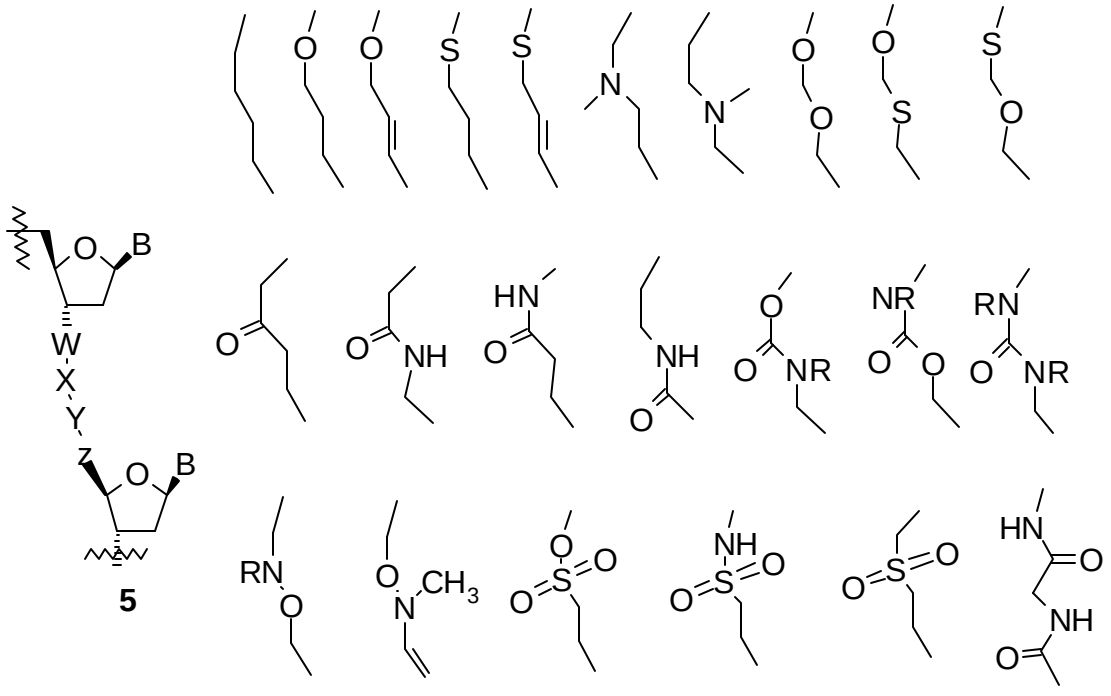

Figure 6. Phosphodiester linkage modifications

been replaced by other combination of atoms[11] (Figure 6). Only a few of these phosphodiester mimics have shown good binding but none showed the potential to be a good drug. In addition to the phosphodiester backbone modification, several modifications

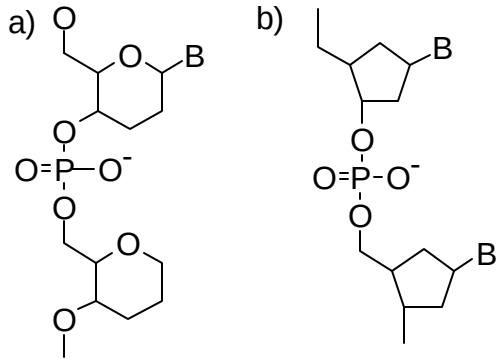

Figure 7. a) Homo DNA b) Carbocyclic DNA

in the sugar moiety[7] as well as nucleobases[12] have also been attempted. The pentose ribose sugar has been replaced by a hexose[13] or carbocycles[14] (Figure 7) and these failed to show any co-operative binding with natural DNA.

So far, only a few attempts to replace the entire (deoxy) ribose phosphate backbone have been successful. One of this is the morpholino oligomer (Figure 8) wherein the monomers are linked through a carbamate linkage.[15] The second generation of morpholino DNA with a phosphoramidate linkage exhibited better stability upon hybridization with complementary DNA and showed potent antisense activity in an *in vitro* assay. To avoid the loss of bioactivity through major structural modifications and impart only the nuclease resistance, oligonucleotides having only 5' or 3' terminal modifications have been studied. These have a central core of unmodified DNA structure, but at the ends have either phosphorothioates or O2'-derivatized nucleosides that are resistant to

5'/3' exonuclease susceptibility. Although these exhibited favorable properties for antisense activity, many phosphorothioates showed non-antisense effects as well leading to adverse clinical side reactions.

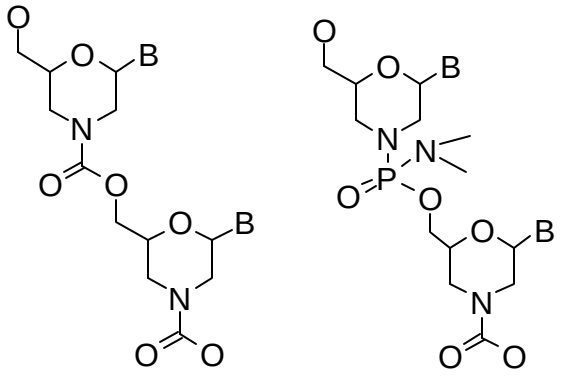

Figure 8. Morpholino derivatives

In a recent approach, conformationally locked nucleic acids (LNAs) introduced by Wengel et al[16] (Figure 8) exhibited unprecedented binding affinity towards complementary DNA and RNA. These are stable to 3' exonucleolytic degradation and showed better water solubility. The furanose ring in LNA nucleoside monomer being part of

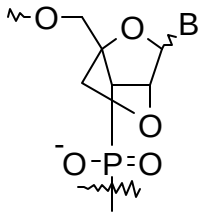

Figure 9. LNA (β-D-ribo)

dioxabicyclo[2,2,1]heptane skeleton is locked in an N-type conformation (C3' endo or ^{3}E conformation). Such a conformational or structural preorganization of the furanose ring is an important factor for the high binding affinity of these modifications.

4.2 PEPTIDE NUCLEIC ACIDS

An interestingly new class of DNA analogues in which entire phosphodiester backbone has been replaced by an amide or peptide like backbones, the Peptide Nucleic Acids (PNA) have recently attracted much attention because of its avidity and interesting DNA recognition properties.[17] PNA is homomorphous (Figure 10) with DNA and has an uncharged backbone composed of N-(2-aminoethyl) glycine units to which the nucleobases are joined via acetyl linkages. These are very potent mimics of DNA since hybrids formed between PNA and complementary DNA/RNA exhibited highest thermal stability. The high binding efficiency of PNA is attributed to the neutral nature of the

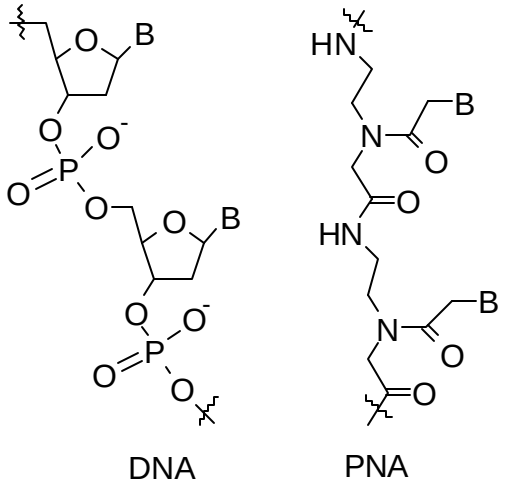

Figure 10. Structures of DNA and PNA

backbone and a conformational adaptability to bind either DNA or RNA. The unique property of PNA is binding to double stranded DNA not by triple helix formation but by strand invasion and this has important biological applications in therapeutics and diagnostics.[18] The drawbacks of PNA are poor solubility in water, poor membrane permeability and an ambiguity in orientation of binding to complementary DNA/RNA.

By convention, PNAs are depicted like peptides with N-terminus at the left and C-terminus at the right. The orientation in which the amino terminus of PNA binds to the 5' end of DNA is designated as *parallel*, while the orientation resulting from the amino terminus of PNA binding to the 3' end of the target DNA is termed *antiparallel* (Figure 11). The PNA:DNA duplex binding is a consequence of Watson-Crick base pairing by oligopurine/oligopyrimidine. PNA is known to bind DNA in both parallel and antiparallel directions. In case of PNA_2:DNA triplexes, the PNA:DNA duplex binding results from Watson-Crick pairing, while the second PNA strand binds the PNA:DNA duplex via the Hoogsteen hydrogen bonding.[19]

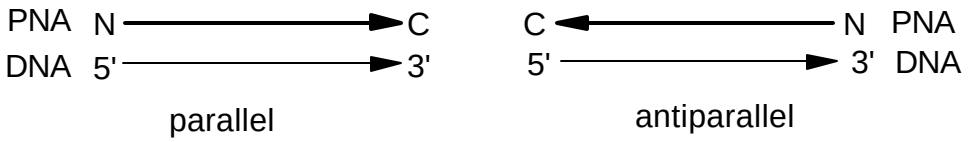

Figure 11. Parallel and antiparallel modes of binding in duplexes

4.2.1 STRUCTURE OF PNA-DNA COMPLEXES

PNA binds to ds DNA by a very unusual mechanism[17a] `strand invasion' different from the one observed with the homomorphous DNA. Homopyrimidine PNAs when targeted to double stranded DNA prefer not to form conventional PNA:DNA_2 triplexes, but

invade by `triplex invasion' (Figure 12a) displacing DNA forming a D-loop or P-loop structure.[20] In addition to base pairing the triplexes are stabilized because of the neutral

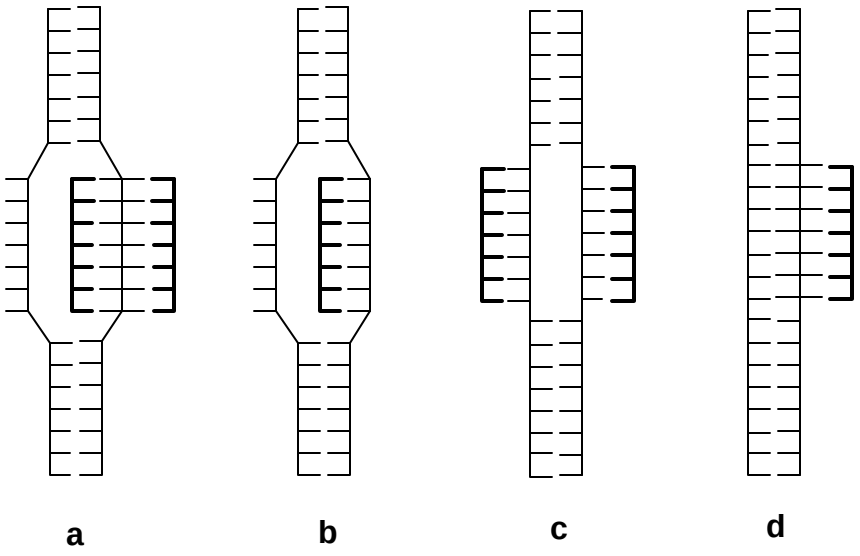

Figure 12. **a**. Triplex invasion **b**. Duplex invasion **c**. Double duplex invasion **d**. Third strand binding forming a PNA:DNA_2 complex

nature of backbone and by hydrogen bonding between each amide –NH of the backbone of Hoogsteen PNA and oxygen of the phosphate in the DNA backbone.[25] Homopurine PNA can invade a target DNA duplex (although with low efficiency) to form a PNA-DNA duplex,[21] displacing one strand of the duplex DNA by 'invasion' (Figure 12b). Pseudocomplementary PNAs invade the target duplex DNA by `double duplex invasion' (Figure 12c) forming two PNA-DNA duplexes where, each PNA strand pairs with its complementary DNA strand by Watson-Crick hydrogen bonding.[22] Cytosine-rich homopyrimidine PNAs bind via Hoogsteen hydrogen bonds to duplex DNA having a complementary sequence, as a third strand (Figure 12d) to form PNA_2:DNA triplexes.[23] The complexes formed by PNA with DNA or RNA are thermally more stable than the

corresponding DNA-DNA or DNA-RNA complexes. For different PNA hybrids, the thermal stability follows the order PNA:PNA > PNA:RNA > PNA:DNA (> RNA:DNA >DNA:DNA).

The structure of PNA:RNA complex determined by NMR methods[24] revealed that the hexameric GAACTC PNA formed a 1:1 antiparallel complex with RNA, that is a right handed double helix held by Watson-Crick base pairing, similar to the A-form of RNA duplexes. It was also shown that the achiral PNA backbone assumed a distinct conformation upon binding to RNA that differed from the previously proposed models.

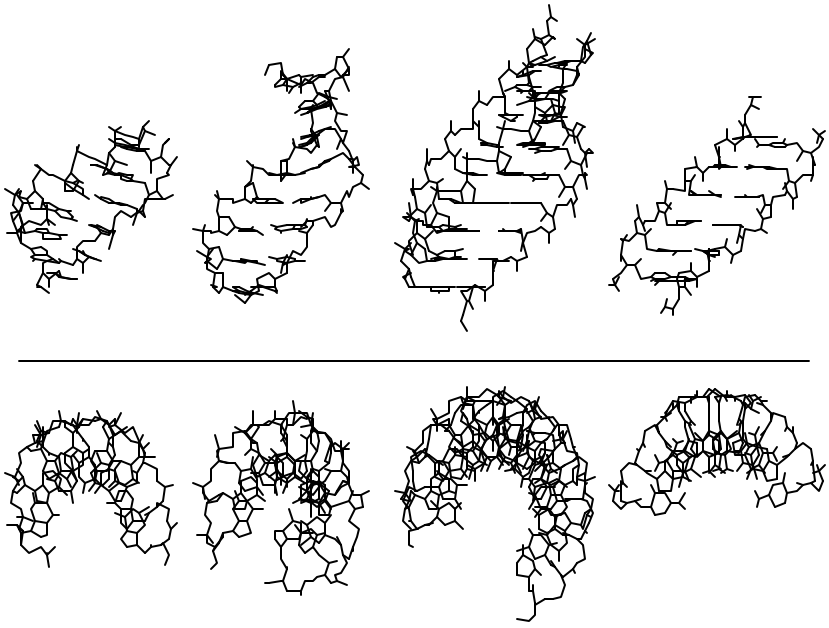

Figure 13. Structures of PNA complexes shown in (a) side view (b) top view. The complexes from left to right PNA-RNA, PNA-DNA duplex, PNA-DNA-PNA and PNA-PNA[30]

The crystal structure of PNA_2:DNA triple helix,[25] designated the P-form, revealed that the hybrid consists of one polypurine DNA strand complexed to a polypyrimidine hairpin PNA. Bis- or hairpin PNAs have been synthesized to promote triplex formation by

tethering two polypyrimidine strands with flexible linkers and were shown to have higher affinity for single stranded DNA and a higher rate of strand invasion for ds DNA relative to single stranded PNA.[17c,26] The structure solved using a PNA with an iodinated U base indicated a P-form that has helical parameters distinct from either A- or B-form DNA. The P-form helix has a large cavity along the helix axis with the base displacement of 6.8Å compared to 4.5Å for A-form DNA. The deoxyribose sugars have C3'-*endo* conformation with an average interphosphate distance of 6Å similar to the A-form DNA. This structure is consistent with the fact that PNAs bind more tightly to RNA than to DNA. The three dimensional structure of an octamer PNA-DNA duplex was solved by NMR methods.[27] A hexamer PNA-PNA duplex structure[28] derived from X-ray crystallography showed that the PNA adopts a helical conformation that is different from the other nucleic acid helices. PNA:PNA duplex structure showed a very wide helix (28Å) with almost twice the pitch (18 base pairs per turn) of an A- or B- form helix (10-11 base pairs per turn).

Circular and linear dichroism studies have indicated that PNA_2-DNA triplexes are very similar to the conventional DNA tiplexes in forming right-handed helix.[29] From the reported structures it is evident that PNA is flexible enough to adapt well to its nucleic acid partner. In the PNA-RNA and PNA-DNA duplexes, the digonucleotide (DNA/RNA) closely adapts to its natural 'A and B' conformations respectively in terms of sugar puckering while the helix parameters have both 'A' and 'B' form (Figure 13) characteristics.[30]

4.3. BIOLOGICAL APPLICATIONS OF PNA

Synthetic molecules that can bind with high sequence specificity to a chosen target in a gene sequence are of major interest in medicinal and biological applications. In this context, PNA has attracted wide attention due to its interesting DNA/RNA recognition features as described above. In addition, the ease of synthesis of PNA monomers using

Boc as well as Fmoc protection strategies followed by solid phase peptide synthesis of the oligomers make PNA ideal for further development as a drug. It is chemically stable and resistant to hydrolytic cleavage[31] and thus not expected to be degraded inside a living cell. The high sequence specificity in DNA recognition and the strand invasion[32] mode of binding to duplex homopurine sequences with a looped-out DNA strand make it attractive for various biological applications.

4.3.1. Transcription arrest

Strand displacement mode of binding of PNA to the dsDNA has been exploited for the transcription arrest.[33] PNA bound to dsDNA target was positioned downstream from phage T3 or T7 promoters in pBluescript KS^+ derived plasmids. RNA polymerases T3 or T7 have been used for the transcription. The strand displacement complex formed between PNA T_{10} and dsDNA A_{10} showed transcription elongation arrest at the target site with PNA bound to the template strand and marginal effect with PNA bound to the non-template strand. With PNA T_{10}, transcription arrest occurred at the first base of the PNA-binding site, while the arrest with PNA T_5CT_4 was 2-3 nucleotides inside the binding site, and with PNA $T_2CT_2CT_4$ (Figure 14) the arrest was less efficient. $(PNA)_2$/DNA.DNA strand displacement 'P-loops' were shown to efficiently block transcription elongation by T_3 RNA polymerase.[34] These loops can function as artificial strong promoters for E.coli RNA polymerase.[35]

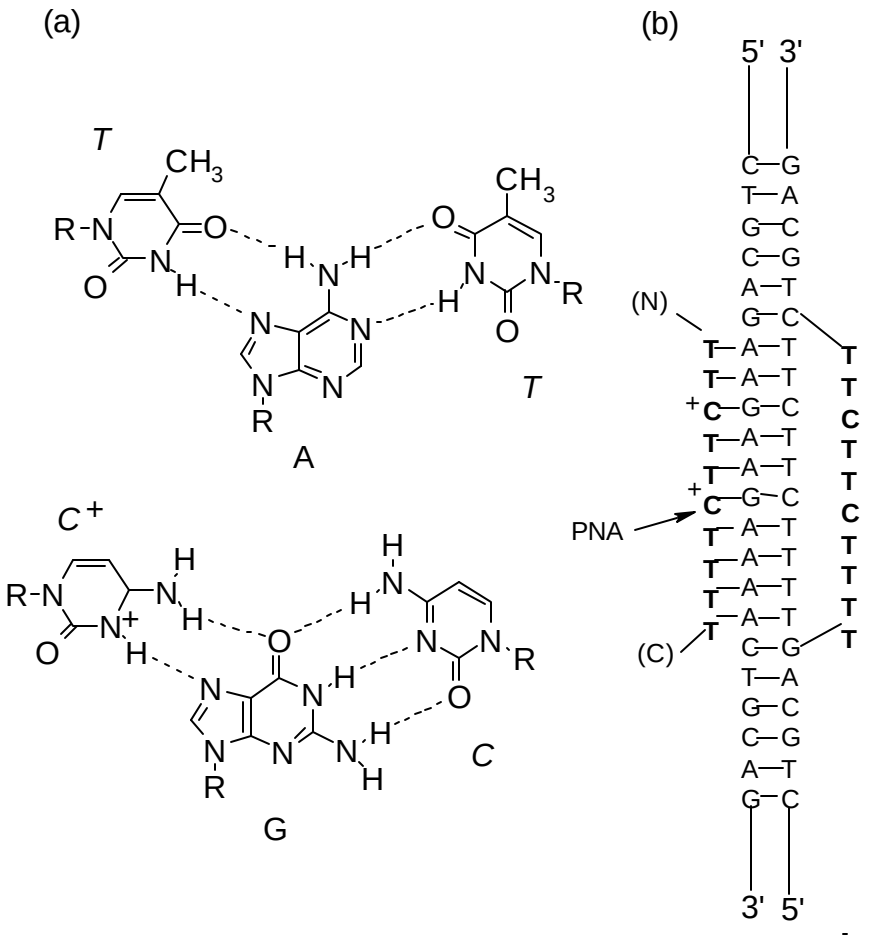

Figure 14. Transcription Elongation Arrest (**a**) Watson-Crick,Hoogsteen type triplex (two PNA strands involved in complex are shown in italics) (**b**) PNA:dsDNA complex and two triplets involved

4.3.2. PNAs as artificial transcription promoters

Strand displacement mode of binding of PNAs has tremendous application as already discussed earlier. RNA polymerase recognizes the D-loops (Figure 15) (formed from PNA when bound to complementary dsDNA by strand displacement) and initiates RNA transcription, at an efficiency comparable to that of *E.coli* lacUV5 promoter. Thus PNA targets can be considered as artificial promoters controlled positively by the

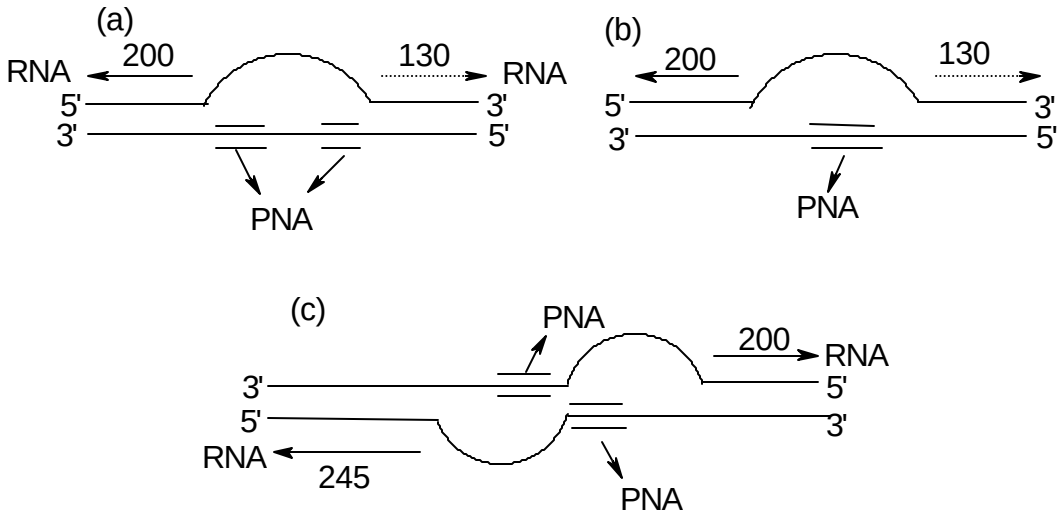

Figure 15. Transcription initiation from PNA:DNA strand displacement loops
Schematic representation of in vitro transcription from purified DNA fragments containing a single **(a)** or a double [two sites in cis] **(b)** or two sites in trans **(c)** PNA target showing the displacement loops and the start and possible directions of RNA synthesis indicated by arrows (full line:observed)

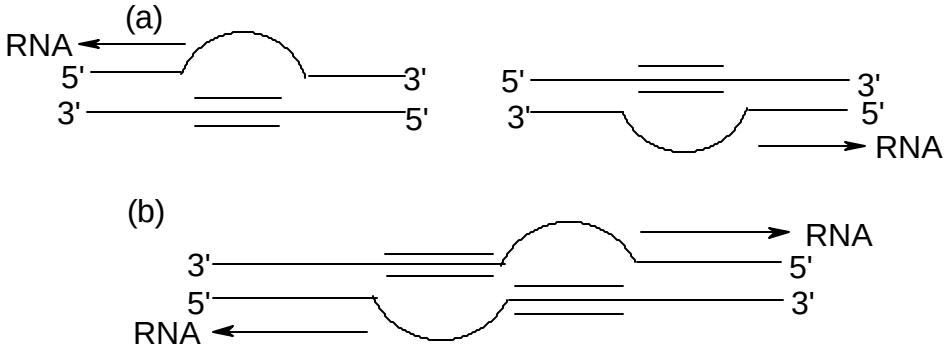

Figure 16. RNA transcription initiation sites by reverse transcriptase primer extension showing the transcribed RNA from the PNA promoters. Schematic representation of the Plasmid DNA restriction fragment containing (**a**) PNA T10 traget on either DNA strand or (**b**) two PNA T5CT4 targets on opposite DNA strands

corresponding PNA as a transcription factor.[35] When two adjacent PNA sites are present on the same DNA strand or on opposite DNA strands, the structures of the type shown in

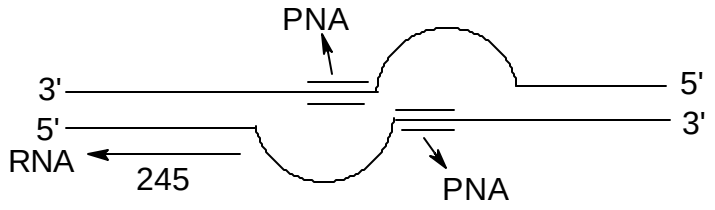

Figure 17. PNA induced transcription in eukaryotic system using extracts from rat spleen cells shows RNA transcription in only one direction

(Figure 16) are formed. Experiments in eukaryotic systems using nuclear extracts from rat spleen cells. (Figure 17) showed specific transcription initiation at the PNA loop.[35]

4.3.3. PNA AND PCR[36]

The high affinity and specificity of PNA for their complementary DNA has been explored in several PCR based applications. Since PNA cannot function as a primer for DNA polymerases, PNA/DNA complex can effectively block the formation of PCR product when the PNA is targeted against one of the PCR primer sites. This blockage allows selective amplification/suppression of target sequences that differ by only one base pair. When the PNA and PCR primer target sites overlap, clamping operates by 'primer exclusion'. When the target site is located at a distance from the PCR primer sites, clamping prevents read-through by the Taq polymerase ('elongation arrest'). Finally when the PNA target is located adjacent to the PCR primer site, clamping is by either mode, preventing polymerase access to the PCR primer and/or by preventing initiation of primer elongation. Clamping with both mix sequence PNA and homopyrimidine PNA showed that

PNA directed PCR clamping is efficient for the detection of even single base mutations which has wide applications.

PNAs were shown to serve as primers[37] for certain DNA polymerases eg. Klenow fragment of DNA polymerase I (*E coli*) and reverse transcriptases, even though they have no phosphate residues to interact with polymerase, which were presumed to be necessary for binding via highly conserved amino acid residues. When PNA carrying a 5'-amino-5'deoxythymidine at the carboxyl terminal end was used as the primer, there was no elongation of PNA primer to yield a PNA-DNA chimera, in cases of phage T4, phage T7 exo (Sequenase 2.0), Thermus aquaticus and Deep Vent exo DNA polymerases, as well as HIV-1 reverse transcriptase. It was also found that the elongation of PNA primer was less efficient for the T^{th} Polymerase (Thermus theromophilus) and the reverse transcriptases from avian myeloblastosis virus (AMV) and moloney murine leukemia virus (M-MuLV).[37,38]

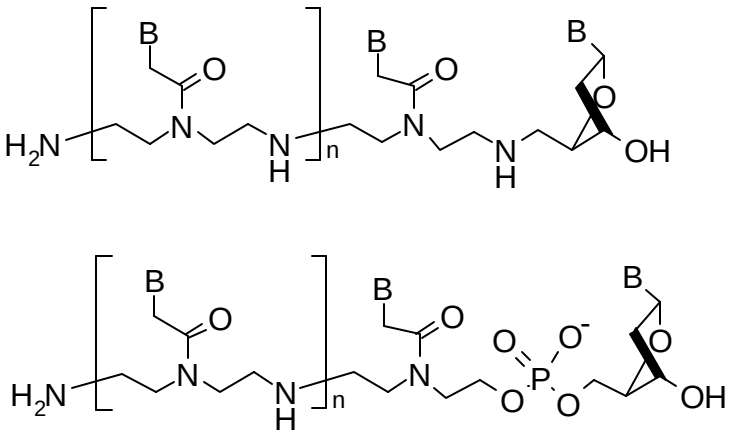

Figure 18. PNA primers

4.3.4. PNA conjugates as artificial restriction enzymes

The strand displacement complexes of PNA have been used for the sequence specific cleavage of double stranded DNA at desired positions. PNA in combination with

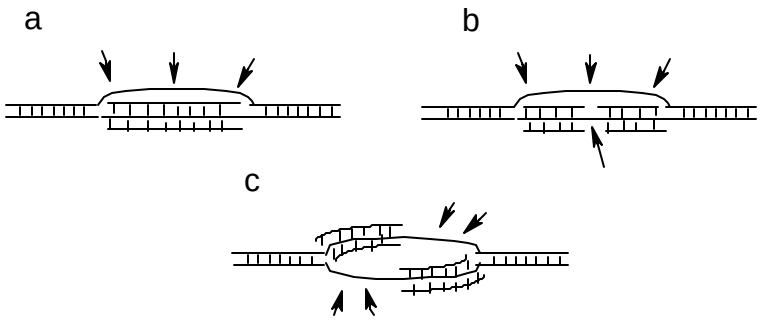

Figure 19. Schematic Representation of PNA-targeted S1 nuclease ds cleavage of DNA **a.** single target **b**. double target in *cis* orientation, **c.** double target in *trans* orientation. Arrows indicate S1 attack

non-specific nucleases such as nuclease S1 have been used as 'artificial restriction enzymes[39] and the cleavage occurs at the displacement site created by PNA. Depending on the strand to be cleaved, the PNA can be selected. The efficiency of this cleavage is more than 10 fold enhanced when a tandem PNA site is targeted and the site is trans oriented. Thus, PNA targeting makes the single strand specific nuclease S1 behave like a pseudorestriction endonuclease. Tethering a metal binding ligand such as Gly-Gly-His tripeptide to bis-PNA has been used to probe the structure of the DNA. Gly-Gly-His tripeptide placed on either the Watson- Crick or Hoogsteen bis-PNA strand forms a nickel complex that mediates cleavage at specific sites on the proximal displaced and hybridized DNA strands.[40]

4.3.5. Inhibition of human telomerase

Human telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the ends of chromosomes known as telomeres.[41] Human telomerase consists of a catalytic protein subunit the telomerase- reverse transcriptase component (hTERT), one or more additional proteins and an integral RNA component (hTR) that serves as a template for the

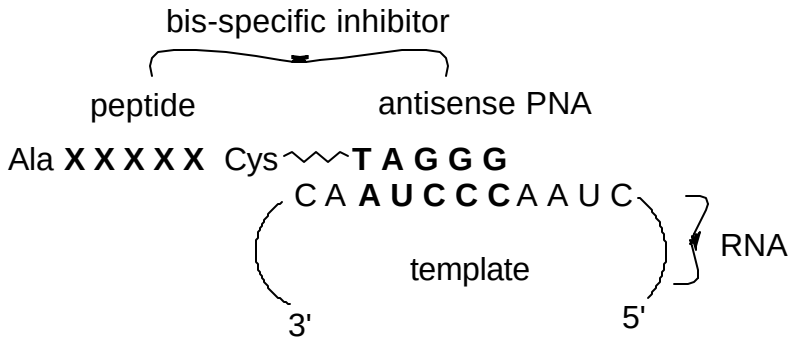

Figure 20. Design of PNA-peptide conjugates for inhibition of human telomerase

synthesis of telomeric repeats.[42] The high telomerase activity found in tumor cells has aroused interest in its use as a potential target for anticancer chemotherapy.[43] Inhibition of telomerase activity by conventional DNA oligomers and phosphorothioates showed poor sequence selectivity of these compounds.[44] *In vitro* studies by Corey *et al*[45] using Telomere Repeat Amplification Protocol (TRAP) showed that PNA can inhibit the telomerase activity by binding to RNA component of enzyme in picomolar to nanomolar range and the inhibition is due to sequence-selective PNA-mediated inhibition of telomerase activity. In another approach PNAs were introduced into the cells by transfection using cationic lipids[46] (lipofection). These PNAs were directed to non-template

regions of the telomerase RNA that can overcome RNA secondary structure and inhibit telomerase by intercepting the RNA component prior to the holoenzyme assembly. The presence of cationic peptides at the N-terminus of the PNA resulted in enhanced inhibition of telomerase activity when targeted to the RNA template.[47]

In addition to these applications, PNAs have been exploited for plasmid labeling[48] and duplex DNA capture,[49] PNAs composed of *trans*-4-hydroxy-L-proline based monomers and phosphono derivatives were used to isolate mRNA free of genomic DNA.[50]

4.4. CHEMICAL MODIFICATIONS OF PNA

Inspite of the dramatic deviations from the phosphodiester backbone of DNA, PNA has emerged as a very good mimic of DNA. However, it does suffer from several limitations such as low aqueous solubility, poor cellular uptake and ambiguity in DNA binding orientation. To address these problems and to understand the structure-activity relationship, as well as to obtain the PNA oligomers with improved properties for various applications in therapeutics and molecular biology, several modifications have been introduced into the classical PNA monomer.

The earliest and simplest modifications involved extension of the PNA structure with a methylene group (Figure 21) into aminoethyl,[51] glycyl[52] or in the base linker unit[52] of the PNA monomer. However, these modifications resulted in less stable PNA:DNA hybrids

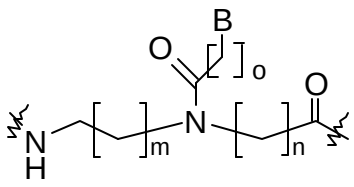

Figure 21. Extension of PNA structure by methylene groups

suggesting the importance of structural preorganization of the classical PNA monomer to interact with DNA.

Aqueous solubility was improved by introducing charges in the PNA monomers or by introducing ether linkages in the backbone. Positive charges were incorporated into the PNA by replacing the acetamide linker with a flexible ethylene linker[53] (Figure 22a) or by the attachment of terminal lysine residues[54] (Figure 22b). Recently, a novel class of

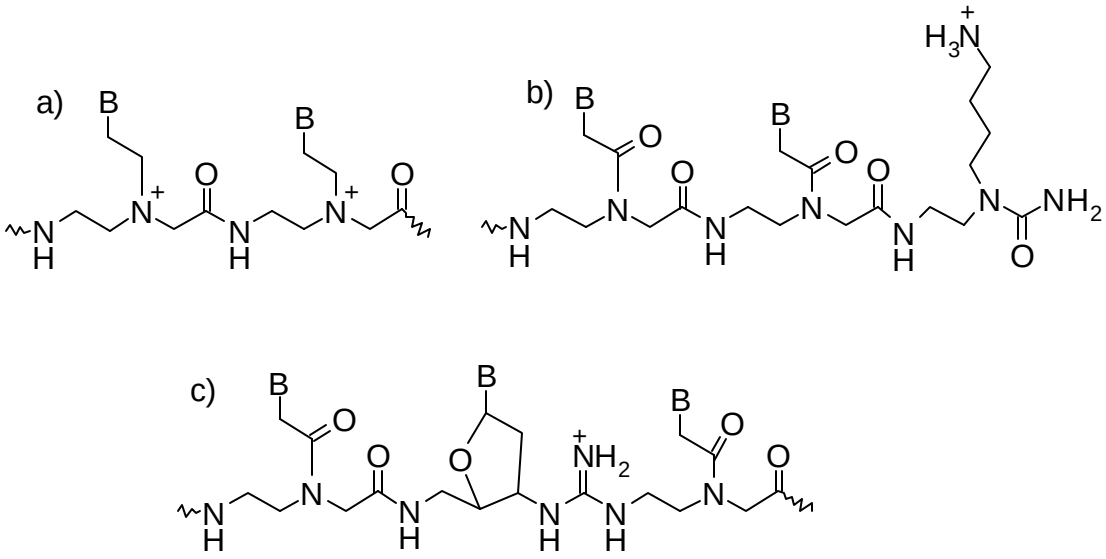

Figure 22. Positively charged PNAs **a** felxible ethylene linker, **b** with lysine residues, **c** guanidinum linkages

cationic PNA (Figure 22c) (DNG/PNA) analogs has been reported.[55] In these alternating PNA /DNG chimeras, the O-(PO_2)-O- linkage of nucleotide was replaced by strongly cationic guanidino [N-C(=N^+H)-N] function. These analogs with neutral and positive linker showed high binding affinity with DNA/RNA targets. Introduction of negative charges in the PNA backbone (Figure 23) improved aqueous solubility and showed good binding with both DNA and RNA. However, these modified complexes were found to be less stable

compared to the unmodified PNA sequences.[56] In a similar approach pPNA-Hyp chimeras were also synthesized.[57]

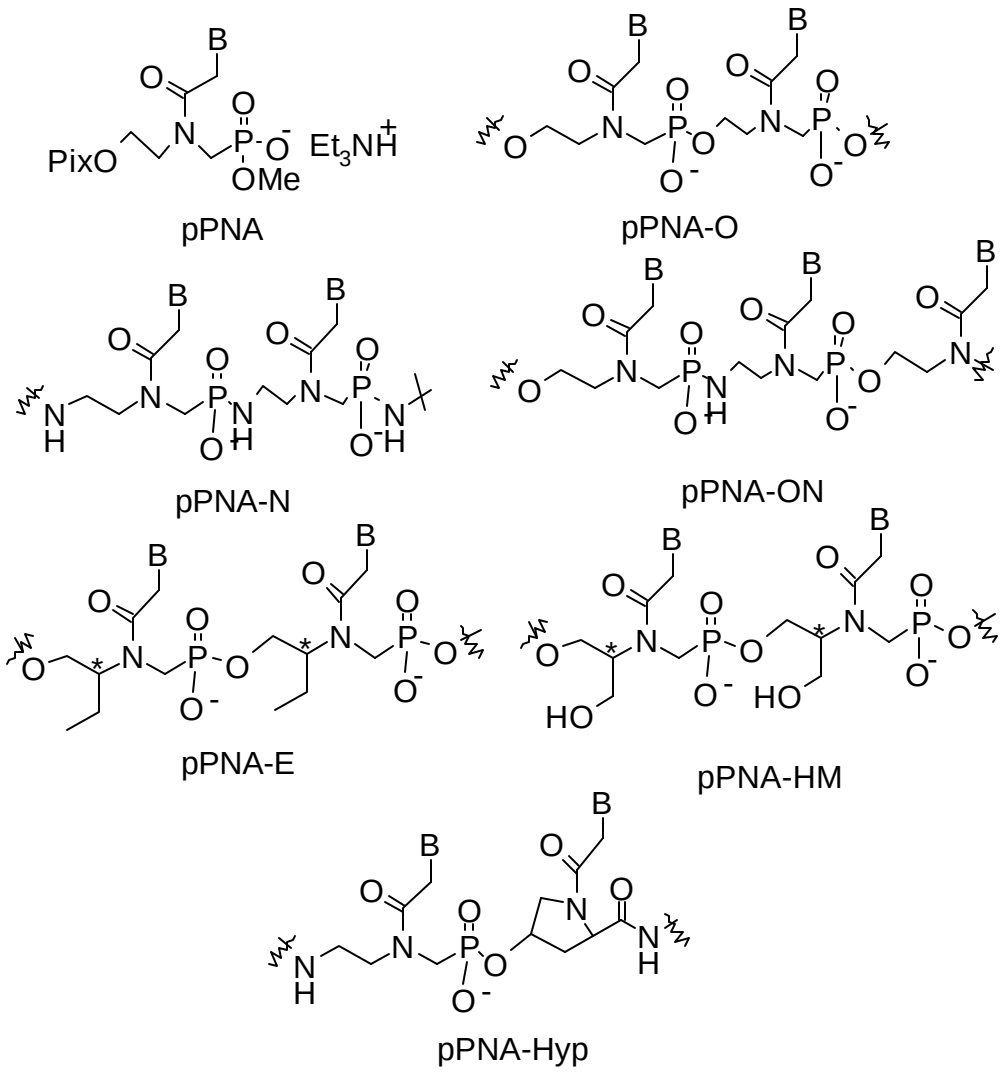

Figure 23. Anionic PNAs

Ether linked PNAs (Figure 24) showed co-operative binding with complementary antiparallel RNA in a sequence specific manner.[58] Oligomers with 24b showed significantly lower affinity than 24a due to the increased flexibility of the side chain homologation. The

replacement of these monomers with α-methylated derivatives led to significant enhancement in RNA binding affinity in case of 2*R* stereoisomer. Whereas, presence of 2*S* isomer resulted in substantial decrease in Tm indicating that the α substitution in case of *S* configuration may sterically interfere with RNA binding. To ensure sufficient water solubility

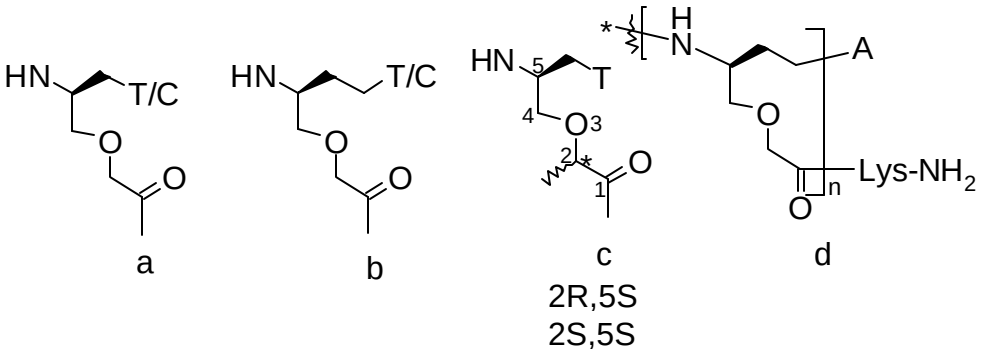

Figure 24. Ether linked (oxy) PNA

for RNA binding experiments, lysine was attached to the N/C terminus of these oligomers.[59] This was followed by another similar report using oxy-PNA oligomers bearing adenine as nucleobase. Binding studies of these oligomers with complementary DNA showed all-or-none type hybridization and with high sequence specificity useful for the detection of single base mismatch DNAs.[60]

Five membered rings have been introduced into the PNA monomers. One of the relatively successful modifications is PNA derived from proline systems which imparted conformational constrain and chirality to the backbone.[61] While PNAs with a single aminoproline unit at the N-terminus of a PNA chain not only enhanced the inherent binding and showed significant discrimination in the orientation of binding.[62] Homooligomers of 4-amino proline backbone failed to show co-operative binding with complementary DNA. Incorporation of 4-aminoprolyl units[63] with L-*trans*, D-*trans* and L-*cis* isomers in

combination with aminoethyl glycine units led to stronger binding properties of L-*trans* isomer compared to unmodified PNA. In contrast to this, the oligomers containing D-*trans* and L-*cis* isomer resulted in a large destabilization of the hybrids.

Conformationally constrained glycyl proline backbones[62a,64] (Figure 25b) showed negative results probably due to the rigidity in the structure arising from the tertiary amide group that is part of the backbone. Interestingly, replacement of the tertiary amide carbonyl on the backbone with a methylene group lead to the generation of N-(2-aminoethyl prolyl (*aep*) PNA[65] and these oligomers showed excellent properties in terms of hybridization and high stability. This modification also improved aqueous solubility and perhaps the most promising modifications to date.

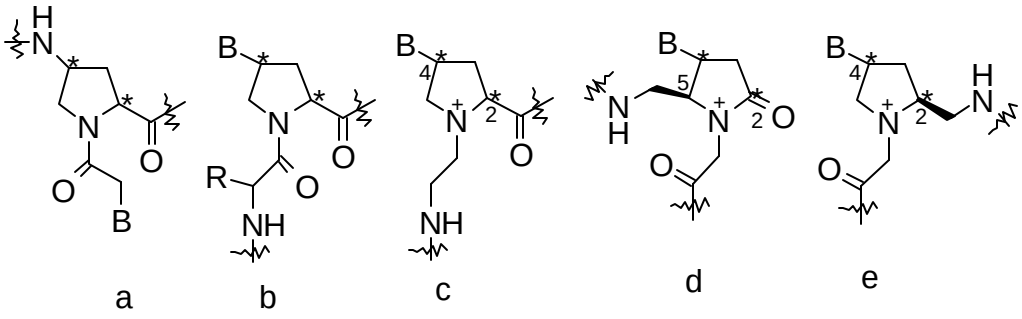

Figure 25. (a) 4-amino (b-e) prolyl/pyrrolidyl PNAs

In a similar approach, synthesis of the monomers with flexible aminoethyl linker (Figure 25c) and the prolyl system with 2*R*,4*R* stereochemistry were reported. This deoxy analog of glycyl prolyl amide[66] oligomers showed good solubility and strong interactions with RNA but not with DNA. Puschl *et al*[67] introduced conformationally restricted 2*R*,4*S* pyrrolidyl PNA monomers bearing adenine. The homooligomers of these positively charged monomers displayed an improved binding affinity to complementary DNA/RNA

oligonucleotides, compared to the parent *aeg* PNA A_{10} whereas the oligomer with single modification showed destabilization. The 2*S*,4*S* pyrrolidyl PNA chimeras destabilized when incorporated at the C-terminus and showed no complexation when present in the middle.[68]

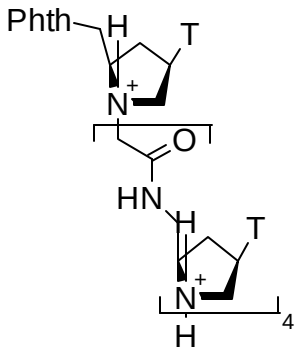

Figure 26. Pyrrolidine amide oligonucleotide mimic

Hickman *et al* [69] reported the synthesis of a pentameric thymidyl pyrrolidine-amide oligonucleotide mimic (POM) (Figure 26) which showed high binding affinity with single stranded RNA and DNA exhibiting kinetic binding selectivity for RNA over DNA.

Cyclohexyl rings have been introduced into the PNA backbone (Figure 27) to get the 1,2-cyclohexylamino[70] and spirocyclohexyl rings[71] in the PNA monomer. PNAs that

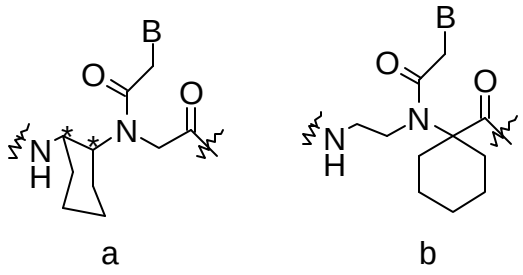

Figure 27. PNA with six membered rings in the backbone

bear (S, S) cyclohexyl rings in the aminoethyl part showed similar hybridization properties as unmodified DNA where as the (*R*, *R*) cyclohexyl moiety lacked such a property.

Novel glucosamine based oligonucleotide analogs (GNA) (Figure 28) derived from conformationally constrained sugar scaffolds have been synthesized.[72] GNA derived oligomers were highly water-soluble. In contrast to the homo DNA and hexose oligonucleotides, these bind to RNA to form stable complexes with an overall affinity comparable to that of DNA to RNA and are sequence selective. Thermodynamic parameters suggested an entropy gain in GNA due to the pre-organized scaffold.

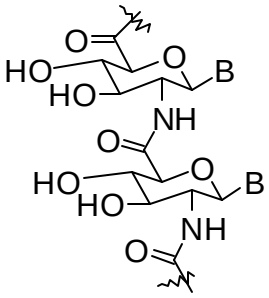

Figure 28. GNA

In other attempts, interchange of various CO and NH groups on the peptide linkages lead to retero inverse[73] peptoid[74] and heterodimeric[75] analogs (Figure 29) which

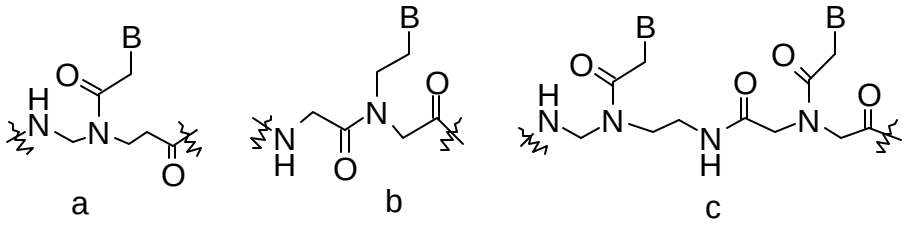

Figure 29. (**a**) Retero inverso (**b**) Peptioid (**c**) Heterodimeric

have interbase/residue separations similar to the unmodified PNA, but accompanied by an inversion of intra and inter residue amide bonds. Except for the heterodimer analog (Figure 29c) the other two exhibited a lower potency for duplex formation with complementary DNA/RNA suggesting that in addition to geometric factors, other subtle requirements such as hydration and dipole-dipole interactions that influence the microenvironment of the backbone may have an important role in efficient hybridization.

Recently, it was reported that the introduction of high chiral constraint in the middle of a PNA sequence strongly affects the direction selectivity, i.e. the antiparallel/parallel preference in DNA complexation.[76] Chiral PNA decamer GTAG**ATC**ACT bearing three D-Lys-based monomers a "D-chiral box" favored highly specific antiparallel DNA binding but failed with parallel DNA.

4.4.1. Modified nucleobases

Incorporation of non-natural nucleobases (Figure 30) would help in understanding the recognition process between natural nucleobases in terms of various factors such as hydrogen bonding and inter-nucleobase stacking. Further, new recognition motifs may also have potential applications in diagnostics.

2-aminopurine[77] hydrogen bonds with U and T in reverse Watson-Crick mode and being inherently fluorescent, it enables the study of the kinetics of PNA-DNA hybridization process. 2,6-Diaminopurine has increased affinity and selectivity for thymine.[78] Pseudo-isocytosine[79] is an efficient mimic of protonated cytosine for triplex formation. E-base[80] was designed for the recognition of T:A base pair in major groove and forms a stable triad with T in central position. Other modifications like hypoxanthine[81] N4-benzoylcytosine[82] and thiouracil[83] have also been incorporated as modified nucleobases. Combination of thiouracil in PNA chain and 2,6-diaminopurine in DNA has been used in the "double duplex

invasion" for the first time. 6-Thioguanine[84] was found to decrease PNA-DNA hybrid stability.

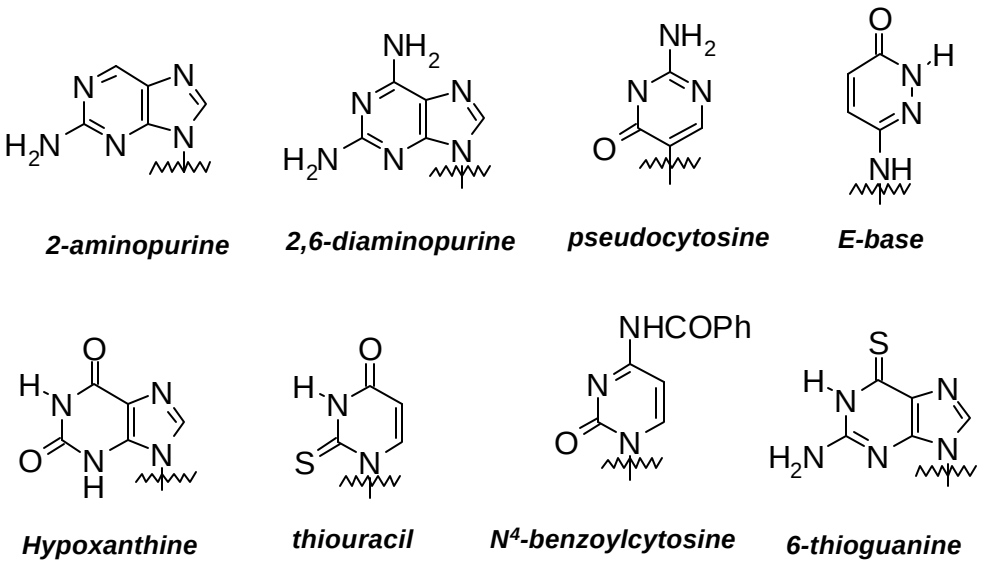

Figure 30. Modified Nucleobases

4.5. PNA CONJUGATES

The covalent linking of PNA hybrids to various other molecules like peptides, DNA, RNA etc. has been exploited to overcome the limitations of PNA such as aggregation, solubility, cellular uptake and resistance to cellular enzymes and to impart better therapeutic applications.

4.5.1. PNA-DNA Chimeras[85]

Despite several interesting features of PNA, it has some drawbacks like poor aqueous solubility[86] and the enzyme RNAse H that cleaves RNA in DNA-RNA complexes does not recognize PNA-RNA duplexes. In order to circumvent these drawbacks and to

improve bioavailability and reduce self-aggregation, several PNA-DNA chimeras (Figure 31) have been synthesized.[87] PNA-DNA chimeras bind with higher affinity to RNA than to

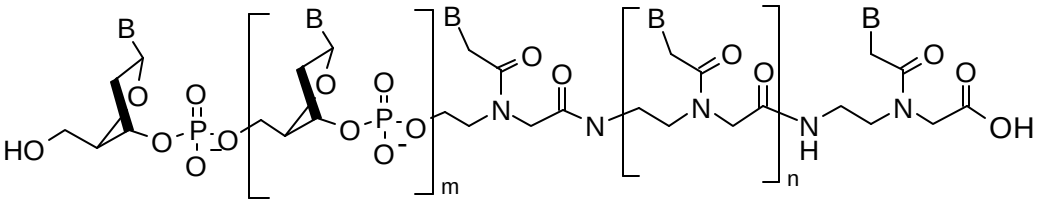

Figure 31. 5'-DNA-3'-PNA

DNA and are used for the cleavage of target RNA by RNAse H on formation of a RNA chimera duplex.[88] The conjugation of PNA to the 5' end of DNA lead to the PNA-(5')DNA chimeras,[89] while conjugation to the 3' end lead to the PNA-(3')DNA chimeras.[90,91] Attaching PNA to the 3' end has the advantage of imparting stability to DNA towards the common 3'-exonucleases.[92] These complexes also improved the aqueous solubility and cellular uptake and were found to have reduced aggregation. However, the thermal stability of these complexes was found to be low, compared to the complexes with PNA. PNA-DNA chimeras were also synthesized from 4-hydroxyproline based linkers[93] amongst which L-*trans* derived chimeras were found to be promising. Chimeras synthesized using cytosine by 5-methylcytosine in DNA-PNA chimera increased duplex stability while 5-bromouracil in place of thymine maintained it. In contrast to DNA, the PNA-DNA chimera as third strand destabilized the derived triple helix.[94]

4.5.2. PNA-Oligopeptide Chimera

The conjugation of PNA with peptides containing basic amino acids like lysine or arginine resulted in an increase in solubility as well as formation of stable complexes with

DNA[95] due to the interaction of the positively charged amino acids of the peptide with the negative anionic phosphate groups of DNA. A PNA-peptide chimera involving linking a 10-mer oligopeptide containing serine, which is a substrate for protein kinase A was used to assay phosphorylation of serine by kinase.[96] The 5-mer PNA sequence H_2N-TAGGG-COOH linked to the N-terminus of various homooligomeric peptides of cationic amino acids lysine, ornithine and arginine are shown to inhibit human telomerase as discussed in section 4.5.7.[97]

4.5.3. PNA-Liposome Chimera

PNAs conjugated with lipophilic groups when introduced into liposomes were found to improve cellular uptake and distribution due to the introduction of amino side chain in the PNA backbone.[98]

4.6. PRESENT WORK

The above sections describe the current literature on Peptide Nucleic Acids with reference to structural variations and biological applications. The strand invasion property along with its high affinity and specificity to complementary DNA/RNA has prompted it as a useful tool in therapeutics and biology. However, due to limitations like poor aqueous solubility, self-aggregation, poor cellular uptake and ambiguity in binding orientation has limited further exploitation of PNA in practical applications. In order to circumvent these problems further modifications and the synthesis of newer PNAs to improve their properties, continue to elicit interest.[99]

In this connection, **Chapter 5** describes the design, synthesis and biophysical studies of a novel conformationally constrained neutral and chiral PNA analog, christened the aminomethylprolyl PNA (*amp*-PNA). The corresponding monomers are derived by

bridging the α-carbon of amino ethyl part to the α' carbon of glycine part by an ethylene bridge to get a new class of hitherto unknown pyrrolidyl polyamide nucleic acids. Nucleobases are attached to the central nitrogen atom by an acetamide linkage. These chiral and neutral monomers are synthesized from commercially available D/L proline and suitably protected monomers without the nucleobase are incorporated into the oligomer by solid phase synthesis.

The nucleobases were attached by 'submonomer' coupling, using corresponding nucleobase acetic acids on solid phase. Preliminary studies on the binding affinity of various *amp*-PNA oligomers to the complementary DNA are reported using UV and CD spectroscopic studies.

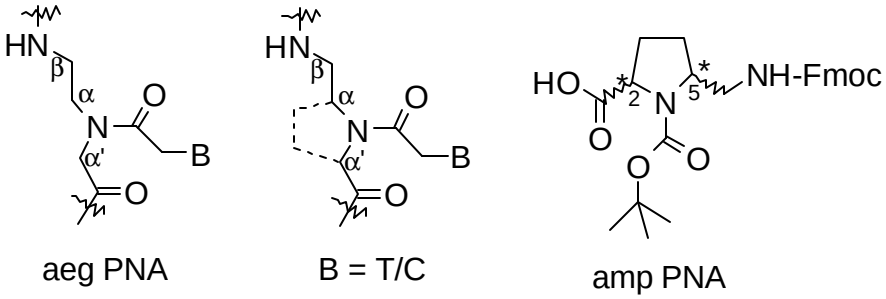

Figure 32. 5-aminomethyl prolyl PNA

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CHAPTER 5

SYNTHESIS AND BIOPHYSICAL STUDIES OF CONFORMATIONALLY CONSTRAINED PNA ANALOGS

5.1. INTRODUCTION

As described in Chapter **4** Peptide Nucleic Acid[1] has emerged as a very potent DNA mimic. PNA has attracted wide attention because of its improved binding properties. PNA is neutral, achiral and can be synthesized easily by solid phase

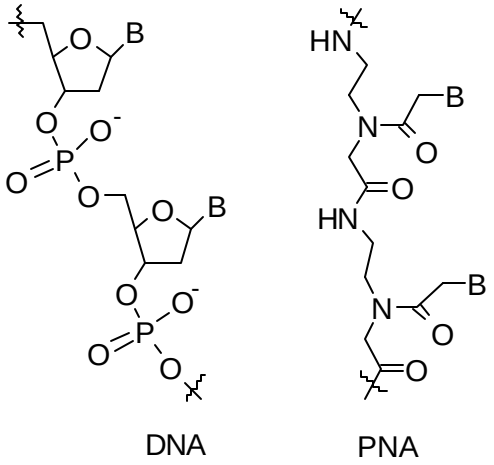


Figure 1: Structure of DNA and PNA

peptide synthesis protocols. In PNA, the inter nucleobase distances are closely similar to that in DNA allowing it to bind to the target DNA/RNA with high sequence specificity and affinity. A combination of these properties that are not found in other modified oligonucleotides, make PNA a useful tool in antisense technology as well as other areas such as diagnostics. To address the limitations of PNA for biological applications several modifications have been introduced into the classical PNA monomer[2] and these modifications resulted in only marginal effects in terms of hybridization properties. These studies have pointed out the importance of rigidity and pre organization of PNA for effective complexation with complementary DNA. From this laboratory there have been reports based on the introduction of five membered pyrrolidine ring to introduce chirality as well as conformational constrain into the PNA backbone[3]resulting in both neutral and positively charged monomers.

5.2. RATIONALE AND OBJECTIVES OF THE PRESENT WORK

Present work is directed towards the introduction of chirality and conformational constrain in the classical PNA backbone to control the orientation selectivity and specificity in binding by preorganization. This can be achieved in several ways as shown in Figure 2 to get the neutral and positively charged monomers. Bridging the β-carbon of ethylene diamine unit and α"-C of glycine unit with a methylene group to get

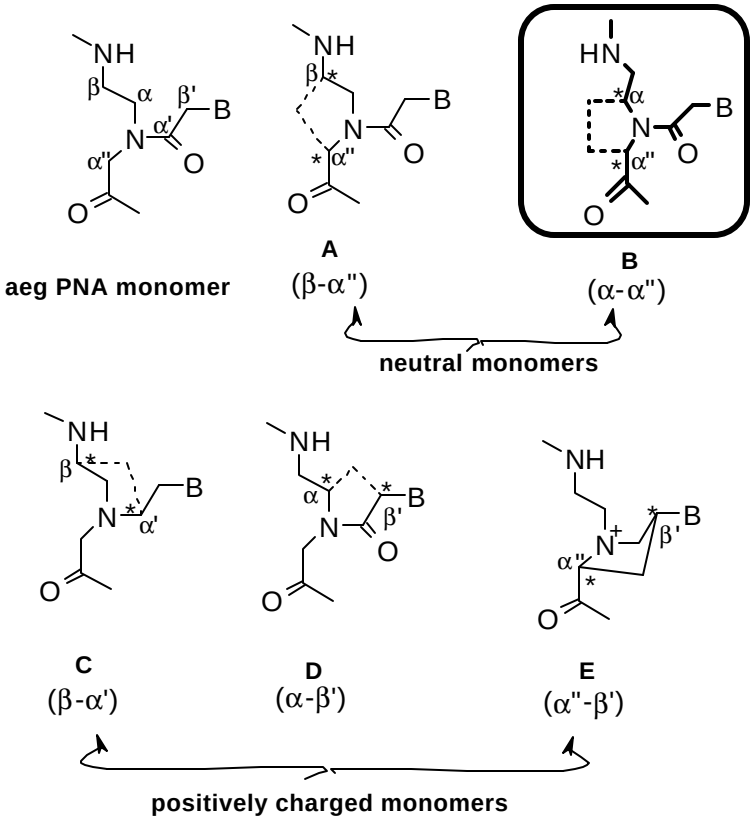


Figure 2. Strategies for conformational constrained PNA

the monomers of type **A** or bridging the α carbon of the aminoethyl unit and α" of the glycine unit by an ethylene group to get the monomers of type **B** (present work of this chapter), bridging the β carbon and α' -carbon on side chain to which nucleobase is

connected to get the monomer **C**, bridging of α carbon with the β' to get monomers of type **D** and bridging α" with the β' carbon to get the monomer **E** (Figure 2). This kind of modifications involves the introduction of rigidity to the flexible aminoethyl glycyl backbone and/or nucleobase side chain, simultaneously introducing chirality in the molecule with the generation of two asymmetric centers. **A**/**B** approach creates two asymmetric centers in the backbone whereas **C**, **D** and **E** introduce one asymmetric center in the backbone and one asymmetric center in the side chain leading to neutral (**A**/**B**) as well as positively charged (**C**, **D** and **E**) monomers. The attractive feature of this approach is that they all can be easily synthesized from a common starting material *trans*-4-hydroxy-L-proline/L/D proline. The present work is directed towards the synthesis of monomers by approach **B**.

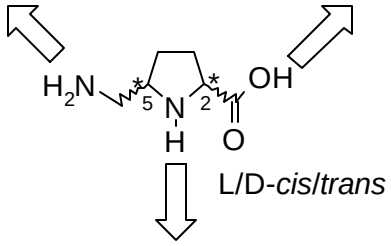


Figure 3

The objectives of this chapter are

a) Functionalization of C5 of the proline ring by electrochemical anodic oxidation to get a methoxy function that can be subsequently used for the conversion to the *aminomethyl* group, which is a part of the aminoethyl segment of the *aeg* PNA backbone (Figure 3).

b) Synthesis of N^{α}-(*tert*-butoxycarbonyl)-fluorenylmethoxycarbonyl-aminomethyl-L/D-proline isomers for the synthesis of the PNA oligomers starting from L/D-Proline.

c) Synthesis of the PNA oligomers using the aminoethyl glycyl (*aeg*) monomers and the modified *amp* PNA monomers by Merrifield Solid Phase Peptide Synthesis (SPPS) protocol.

d) Attaching the nucleobase on the solid support using sub-monomer strategy.

e) Cleavage of the oligomers from the solid support, purification and characterization of the modified PNA oliogmers.

f) Biophysical studies of the synthesized PNAs using UV-spectroscopy and CD Spectrophotometry.

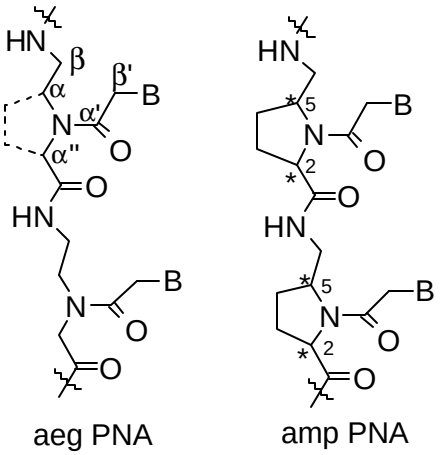


Figure 4

5.3. RESULTS AND DISCUSSION

5.3.1. General Strategy

The desired aminomethylprolyl (*amp*) monomers were synthesized from the commercially available L/D proline. The additional functionality at the C5-position of the proline ring was introduced by anodic oxidation. The nucleobases thymine and cytosine

were coupled to the ring nitrogen on the solid support using submonomer strategy by converting to their N1-acetic acids. During the solid phase assembly thymine does not require any protection of groups on the ring whereas the exocyclic amino group of cytosine requires protection as benzyloxycarbonyl (*Z*) group. Thymine and protected cytosine were esterified as their N-ethyl esters followed by hydrolysis of the esters to get the corresponding nucleobase acetic acids required for coupling with the modified *amp* PNA monomers using DCC and HOBt by submonomer coupling. The syntheses were carried out on Merrifield resin derivatized with β-alanine. In the sub monomer method[4] presented here, the modified *amp* PNA oligomers were assembled from two submonomers (i) the bifunctional modified *amp* PNA monomers and (ii) the nucleobase acetic acids. Each cycle consists of two steps, coupling of the modified monomer followed by coupling of the nucleobase acetic acids using carbodiimide as an activating reagent.

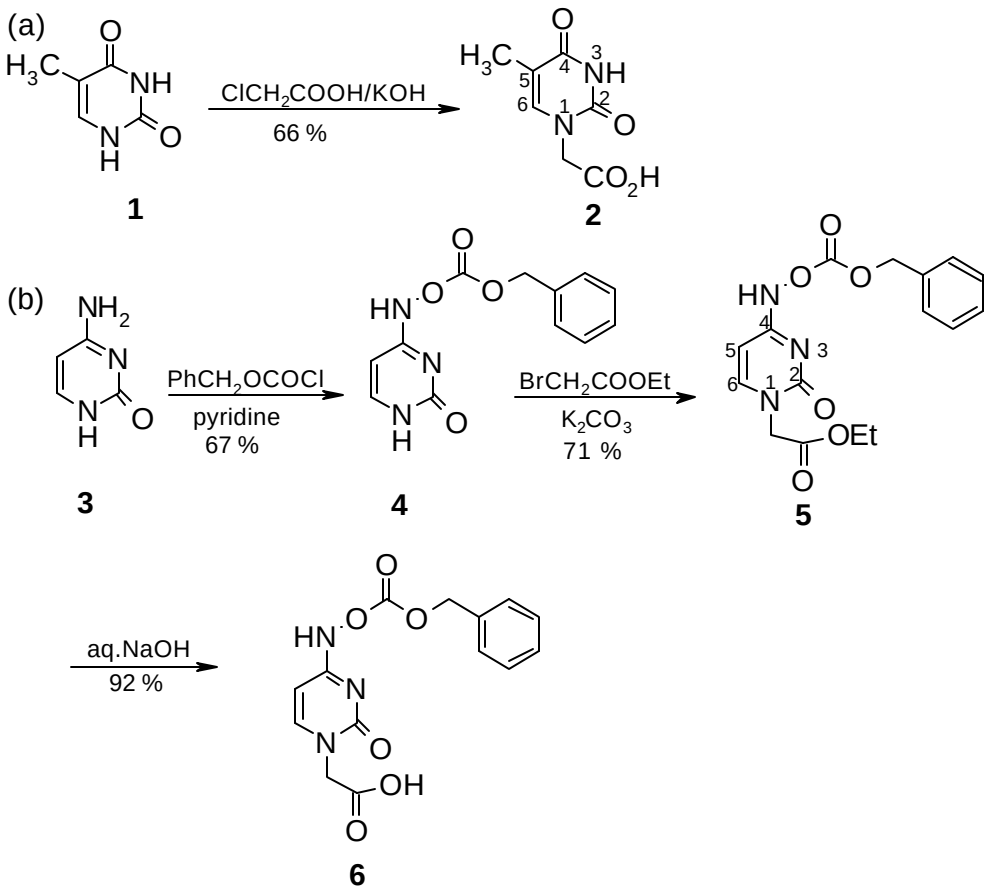
5.3.1a. Synthesis of N^1-Thyminyl Acetic Acid 2

Thymine was alkylated[5] using chloroacetic acid and KOH to get the desired N-thyminyl acetic acid **2** (Scheme 1a).

5.3.1b. Synthesis of N^4-Benzyloxycarbonyl N^1-Cytosinyl Acetic Acid 6

In the case of cytosine **3**, the exocyclic amino group required protection in order to prevent chain extension from this position. The protection was done via its benzyloxycarbonyl derivative, [6] using benzyl chloroformate in anhydrous pyridine to get the protected derivative N^4-benzyloxycarbonyl cytosine (C^{Cbz}) **4** (Scheme 1b).

The N^1-alkylation of C^{Cbz} **4** was carried out using ethylbromo acetate and potassium carbonate in dry DMF to get the ester **5** that was hydrolysed using 2N NaOH to give the required N^4-(benzyloxycarbonyl)cytosin-1-yl acetic acid **6**.



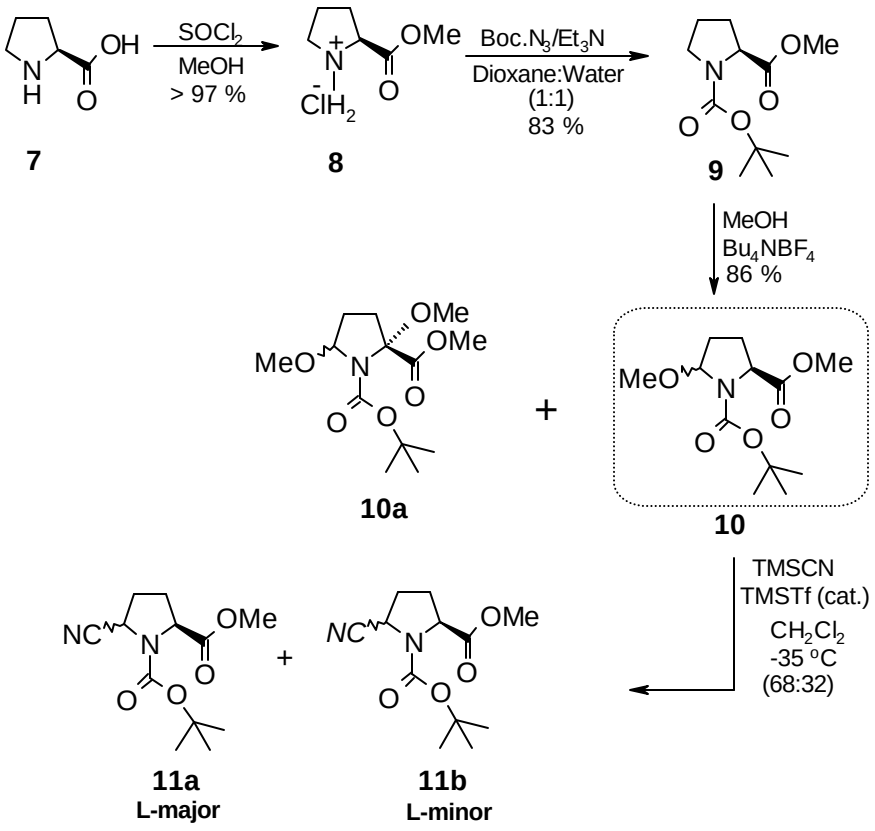
Scheme 1. Alkylation of Nucleobases

5.3.2. Synthesis of 5-aminomethyl prolyl (*amp*) monomers

5.3.2a. Synthesis of (2*S*) -N1-(*tert*-Butoxycarbonyl)-5-[(N-Fluorenylmethoxycarbonyl)aminomethyl] - Proline (14):

The synthesis of *amp* PNA monomer **14** can be achieved from L-proline **7** (Scheme 2), which on treatment with thionyl chloride in methanol afforded the 2-carboxymethyl ester **8** as its hydrochloride salt. The ring nitrogen (N1) of the ester **8** was protected as *tert*-butoxycarbonyl by treatment with *tert*-butyl carbazide and triethyl amine in dioxane/water to get the N-(*tert*-butoxycarbonyl) proline carboxymethyl ester

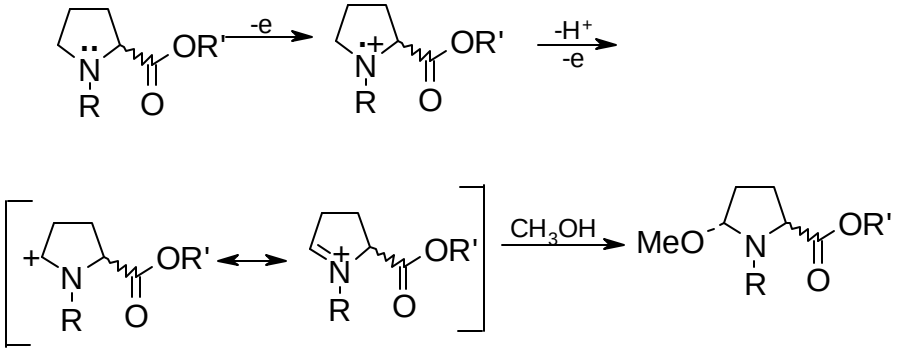
9. This was then subjected to electrochemical oxidation employing Ross-Eberson-Nyberg reaction.[7] This is a unique method for the synthesis of α-methoxy carbamates from the unsubstituted amino compounds. Earlier reports on use of this reaction have



Scheme 2. Synthesis of (2*S*) Nitrile

revealed that the methoxylation should occur at the least substituted site α to the nitrogen atom. The mechanism of α-methoxylation (Scheme 3) involves electrochemical removal of one electron from the lone pair on nitrogen in the initial step when inert supporting electrolytes are used.[8] This reaction was explored in the synthesis of C5-methoxy proline, which is the key intermediate in the synthesis of *amp* monomers. The anodic oxidation involved treatment of the ester **9** with methanol as a

solvent-reagent and tetrabutylammoniumtetrafluoroborate as a supporting electrolyte and passing a constant current of 0.06 F/cm^2 using graphite electrodes. The reaction



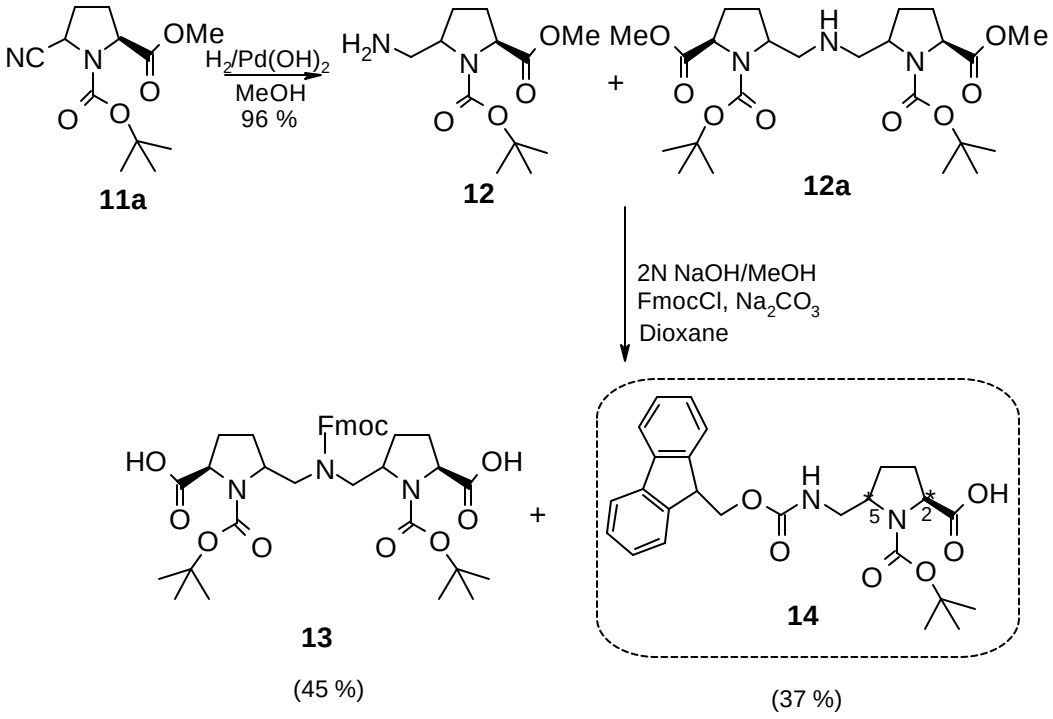
Scheme 3. Mechanism of anodic oxidation

mixture was cooled to 5 ^{o}C before passing the current and the progress of the reaction as followed by TLC, indicated formation of the C5-methoxylated product **10** as a diastereomeric mixture. The efficiency of the reaction depends on controlling the current optimally to avoid the formation of C2 and C5-dimethoxylated product **10a**. It was difficult to separate the C5-methoxylated diastereomeric mixture since the R_f values of both the diastereomers is exactly the same.

The formation of C5-methoxy product **10** was characterized by the appearance of multiple peaks at δ 3.45-3.30 in the proton NMR spectrum. The diastereomeric mixture N,O-acetal **10** was treated with TMSCN and catalytic amounts of TMSOTf at -35^{o} C and transformed to the diastereomeric mixture of C5- nitriles[9] **11** (**11a**& **11b** 68:32). A complete displacement of the methoxy group by cyano group was confirmed by the disappearance of methoxy peaks in the proton NMR spectrum at δ3.45-3.30 and appearance of a new peak in the IR spectrum at 2260 cm^{-1} and a peak at δ 118 in the ^{13}C NMR of the cyano compound. The major isomer **11a** (lower spot on tlc) was used

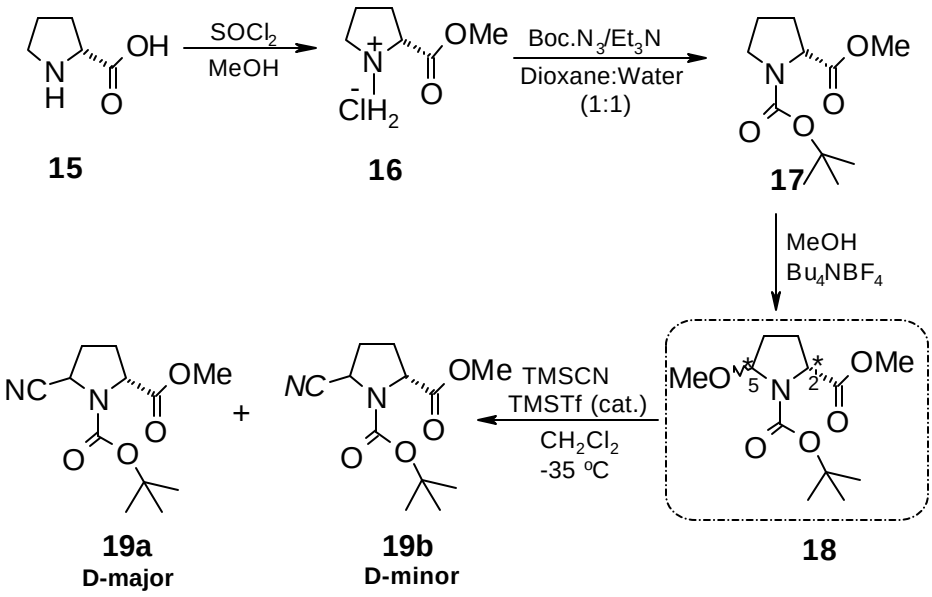
for further transformation into aminomethyl prolyl (*amp*) monomers and *amp* PNA oligomers. The diastereomers **11a** & **11b** showed a different sign and magnitude of optical rotation **11a** +41.8° and **11b** -93.7°. However, assignment of the relative/absolute configuration at the new stereogenic center C5 of the diastereomers was found to be difficult at this stage as evidenced from the data presented in the next section and the scheme was continued with the major isomer (**11a**) in the absence of its stereochemical details.

The nitrile **11a** upon hydrogenation using 50 psi H_2 pressure at room temperature and $Pd(OH)_2$ as a catalyst in methanol yielded the 5-aminomethyl-N^1-(*tert*-butoxycarbonyl) proline methyl ester **12** (Scheme 4). The complete reduction of the nitrile to the amine was indicated by the disappearance of cyano peak at 2260 cm^{-1} in the IR spectrum, at δ 118 in ^{13}C NMR spectrum accompanied by the appearance of a



Scheme 4. Synthesis of (2S) major *amp* PNA monomer

new peak for the amine at 3350 cm^{-1} in the IR spectrum and a CH_2 multiplet at δ 3.01-2.65 in the proton NMR spectrum of **12**. This upon hydrolysis using 2N NaOH in methanol gave the sodium salt of the acid, which was directly treated with fluorenyl methyl chloroformate in dioxane and sodium carbonate as a base to get the fluorenylmethoxy carbonyl derivative **14** in 37% yield along with a major byproduct



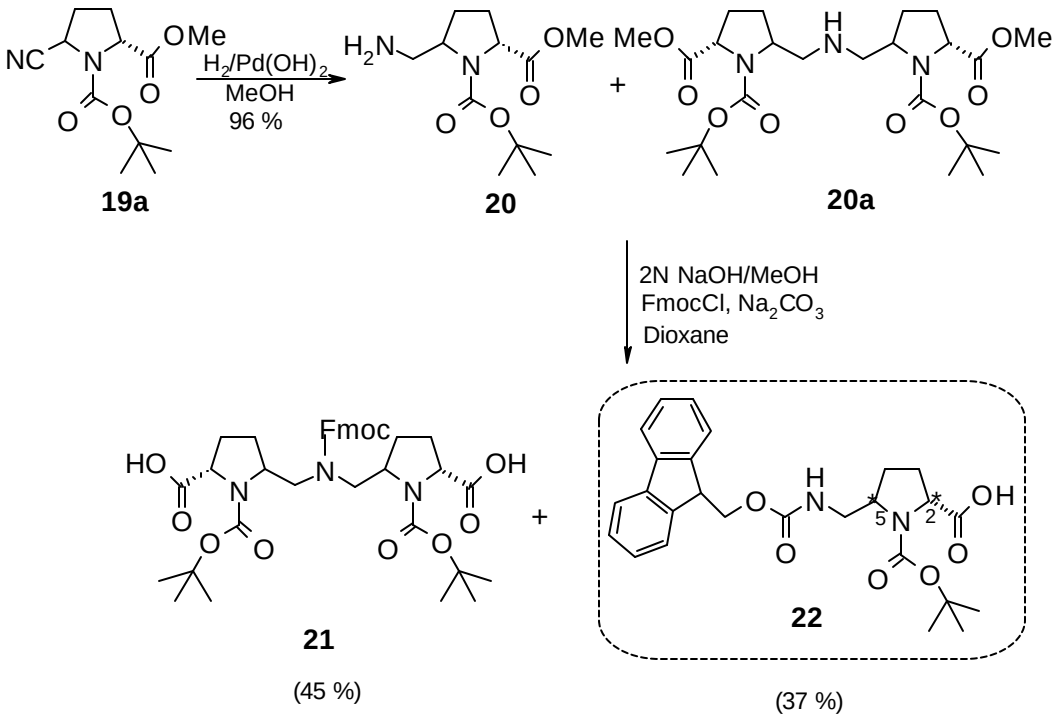
Scheme 5. Synthesis of (2R) Nitrile

identified as protected secondary amine **13**. This compound is perhaps formed during the reduction step by the reaction of the amine with the imine intermediate. The bifunctional amino acid **14** was characterized by NMR and mass spectral analysis.

5.3.2b. Synthesis of (2*R*)-5-[(N-Fluorenylmethoxycarbonyl)-aminomethyl]-N1-(*tert*-Butoxycarbonyl)-D-Proline (22):

The synthesis of (2*R*)-5-[(N-fluorenylmethoxycarbony)aminomethyl-N1-(*tert*-butoxycarbonyl) proline **22** was similarly achieved starting from D-proline **15** (Scheme

5). The steps involving esterification, protection of the ring nitrogen, C5-functionalization by anodic oxidation and convertion of the 5-methoxy-N1-(tert-butoxycarbonyl) proline methyl ester **18** to the separable diastereomeric nitriles **19a** & **19b**. The major product i.e. (2*R*)-D-major **19a** was used for reduction to the aminomethyl ester **20** that was hydrolyzed using 2N NaOH in methanol to get the sodium salt of the amino acid. The product upon protection (Scheme 6) of the primary amine group using fluorenyl methylchloroformate and sodium carbonate gave the bifunctional amino acid D-*amp* monomer **22** containing both Boc and Fmoc groups suitable for the solid phase peptide synthesis.



Scheme 6. Synthesis of (2R) major *amp* PNA monomer

5.3.3. Attempts towards the assignment of Absolute Stereochemistry at C5-position of *amp* PNA monomers

Though the synthesis of *amp* monomers **14 & 22**, have been described in the previous section, their absolute stereochemistry at C5 have not been discussed. For

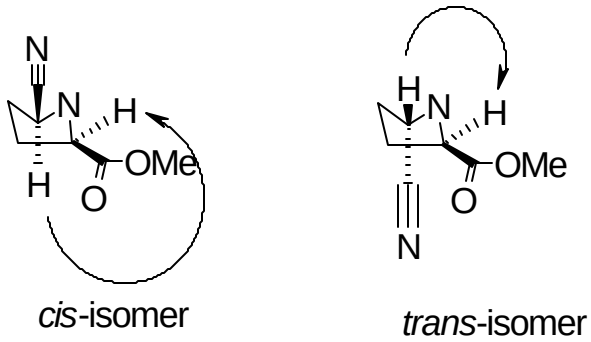
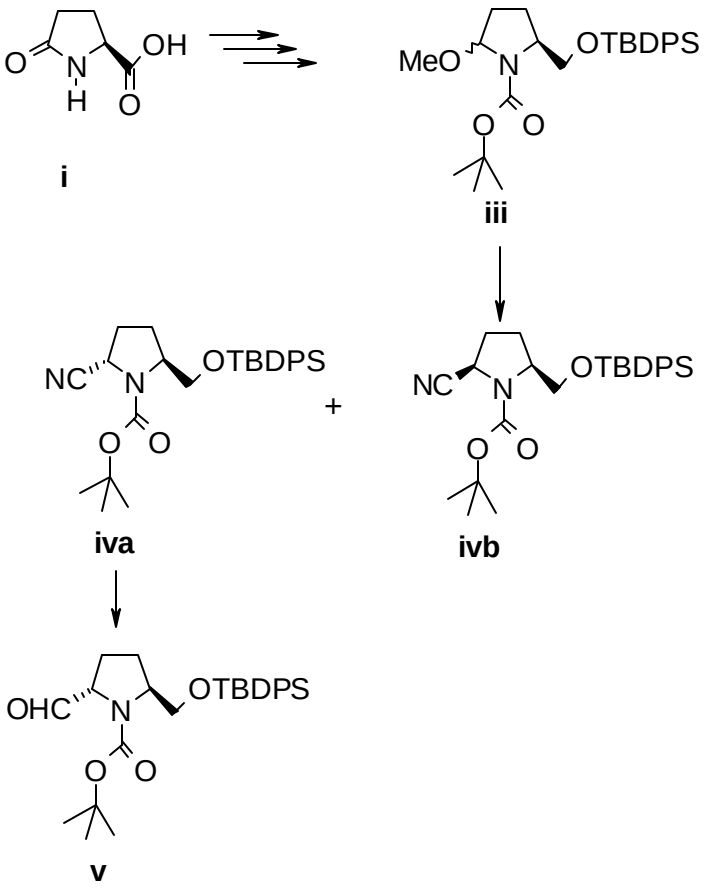


Figure 5

the *amp* PNA oligomer synthesis, only the major product obtained in the conversion of 2*S* and 2*R* C5-OMe to C5-CN reactions was used without the knowledge of the stereochemistry. Since the absolute stereochemistry at C2 is known in **14 & 22**, determination of absolute stereochemistry at C5 is straightforward if the relative stereochemistry of C2-C5 are determined in **14 & 22**. This was thought to be feasible by H2/H5 NOESY correlation in ^{1}H NMR, since the distance of H2-H5 in *cis* geometry is more than that in *trans* geometry. One-dimensional difference NOE experiments were attempted by irradiation of either H2 or H5 (Figure 5). However, no significant NOEs were observed among these in either the major or minor components with both L and D series, inspite of the fact that the distances of H2 and H5 is less than 5 Å in both the isomers. Hence 2D NOESY experiments were attempted on the isomers **19a** & **19b**. However, no cross peaks were observed corresponding to the C2 and C5 protons. But a complicating factor in analysis arising from the presence of rotamers due to the restricted rotation of the tertiary amide group of N1-(*tert*-butoxycarbonyl)

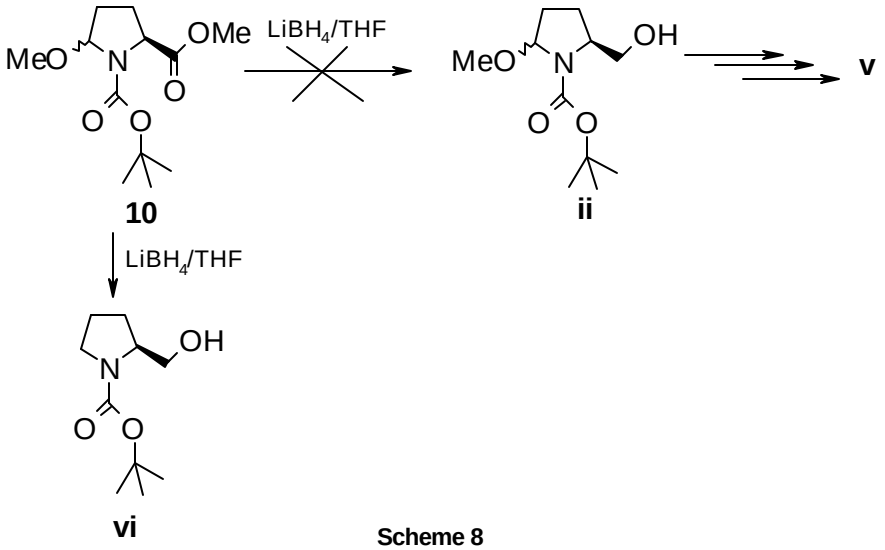
involving the ring nitrogen, lead to the observance of two peaks for both H2 (δ 4.7-4.5) and H5 (δ 4.45-4.20). The two possible isomers were almost in equal population as seen from the intensity of their peaks and all these showed crosspeaks with their respective coupled protons, but no cross peaks were seen. Since absence of NOE cross peaks is no positive proof, no information on their mutual relationship could be obtained from 2D NOESY NMR.

It was found in literature that the X-ray structure of protected derivative of (2S,5R) *trans*-N1-(*tert*-butoxycarbonyl)-L-pyrrolidine-5-aldehyde **v** prepared from pyroglutamic acid **i** (Scheme 7) is known.[9] By converting one of the present series of 5-



Scheme 7

cyano compound into the aldehyde **v** it should be possible to establish the stereochemical relationship by correlation. This can be achieved in principle by the route shown in Scheme 8. However, the initial reaction with $LiBH_4$ led to the

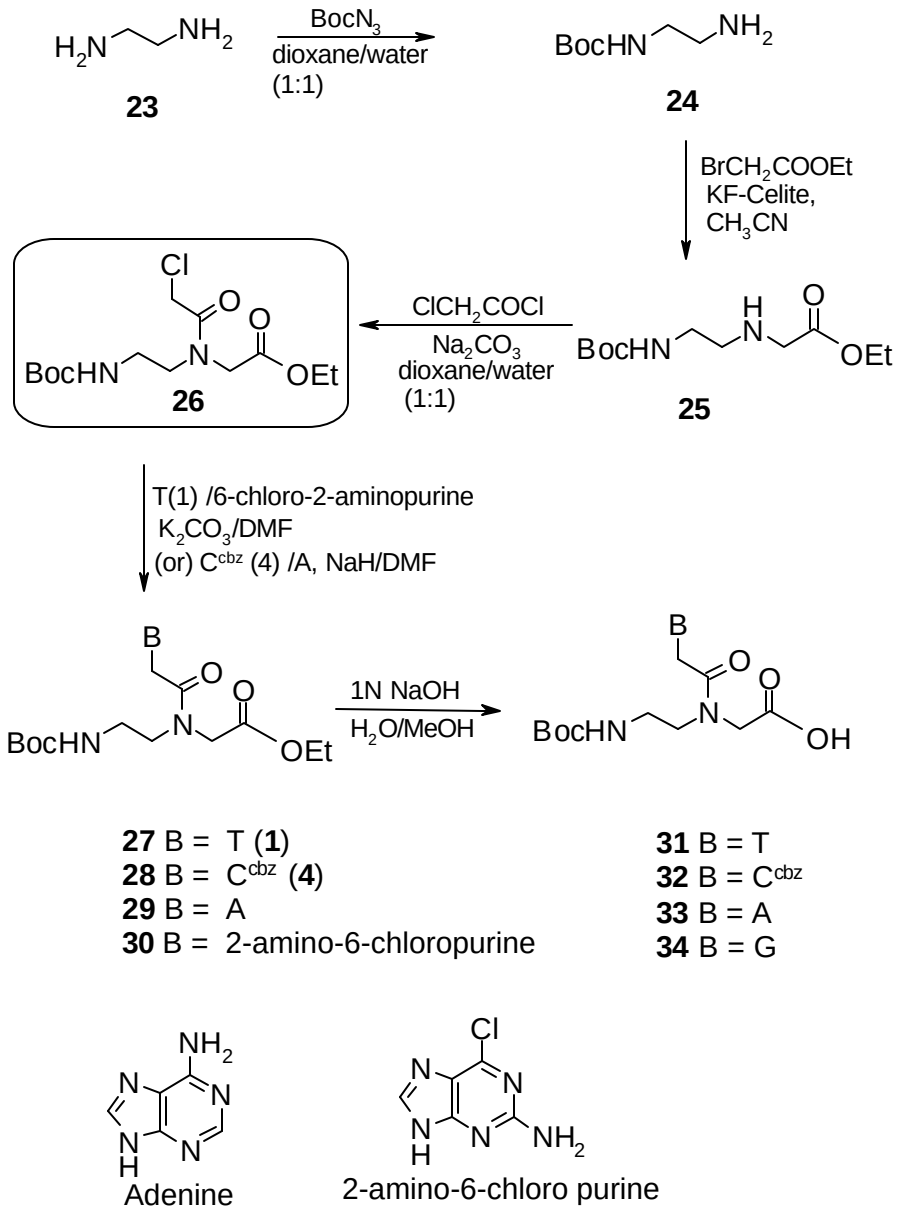


Scheme 8

demethoxylation and ester reduction to the alcohol giving the demethoxylated **vi** instead of the desired product **ii** during the reduction process. Hence this necessitates alternative approaches to be explored in future.

5.3.4. Synthesis of aminoethylglycyl (*aeg*) PNA monomers

In order to study the effect of the aminomethylprolyl (*amp*) PNA monomers on hybridization and stability of PNA they were incorporated into the PNA oligomers along with the unmodified *aeg* PNA monomers which were synthesized according to the reported procedures[10] (Scheme 9). 1,2-Diaminoethane **23** was treated with Boc-azide under high dilution conditions, to get the mono-N-*tert*-butoxycarbonyl derivative **24**. The di-*tert*-butoxycarbonyl derivative, which was formed in small quantity, could be removed easily because of its insolubility in water. N1-*tert*-butoxycarbonyl-1,2-diamino ethane was N-alkylated using ethyl bromoacetate and KF- Celite in dry acetonitrile. The



Scheme 9. Synthesis of *aeg* PNA monomers

use of KF-Celite[11] was found to be advantageous over K_2CO_3 due to better yield of the product and the ease of work-up. After the completion of the reaction, KF-Celite was

filtered off and acetonitrile was evaporated to get the N-(*tert*-butoxycarbonyl)-aminoethyl glycinate **25** in good yield. This was used as such for the next step without further purification. Compound **25** on treatment with chloroacetyl chloride (obtained from chloroacetic acid and thionyl chloride) in presence of Na_2CO_3 in dioxane gave **26** in good yield. The use of triethylamine[12] as a base gave poor yields and highly colored products. Alternatively, using aq. Na_2CO_3 in dioxane as a base and using excess of chloroacetyl chloride gave the corresponding chloroacetyl derivative in good yield.[13] The glycinate **26** thus obtained was purified by column chromatography and used as a common intermediate for the alkylation of nucleobases (Scheme 9).

The alkylation of nucleobases thymine and cytosine using chloroacetyl derivative **26** was found to be regiospecific at N1. Thymine was reacted with **26** using K_2CO_3 as base to obtain N-(*tert*-butoxycarbonyl-aminoethyl) glycyl-thymine ethyl ester **27** in high yield. In case of cytosine, the exocyclic amino group was protected with benzyloxycarbonyl group and alkylated using **26** and NaH as the base to provide N1-substituted product **28**. Although adenine is known to undergo both N-7 and N-9 substitution, N-7 alkylation product was absent when NaH was used as a base and only N1-[(*tert*-butoxycarbonyl-aminoethylglycyl] adenine ethyl ester **29** was obtained in moderate yield. Alkylation of 2-amino-6-chloropurine with **26** was facile with K_2CO_3 as base and yielded the corresponding N1-[(*tert*-butoxycarbonyl)aminoethylglycyl]-(2-amino-6-chloropurine) ethyl ester **30** in good yield. The exocyclic amino group in adenine and guanine were left unprotected, as these were unreactive under the conditions employed for the subsequent coupling reactions on solid phase. All the compounds exhibited satisfactory ^1H and ^{13}C NMR data consistent with the literature data.[14]

All the esters were hydrolyzed with aq. NaOH to get the corresponding *aeg* monomeric PNA acids **31-34** that were used for the SPPS.

5.4. Solid Phase Peptide Synthesis (SPPS) OF PNA

The *aeg*-PNA oligomers and the modified *amp* PNA oligomers were synthesized by standard Solid Phase Peptide Synthesis[15] protocols using both Boc and Fmoc strategies.

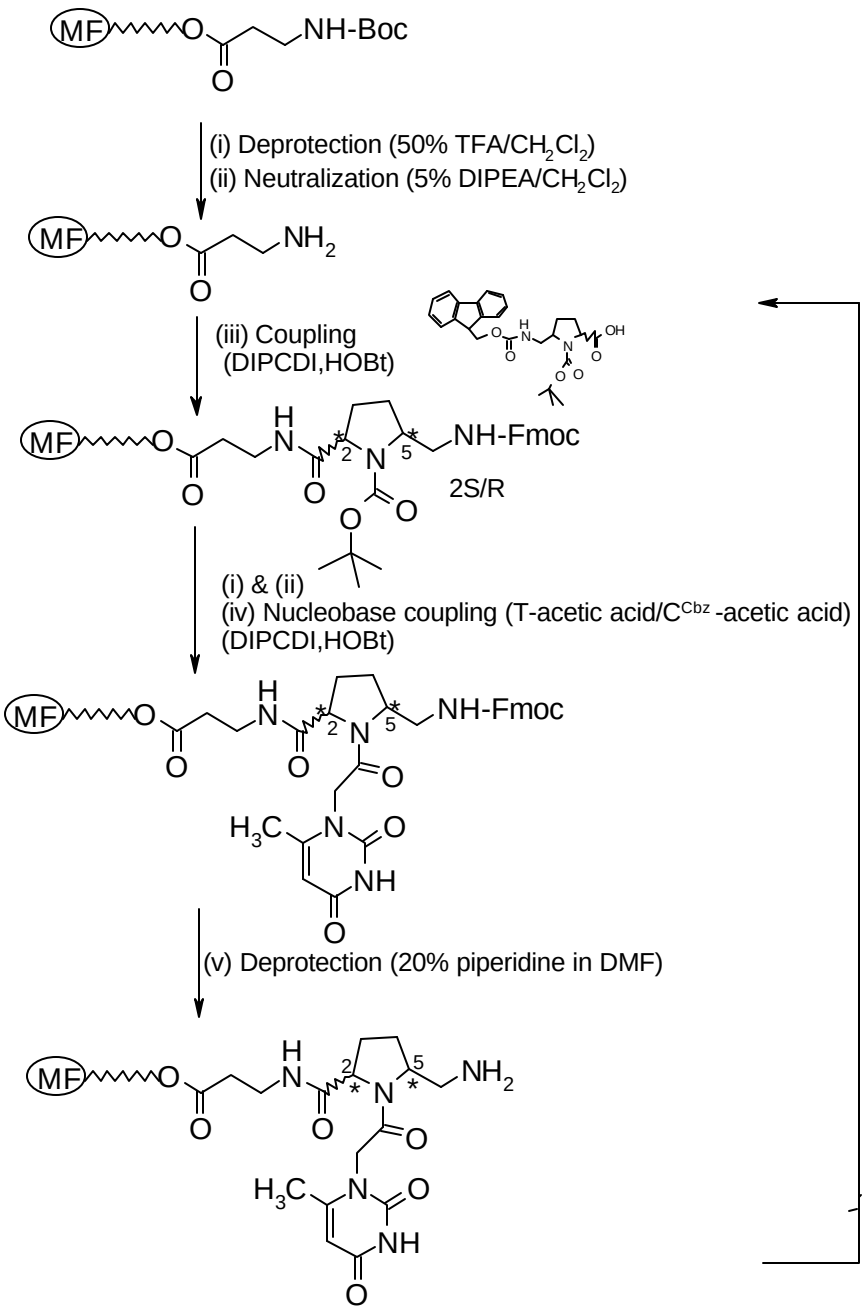
Principle. The synthesis can be conveniently carried out using suitably protected amino functionalities with the free carboxylic groups from the C-terminus to the N-terminus. The readily available Merrifield resin was chosen as the polymer matrix on which the *aeg* PNA oligomers as well as the *amp* PNA oligomers were built. The first amino acid is attached via benzyl ester that can be cleaved easily at the end of the synthesis either by acidolysis to get the free peptide acid or by aminolysis to get the carboxyamide. After the attachment of the first amino acid β-alanine, both Boc and Fmoc strategies were used on the resin for extending the oligomers. The N-protecting groups employed in this process are generally Boc, Fmoc or benzyloxycarbonyl (*Z*) groups. Although Merrifield used benzyloxycarbonyl group in the protection of α-amino group, it has the disadvantages of unwanted side reactions and cleavage of the oligomers from the solid support. A milder and convenient protecting group *tert*-butoxycarbonyl has become very useful and can be used in combination with other protecting groups in differential (Boc/Cbz) as well as orthogonal strategies (Boc/Fmoc). In the present work the orthogonal strategy has been employed to construct the oligomers followed by subsequent attachment of the nucleobases. The *aeg* PNA monomers used have the amino function protected as the *tert*-butoxycarbonyl group and the modified bifunctional *amp* monomers (**14** & **22**) have the primary amino function protected as the Fmoc group and the ring nitrogen *tert*-butoxycarbonyl group. In both the cases DCC/HOBt activation coupling strategy was employed.[16] The use of Fmoc protection has drawback in PNA synthesis, as small amount of acyl migration has been observed under basic conditions from the tertiary amide to the free amine

liberated during the piperidine deprotection step.[17] However, Fmoc protection employed in case of the *amp* PNA monomers was found to be convenient for extending the oligomers.

In the present study all the oligomers were built on Merrifield's resin using β-alanine as the amino acid linker. Being achiral and small in size its interference in spectral properties and hydrophobicity of the resulting PNA oligomers was negligible. The resin was functionalized with N-(*tert*-butoxycarbonyl)-β-alanine by cesium salt method.[18] The functionalized resin after the TFA treatment was assayed to determine the loading value (free amino groups) of the first amino acid the β-alanine by picrate method[19] (0.61 meq/g). The resin was then partially capped by acetylation after the Boc deprotection to avoid the aggregation of the growing oligomers, followed by the picric acid assay to estimate the loading value (0.27 meq/g). PNA oligomers were then built on the resin by repetitive cycles consisting of

a) Deprotection of the N1-*tert*-butoxycarbonyl function using 50% TFA in dichloromethane.
b) Neutralization of the TFA salt to get the free amine using 5% DIPEA in dichloromethane.
c) In case of the Fmoc amino acid it was deprotected using 20% piperidine in DMF.
d) Coupling of the amine with 3 to 4 equivalents of free carboxylic function of the incoming amino acid using diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzo triazole in DMF as solvent.

The completion of deprotection and the coupling reaction were monitored by Kaiser's test,[20] in which beads show intense blue color for the free amine and remain colorless after the coupling. A typical cycle for the SPPS is described in Scheme 9.



Scheme 10. Schematic representation of SPPS using both Boc. and Fmoc. strategy

5.4.1. SYNTHESIS OF *amp* PNA OLIGOMERS

5.4.1a. Homopyrimidine Oligomers

The various oligomers synthesized in the present study are shown in Table 1. The unmodified PNA oliogomers T_8 (**35**) and T_2CTCTT_2 (**40**) were synthesized using the Boc protected *aeg* PNA monomers (**31** and **32**). These were used as the control sequences for comparing the properties of the *amp* PNA oligomers. The synthesis of the oligomers (**36-39** and **41-44**) incorporating the chiral, conformationally constrained modified *amp* PNA monomers at specific positions in the *aeg* PNA oligomers was done on solid support in a similar way, but using Fmoc chemistry for the *amp* monomer coupling and Boc chemistry for the *aeg* PNA monomer coupling to extend the oligomers. Since the *amp* monomers do not carry the nucleobases, these were introduced at desired positions by sub monomer coupling. After this, the amp amino acid that has acid stable Fmoc group at amino methyl terminus was treated with TFA to deprotect the ring nitrogen. The thyminyl **2** or cytosinyl **6** acetic acid was then reacted with the resin using DIPCDI/HOBt as coupling agent to attach the nucleobases to the ring nitrogen. This was followed by treatment with 20% piperidine to remove base labile Fmoc group from aminomethyl terminus for initiating the next cycle (Scheme 8). This method involving orthogonal coupling proceeded with good efficiency leading to high purity products. It also circumvented the problem of pre-synthesis of *amp* monomers for each of the bases.

5.4.1b. Purine-Pyrimidine Mix Sequences

The polypyrimidine sequences discussed in the previous section are known to form triplexes with the complementary DNA in 2:1 (PNA_2:DNA) stoichiometry. With the aim of studying the duplex forming potential of the present modification, *amp* monomers were also incorporated into a mixed decamer sequence **46** containing both

the purines and pyrimidines using the Boc and Fmoc chemistry. The corresponding unmodified control *aeg* PNA sequence is **45**.

Table 1. **PNA sequences**

Entry	Sequence
35	H-T T T T T T T T-NH-$(CH_2)_2$-COOH
36	H-T T T T T T T- **T** -NH-$(CH_2)_2$-COOH
37	H -T T T **T** T T T **T**- NH-$(CH_2)_2$-COOH
38	H- **T** T T T T T T T - NH-$(CH_2)_2$-COOH
39	H-**T T T T T T T T**- NH-$(CH_2)_2$-COOH
40	H-T T T C T C T T- NH-$(CH_2)_2$-COOH
41	H-T **T** T **C** T **C** T **T**- NH-$(CH_2)_2$-COOH
42	H-TT T T T T T **t** - NH-$(CH_2)_2$-COOH
43	H -T T T **t** T T T **t** - NH-$(CH_2)_2$-COOH
44	H-T **t** T **c** T **c** T **t**- NH-$(CH_2)_2$-COOH
45	H-G T A G A T C A C T- NH-$(CH_2)_2$-COOH
46	H-G **T** A G A **T** C A C **T**- NH-$(CH_2)_2$-COOH
	Oligonucleotide Sequences
47	5'-G C A A A A A A A A C G-3'
48	5'-G C A A G A G A A A C G-3'
49	5'-A G T G A T C T A C-3'

Unmodified control *aeg* PNA monomers are shown in upper case letters, *amp* PNA monomers with L-major are shown in upper case letters bold, *amp* PNA monomers with D-major are shown in lower case letters bold.

5.4.1c. Cleavage of the PNA oligomers from the solid support

The cleavage of the PNA oligomers from the resin was accomplished by the use of trifluoromethane sulphonic acid (TFMSA)-trifluoroacetic acid (TFA) mixture in presence of scavengers like ethane dithiol and thioanisole that react with the liberated tertirarybutyl cations.[21] This cleavage reaction yields the peptide with free carboxylic acids at their C-terminus[22] and was monitored by removal of aliquots at different time intervals and analyzed by HPLC. The benzyloxycarbonyl protecting group of the exocyclic amino groups of cytosine, adenine and guanine were also found to be cleaved during this process. In case of the *amp* PNA oligomers, N-terminus Fmoc group after the last coupling was deprotected using 20% piperidine in DMF prior to the TFMSA-TFA cleavage.

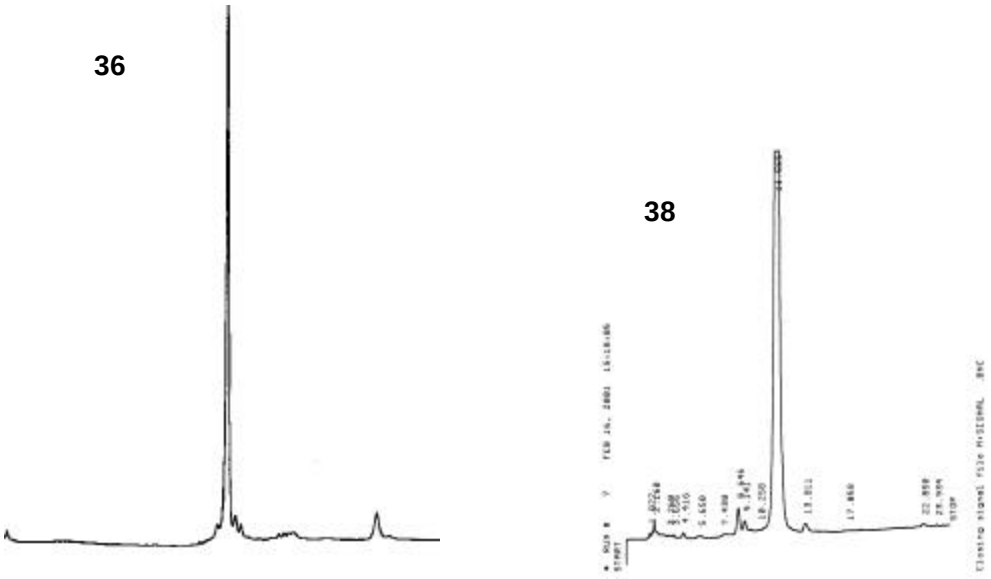


Figure 6. HPLC profiles of oligomers **36** and **38**

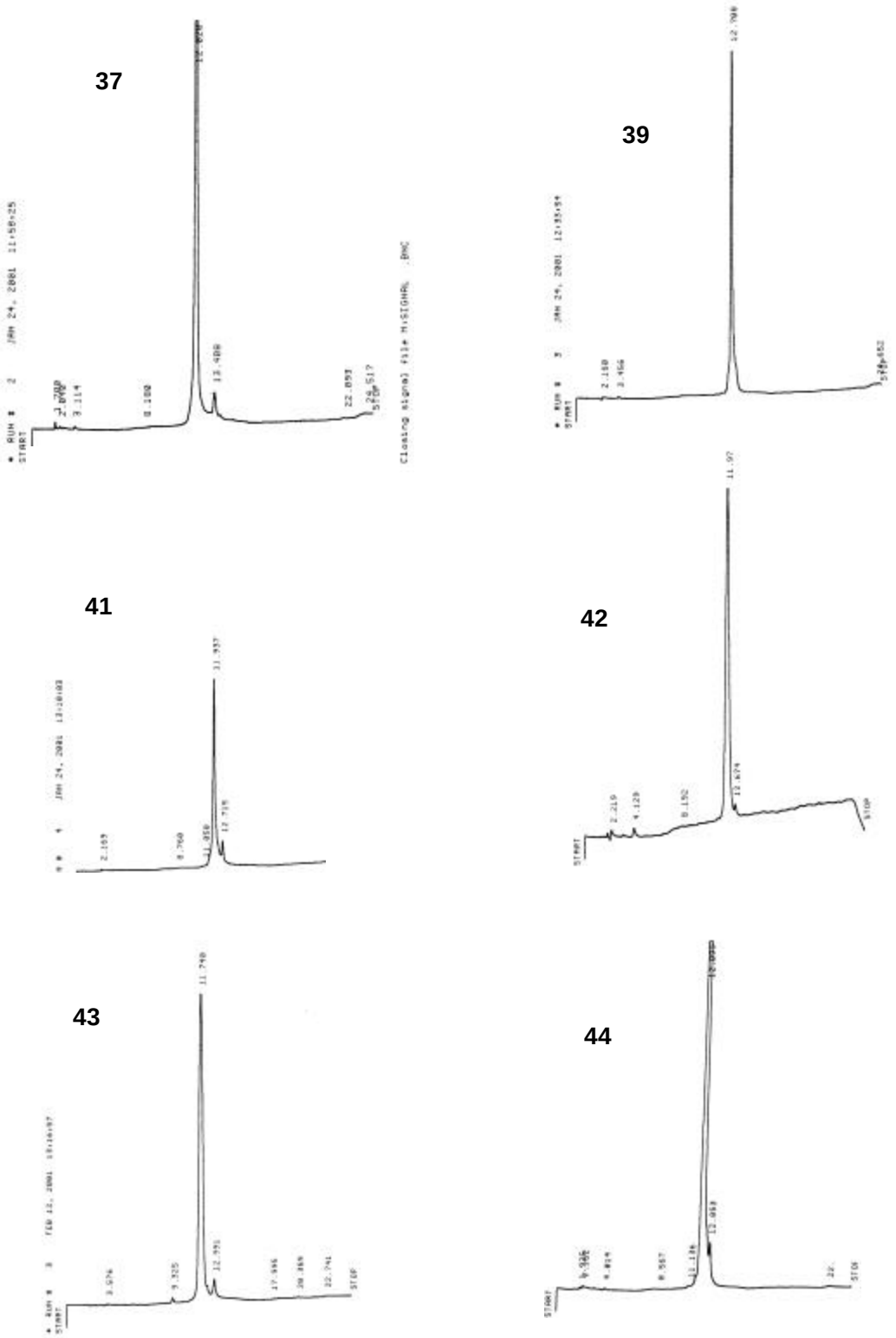


Figure 7. HPLC profiles of *amp* PNA oligomers

5.4.1d. Purification of PNA oligomers

The cleaved oligomers (Table 1) were subjected to the gel filtration followed by purification using RP-HPLC. The molecular ion identity was confirmed by MALDI-TOF mass spectral analysis. Some of the representative HPLC profiles (Figure 6 & 7) and mass spectra are shown in Figures 8-12.

5.4.2. Synthesis of complementary oligodeoxynucleotides

The oligodeoxynucleotides **47-49** (Table 1) required for the biophysical studies were synthesized on Pharmacia Gene Assembler Plus automated DNA synthesizer, cleaved as described before (Chapter 3) and used for the biophysical studies with PNA.

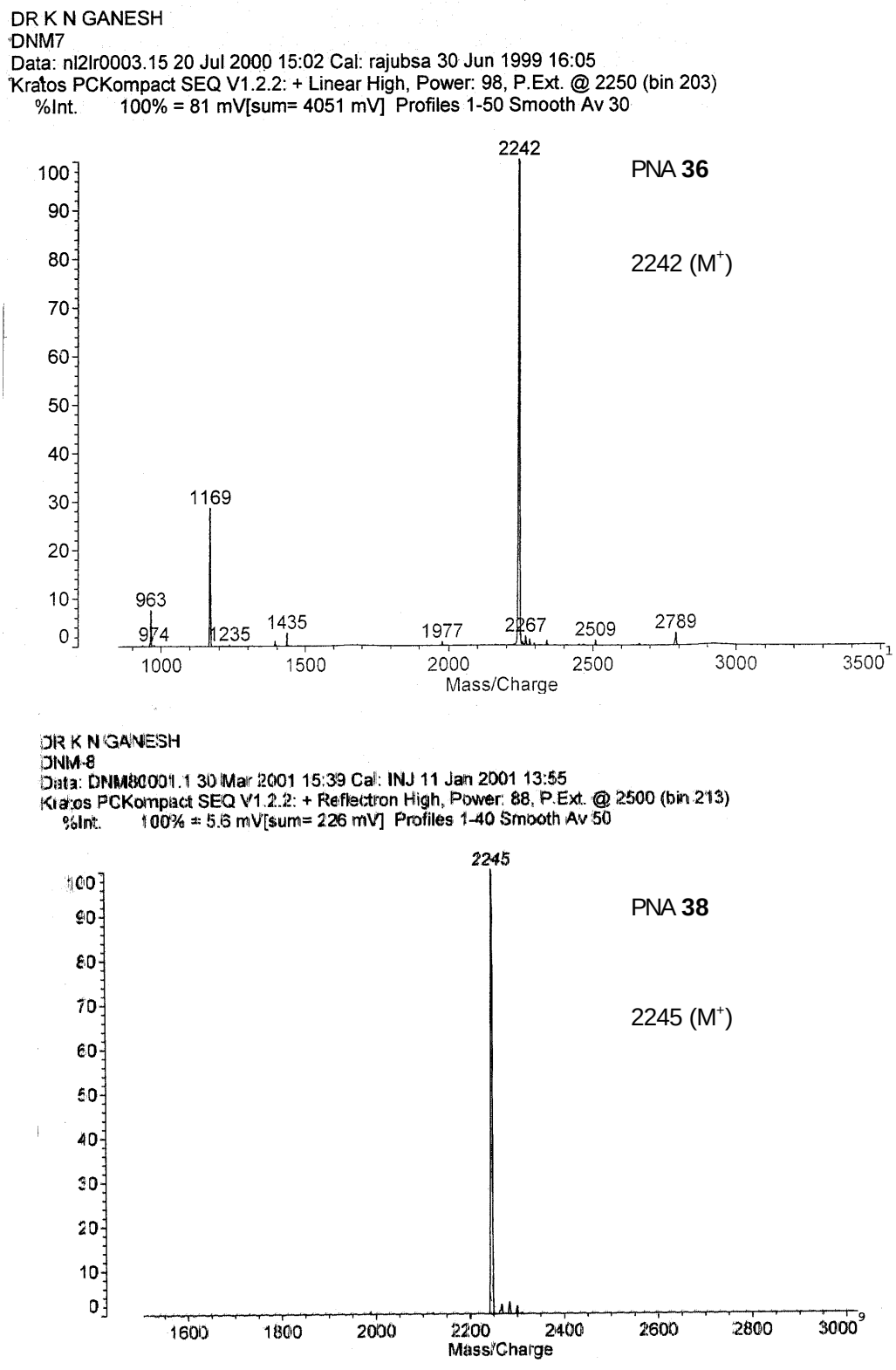
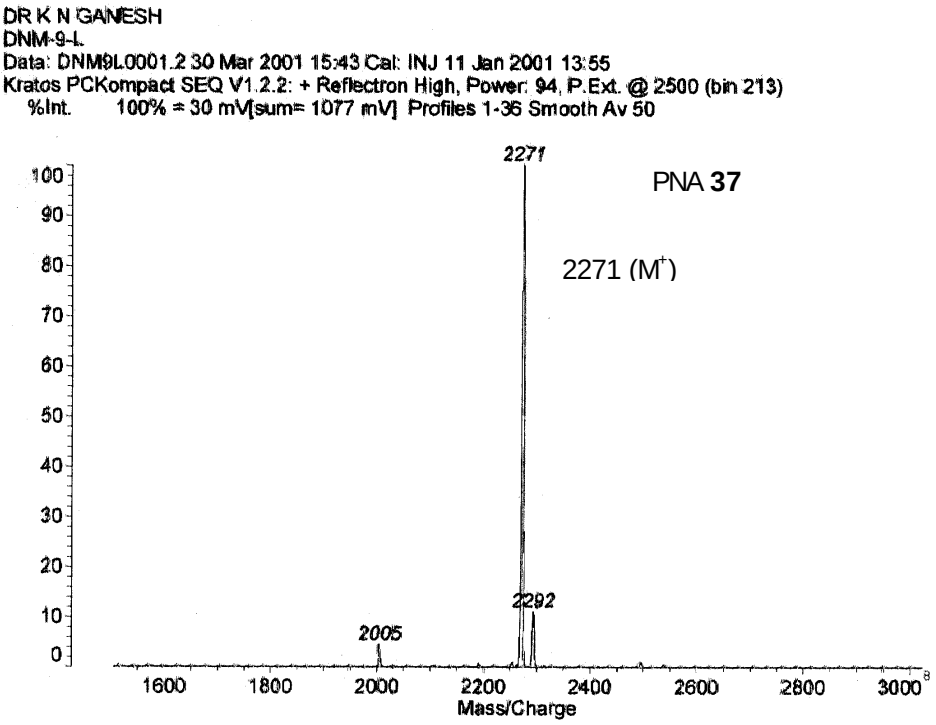


Figure 8. MALDI-TOF of oligomers **36** and **38**



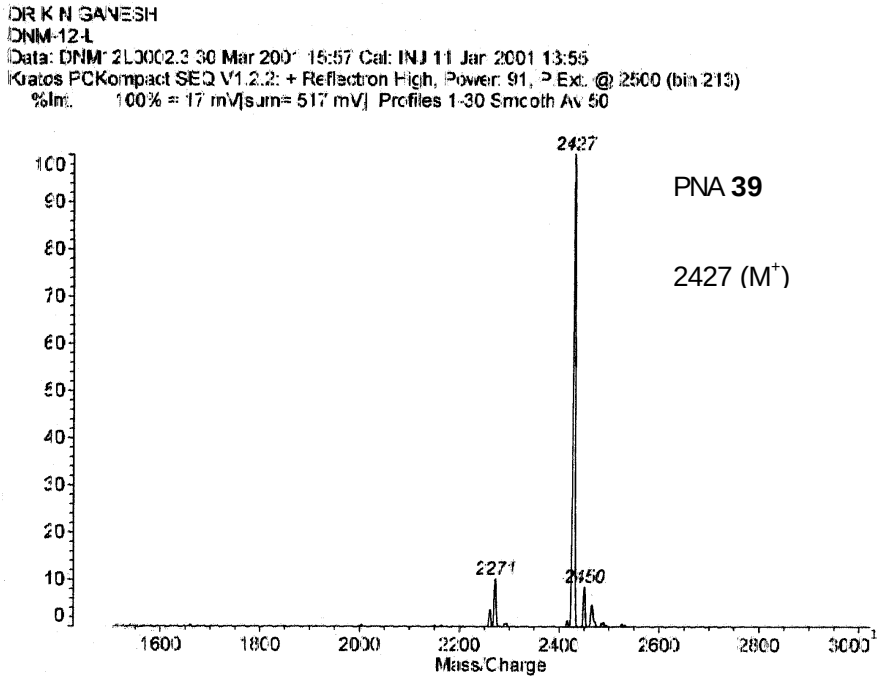
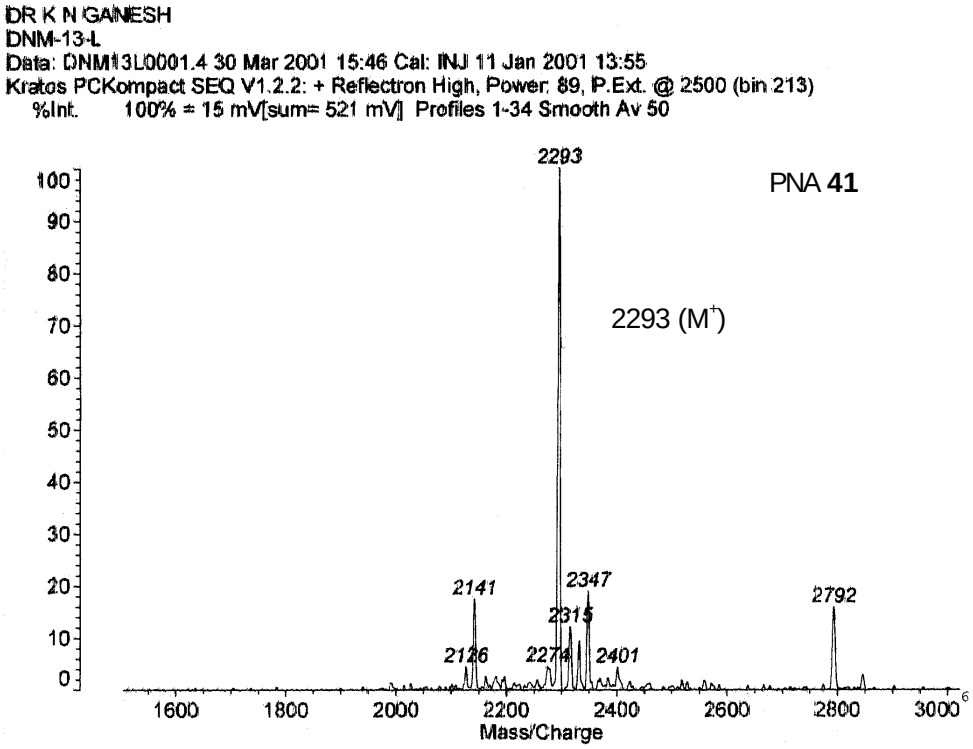


Figure 9. MALDI-TOF of oligomers **37** & **39**



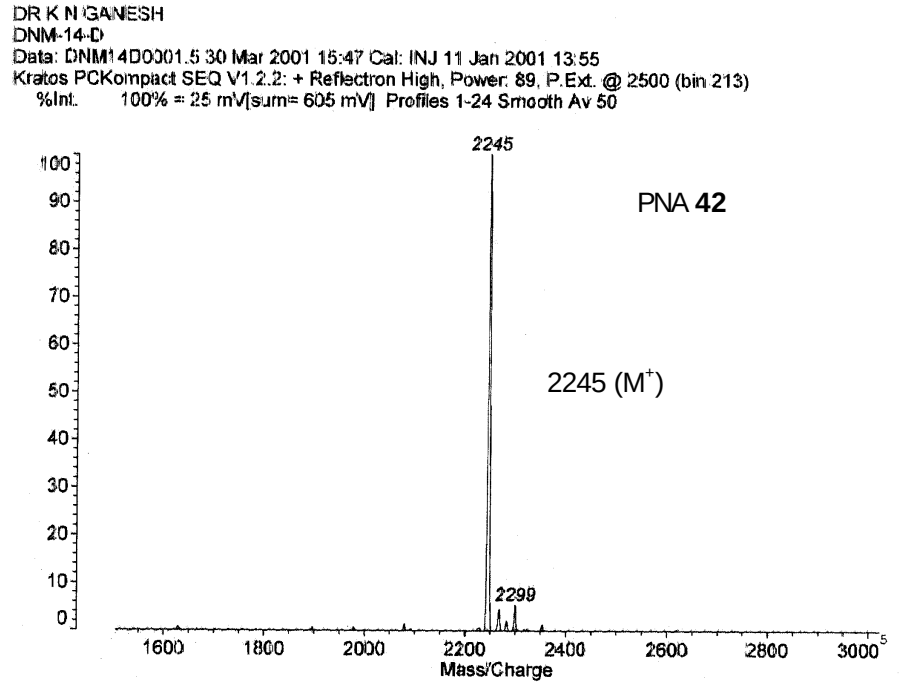
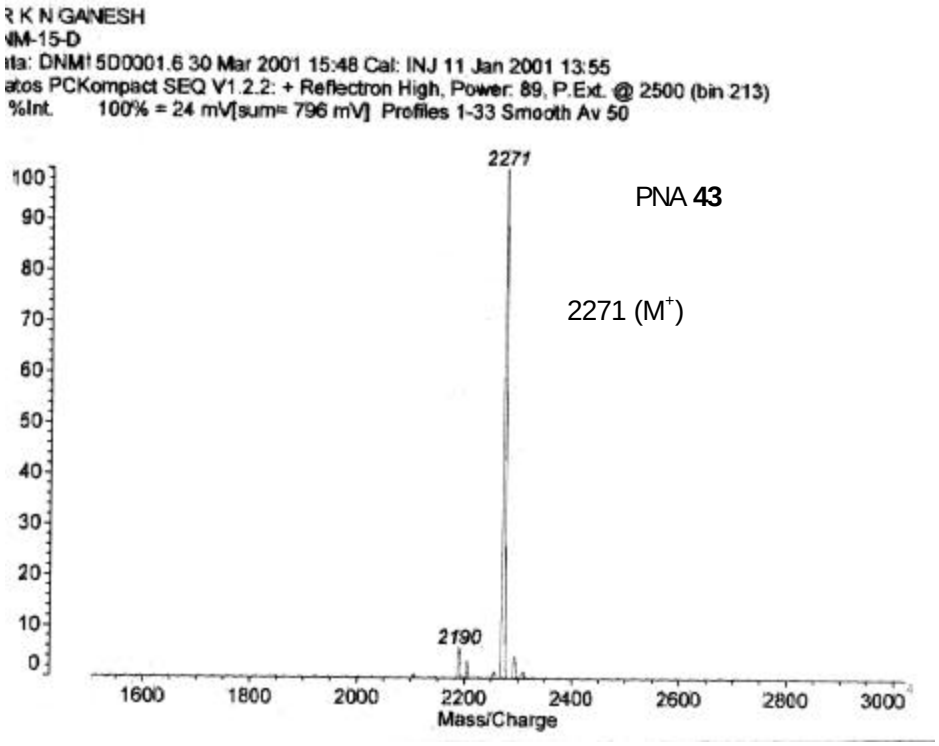


Figure 10. MALDI TOF of oligomers **41** and**42**



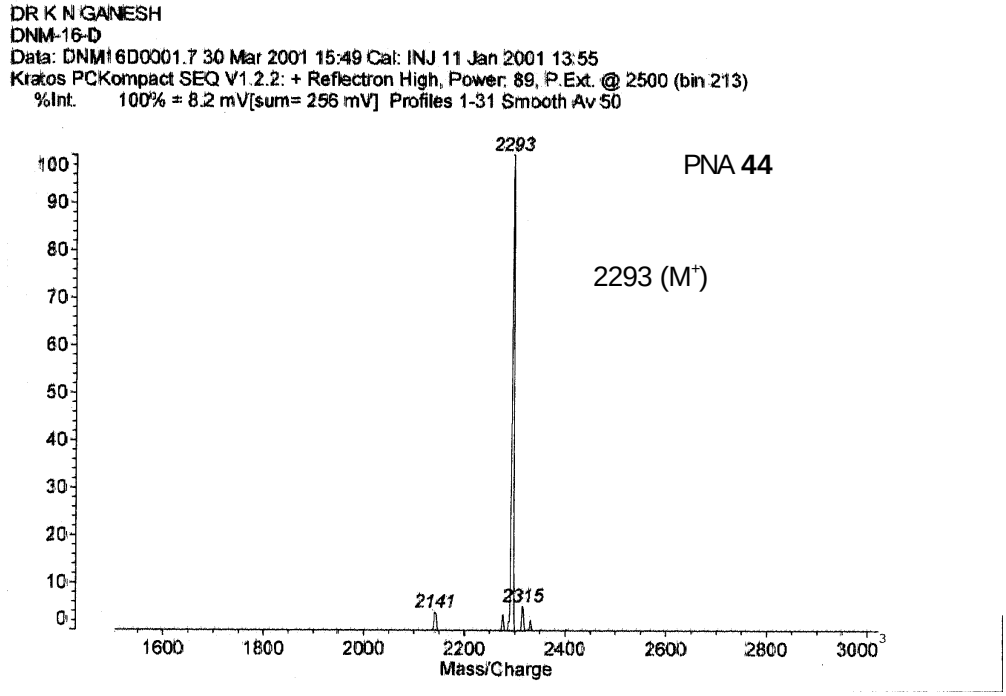


Figure 11. MALDI-TOF of oligomers **43** and **44**

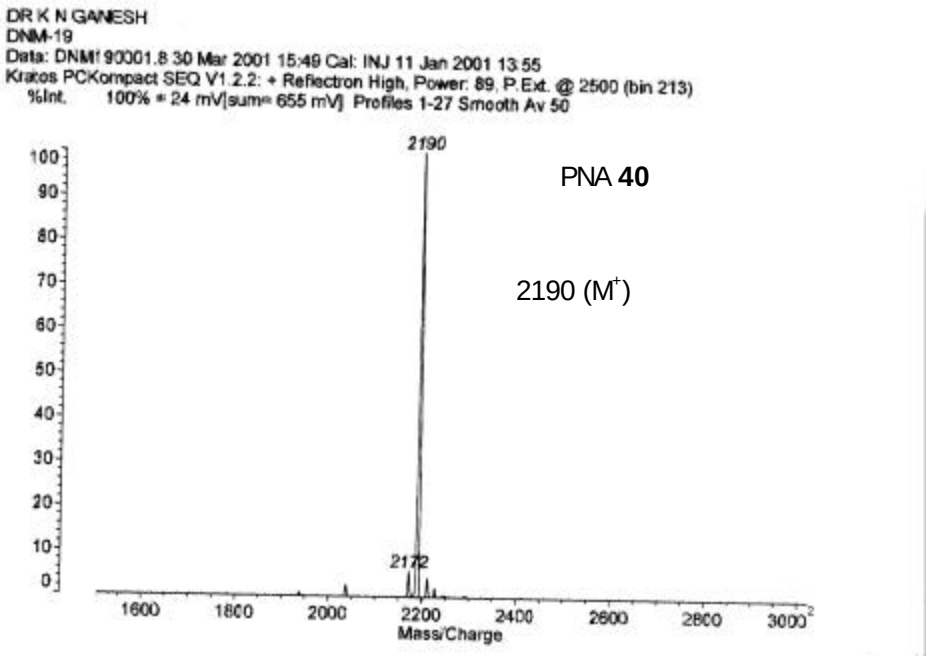


Figure 12. MALDI-TOF of oligomer **40**

5.5. BIOPHYSICAL STUDIES OF *amp* PNA:DNA HYBRIDS

5.5.1. UV melting studies of *aeg* PNA:DNA and *amp* PNA:DNA hybrids

Stoichiometry of binding. Since homopyrimidine PNAs bind DNA in a 2:1 ratio, the binding stoichiometry of *amp* PNA:DNA hybrids was determined by UV titration experiments. This was done by titration experiment in which the UV absorbance of the sample is plotted as a function of mole fraction of one of the constituents. The absorbance initially decreases because of the complex formation followed by increase as the concentration of one of the strands starts increasing. The stoichiometry of the complexes is derived from the minimum in such plots.

The results of such a UV titration experiment done by stoichiometric addition of homopyrimidine T/C *aeg* PNA oligomer **40** or homopyrimidine T/C *amp* PNA oligomer **44** to the cDNA **48** is shown in Figure 13. The stoichiometry as determined from the minima at 50% in both the cases indicated a 1:1duplex formation.

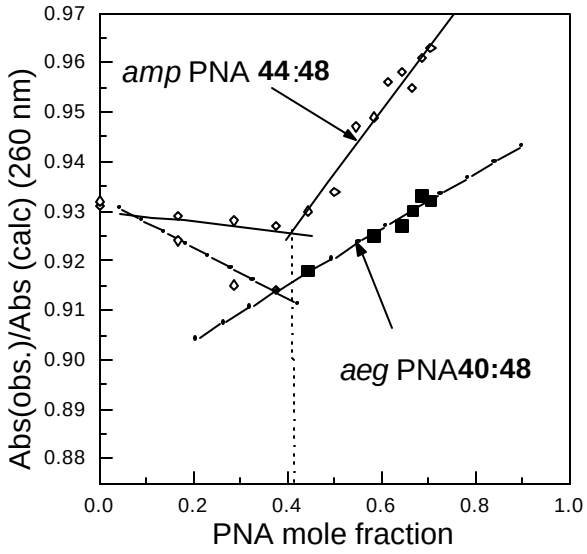


Figure 13. UV-titration of PNA:DNA cmplexes **40:48** and **44:48**

UV-T_m of PNA:DNA complexes. The UV-T_m data of the synthesized homooligomeric-T *amp* PNA ologomers (**36-39** & **42**,**43**) with complementary DNA sequences at different stoichiometry and at different pH were examined. PNAs composed of homooligomeric T bind to complementary DNA (homooligomeric A) in (2:1) PNA_2:DNA stoichiometry and hence melting experiments were done in both 1:1 and 2:1 PNA_2:DNA stoichiometry. This is not the case with PNA sequences containing mixed pyrimidine or purine bases, which form only duplex (1:1 complexes) with complementary DNA. Further, PNA sequences containing only pyrimidines (T and C) can form PNA_2:DNA triplexes which become more stable at acidic pH due to N3 protonation of C (Figure 3, Chapter 4) in the Hoogsteen strand. To investigate such an effect, UV-T_m experiments of PNA_2:DNA complexes were also measured at acidic pH. The accurate UV-T_m values in all the cases were obtained from the peaks in the first derivative plots.

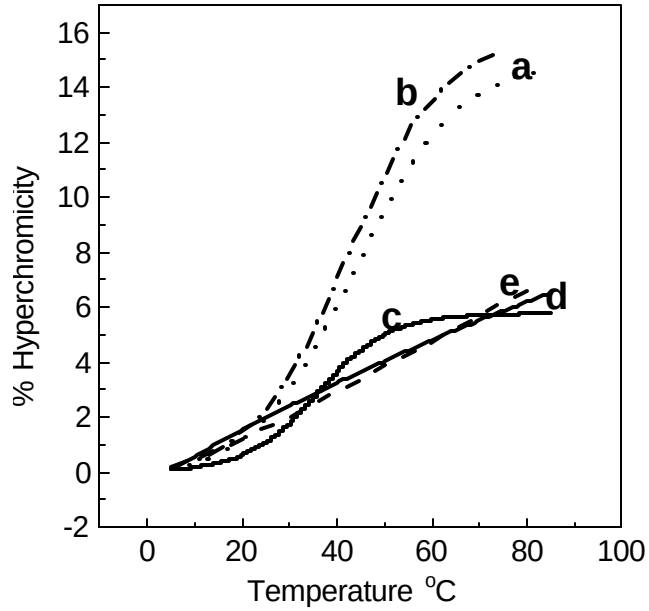


Figure 14. UV-Tm plots of PNA_2:DNA complexes (**a**) **35:47** (control), (**b**) **38:47** (N-terminal) (**c**) **36:47** (C-terminal), (**d**) **37:47** (C-terminal and interior), (**d**) **39:47** (homooligomer)

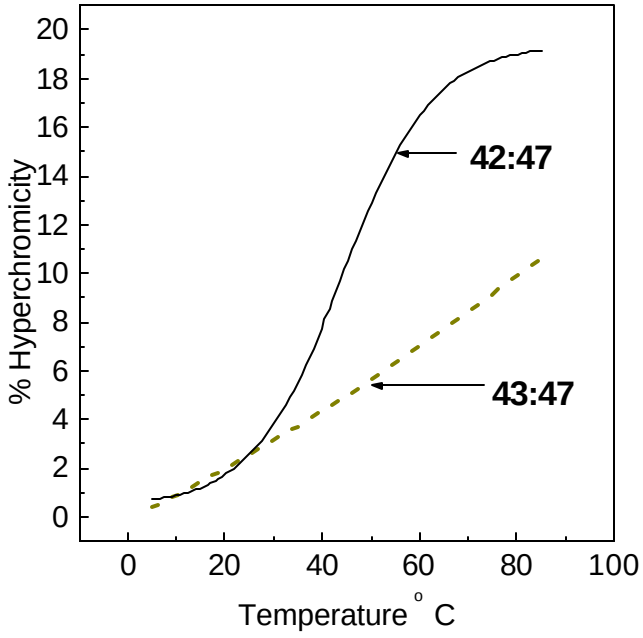


Figure 15. UV-Tm plots of the PNA_2:DNA complex **42:47** (one unit of *amp* PNA D-isomer at the C-terminus) and **43:47** (two units of *amp* PNA with D-isomer at C-terminus and at the interior)

Table 2. UV T_m of PNA_2:DNA complexes

Entry	L-major		D-major	
	PNA_2:DNA	**T_m(^oC)**	**PNA_2:DNA**	**T_m(^oC)**
1	**35:47**	43.4		
2	**36:47**	36.5	**42:47**	45.4
3	**37:47**	50.3	**43:47**	nb
4	**38:47**	41.8		
5	**39:47**	nb		

Buffer: 10 mM Na phosphate pH 7.4. Tm values are accurate to (±) 0.5^{o}C. Experiments were repeated at least thrice and the Tm values were obatined from the peaks in the first derivative plots.

The stabilities of various *aeg/amp* PNA_2:DNA complexes differed depending on the stereochemistry and position of the *amp* PNA monomer incorporated. Some representative UV melting profiles are shown in Figure 14 & 15 and the data in Table 2. The homopyrimidine T_8 *aeg* PNA (**35**) formed complex with complementary DNA **47** at pH 7.3 in both 1:1 and 1:2 stoichiometry and no differences were seen in relative T_m values (entry 1). These PNA_2:DNA triplexes show a single step dissociation with only one T_m due to the simultaneous dissociation of both the PNA strands of triplex and the T_m was 43.5 ^{o} C (Figure 14). Upon incorporation of one L-*amp* T monomer at the C-terminus the Tm of the *amp*-PNA:DNA complex **36**:**47** (entry 2) lowered to 36.5 ^{o} ($\triangle T_m$ -7^o). However, incorporation of D-*amp* T monomer at the C-terminus as in the complex **42**:**47** led to a slight stabilization (entry 2, $\triangle T_m$ +2^o) of the corresponding complex over the control indicating the possible effect of stereochemistry. The presence of the L-*amp* T monomer at the N-terminus (**38**:**47**) showed less destabilization over the control by 1.5^o (entry 4).

Thus the L-*amp*-T modification either at the N or C terminal destabilized the triplex over the control. Interestingly, when two L-*amp*-T monomers were incorporated into the *aeg* PNA oligomers, one at the C-terminus and another in the center, an increase in the UV-T_m of the complex **37**:**47** was seen (entry 3, ΔTm = +6.5°, 3.2°/modification). But a similar increase in modification level with D-*amp*-T did not show any binding (**43**:**47**, entry 3). A complete replacement of the *aeg* PNA monomers with the modified *amp* PNA T monomers resulting in a *amp*-homooligomer also showed no binding with the complementary DNA (**39**:**47**, entry 5).

The combined effect of *amp*-C and *amp*-T monomers on PNA:DNA hybridization was studied by employing *amp*-PNA sequences (**41:44**) containing both

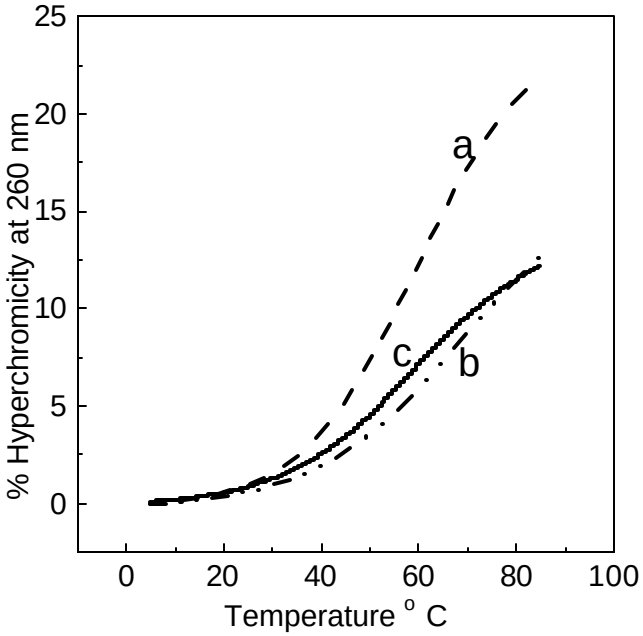


Figure 16. UV melting profile of (**a**) **40:48** (control), (**b**) **44:48** (with D-isomer), (**c**) **41:48** (with L-isomer)

modifications. The stoichiometry of complex as assessed by UV titration experiment indicated a 1:1 binding for DNA complexes with both control *aeg* and modified *amp*

PNA oligomers. (Figure 13). The UV-T_m plots for melting of mixed pyrimidine *amp*-PNA:DNA duplexes are shown in Figure 16 and the data in Table 3. The *aeg*-PNA:DNA complex exhibited a T_m of 55.1° at pH 7.3 (entry 1) while the tetramodified (2xT, 2xC) D-*amp*-T PNA:DNA complex (**44:48**) (entry 2) showed a significant enhancement in UV-T_m (65.6°, ΔT_m= +10°, 2.5° /modification). On the other hand, the corresponding L-*amp*-PNA:DNA duplex (**41:48**) exhibited a T_m of 59.1°, corresponding to a ΔT_m= +4° (entry 2). Either changing the stoichiometry to PNA:DNA 2:1 or lowering the pH did not have any effect on T_m suggesting the formation of 1:1PNA:DNA duplexes in these cases.

These results with mixed pyrimidine *amp*-PNA oligomers clearly indicate that the designed *amp* monomers stabilize the derived PNA:DNA duplexes, while homooligomeric *amp*-T PNAs capable of forming triplexes do not show any such stabilizations. The limited and preliminary data presented here point to the possibility that the stereochemical consequences of *amp* modifications are not conducive for

Table 3. UV-T_m (° C) of PNA:DNA complexes

Entry	L-major		D-major	
	PNA:DNA	**T_m (°C)**	**PNA:DNA**	**T_m(°C)**
1	**40:48**	55.1		
2	**41:48**	59.1	**44:48**	65.6

Buffer: 10 mM Na phosphate pH 7.3 Tm values are accurate to (±) 0.5°C. Experiments were repeated at least thrice and the Tm values were obtained from the peaks in the first derivative plots.

triplex formation. However, the derived PNA:DNA duplexes are stabilized even at the level of 4 modifications. Since the relative/absolute stereochemistries of amp-PNA monomers are not known at this stage, further experiments are needed for a

conclusive view on the *amp*-PNA oligomeric interactions. Nevertheless, the results presented here are quite encouraging to pursue further investigations.

5.5.2. Circular Dichroism Studies[23]

The CD spectrum gives information on overall conformational state of the nucleic acids.[24,25] PNA is achiral and hence not expected to show any CD, however when complexed with DNA/RNA which are chiral, the CD of PNA:DNA hybrids are amenable for analysis and can be used as a useful tool to study the PNA:DNA hybrids.[26]

PNAs containing chiral units show CD signals.[27] The CD spectra of PNA:DNA complexes are dominated by the chiral DNA component. The CD spectra of enantiomeric L-*amp* PNA single strand (Figure 17A) showed a somewhat mirror image relationship in 260-280 region with the D-*amp* PNA (**42**) showing a positive band at 270-280 nm and L-*amp* PNA (**36**) a negative band at 270-280 nm. However these bands are very weak. Upon complexation with DNA, both the *amp*-PNA and *aeg*-PNA hybrids showed similar CD patterns. The appearance of a band at 260 nm in both the cases is characteristic of PNA_2:DNA triplex formation.[26]

Figure 17B shows strong CD bands for DNA complexes with control *aeg* PNA oligomers as well as with *amp* PNA oligomers did not exhibit any difference in the band patterns or cross over points. The amplitude and sign of the CD bands are influenced by the stereochemistry of *amp* PNA monomers that the amplitude of CD bands is significantly influenced by the stereochemistry of *amp* PNA monomeric units.

The CD spectra of the *amp*-PNA:DNA triplexes exhibited similar CD characteristics as *aeg*-PNA:DNA triplexes with positive bands at 218 and 270 nm and negative bands at 205 and at 248 nm. (Figure 17B). Further the *amp*-PNA:DNA hybrid also showed an additional +ve band at 280 nm which was slightly more prominent than that of *aeg*-PNA. The mixed base *aeg/amp* PNA:DNA duplexes also exhibited

significant CD patterns, and in these as expected, no shoulders or distinct bands were seen at 260 nm attributed to triplex formation. The results over all suggested that in *amp*-PNA, no major alteration in chiral base stacking was seen as compared to *aeg*-

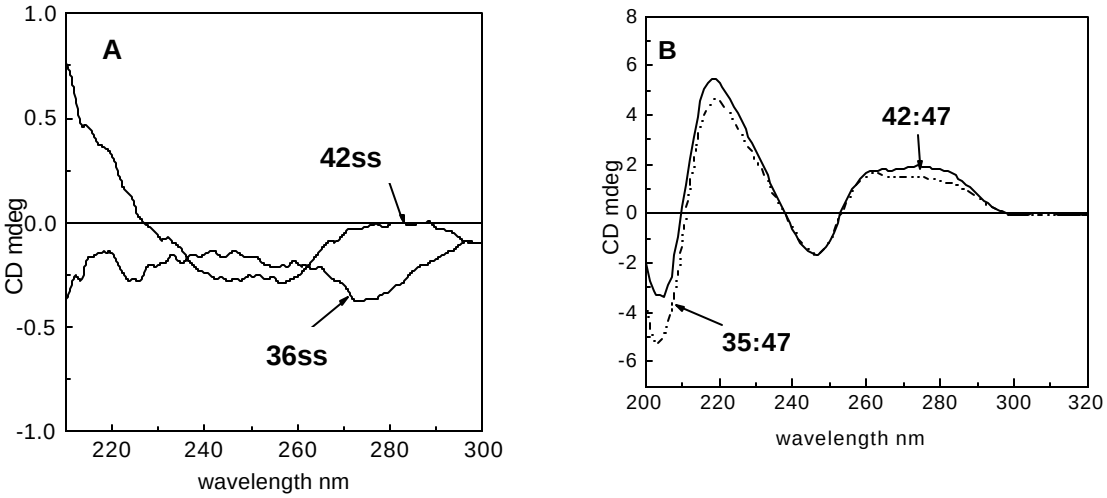


Figure 17. CD profile of (**A**) enantiomeric *amp* PNA single strands **36** and **42** (**B**) PNA_2:DNA complexes of control *aeg* PNA **35**, *amp* PNA **42** with cDNA **47**

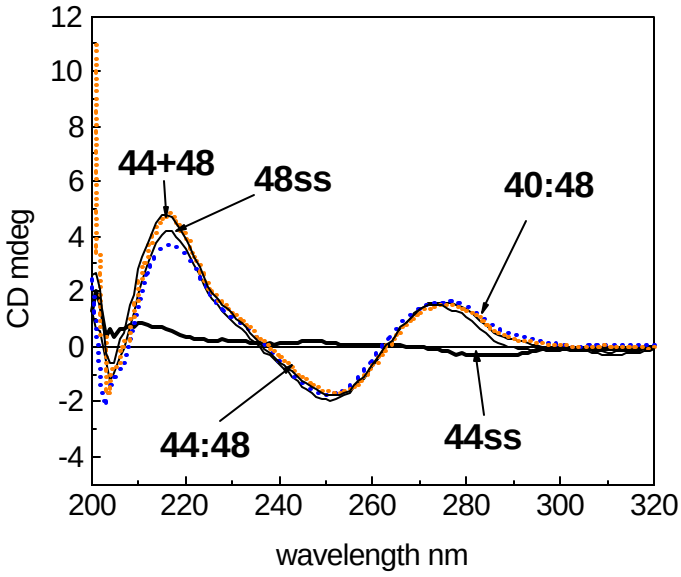


Figure 18. CD profile of duplexes with control **40:48**, **44:48** dna **48** single strand, *amp* PNA oligomer **44** and the addition spectrum of the constituent single strands **44** and **48.**

5.6. CONCLUSIONS

This chapter describes the synthesis and studies on a new PNA analog, chiral and conformationally constrained aminomethyl prolyl (*amp*) PNA. The monomers were synthesized starting from L/D proline, with additonal methoxy functionality at C5 being introduced by electrochemical anodic oxidation. This was then transformed to the aminomethyl group to form part of the chiral PNA backbone. The modified PNA monomers were introduced at desired places into the PNA oligomers by solid phase synthesis using submonomer strategy. This has the advantage for introduction of any of the bases during the solid phase synthesis, without making all pre-formed monomers. The biophysical studies with complementary DNA sequences carried out using UV and CD spectroscopic techniques indicated that these form compatible duplexes and triplexes with no major departure in conformation from unmodified *aeg*-PNA:DNA complexes. With mixed base pyrimidine (*amp*-T/C) sequences duplex stabilization was observed. More work is needed to develop these into useful PNA analogues.

5.7. EXPERIMENTAL

All reagents were obtained from commercial sources and used without further purification. NaH was obtained from Alrdich as 60% suspension in paraffin oil and the paraffin coating was washed off with pet-ether before use to remove the oil. The supporting electrolyte tetrabutyl ammonium tetrafluoroborate was obtained from alrdich and used as such without further purification. All the solvents were dried according to literature procedures. IR spectra were recorded on a Perkin Elmer 599B instrument.1H NMR (200MHz), 13C NMR (50 MHz) spectra were recorded on Bruker ACF200 spectrometer fitted with an Aspect 3000 computer. NOESY spectra were recorded on DRX 500 spectrometer with the mixing time 2.2 seconds. All chemical shifts are with

reference to TMS as an internal standard and are expressed in δ scale (ppm). The values given are directly from the computer printout. TLCs were carried out on (E.Merck 5554) precoated silicagel 60 F254 plates. TLCs were visualized with UV light and/or ninhydrin spray, followed by heating after exposing the HCl for the deprotection of the tert-butoxycarbonyl group. Optical rotations were measured on JASCODIP-181 polarimeter and CD spectra were recorded on a .All TLCs were run in pet-ether containing appropriate amount of ethyl acetate or dichloromethane containing appropriate amount of methanol to get the rf value 0.3. All the compounds were purified by flash column chromatography using 230-400 silical gel obtained from Sisco Research Laboratory. In NMR spectra that show splitting of peaks due to the presence of rotameric mixtures, arising from the tertiary amide linkage, the major rotamer is designated as maj. and the minor rotamer as min. The ratio of major:minor rotamers is 80:20 unless otherwise mentioned. In cases, where minor isomer is <10% only the peaks of major rotamer are reported. Meltings points of the compounds reported are uncorrected.

Thymine N1-acetic acid 2

To thymine **1** (5 g, 39.7 mmol) and potassium hydroxide (3.8 g, 79.4 mmol) in H_2O (30 mL) was added chloroacetic acid slowly (3.1 g, 39.7 mmol) in water (12 mL). The pH of the solution was adjusted and kept at 10 by dropwise addition of aq. KOH solution. After refluxing for 2h, the solution was cooled and acidified to pH 2 with conc. HCl. The resulting precipitate was filtered, washed with cold water and dried to obtain the crude product, recrystallized from water to get pure **2** (4.9 g, 66%) as a white solid. mp 258°C.(lit.[5] 260-261°).

N^4-Benzyloxycarbonyl cytosine 4

Cytosine **3** (1 g, 9 mmol) was suspended in dry pyridine (15 mL) and cooled to 0°C. To this was added bennzyloxycarbonyl chloroformate (3.0 mL, 18 mmol, 2 eq.) dropwise over a period of 15 min. under nitrogen atmosphere. The reaction mixture was stirred overnight. The pyridine suspension was evaporated to dryness in vacuo. To this were added water (10 mL) and 4N hydrochloric acid to bring the pH to 1. The resulting white precipitate was filtered off, washed with water and partially dried. The wet precipitate was boiled in absolute ethanol (25 mL) for 10 min. cooled to 0°C, filtered, washed thoroughly with ether, and dried, in vacuo to get **4** as a white solid. Yield 1.45 g, 67%.

^{1}H NMR (DMSO D_6): δ 7.85-7.70 (d, 1H, H6), 7.45-7.20 (m, 5H, aromatic), 7.00-6.80 (d, 1H, H5), 5.15 (s, 2H, benzyl CH_2). Mass (m/e) 245 (M^+).

Ethyl (N^4-Benzyloxycarbonyl- N^1cytosinyl) acetate 5

A suspension of N^4-Benzyloxycarbonyl cytosine **4** (0.5 g, 2 mmol) and K_2CO_3 (0.28 g, 2 mmol) in dry DMF was cooled to 0°C and ethyl bromoacetate (0.133 mL, 1.2 mmol, 0.6 eq.) was added dropwise and the mixture was stirred vigorously overnight, filtered and evaporated to dryness. Water (7 mL) and 4 N HCl (0.2 mL) were added and the mixture was stirred for 15 minutes at 0° C, filtered, and washed with water (2 x 10 mL). The isolated precipitate was purified by column chromatography on silica gel using ethylacetate/petether as an eluent to get the pure ester **5** as a white solid. Yileld 0.48g, 71%.

^{1}H NMR ($CDCl_3$): δ 7.75 (bs. NH), 7.60-7.45 (d, 1H, H6), 7.45-7.30 (m, 5H, aromatic), 7.30-7.20 (d, 1H, H5), 5.20 (s, 2H, benzyl CH_2), 4.60 (s, 2H, $N-CH_2$), 4.35-4.15 (q, 2H, $-CH_2$), 1.35-1.20 (t, 3H, CH_3). Mass (m/e) 331 (M^+).

N^4-Benzyloxycarbonyl cytosine-N^1-acetic acid 6

Ester **5** (0.25 g, 0.74 mmol) in water (1.5 mL) was treated with 2N NaOH (1.5 mL) for 15 min. filtered, cooled to 0°C and neutralized with 4N HCl (0.5 mL). The product acid was isolated by filtration and the precipitate was washed thoroughly with water to get the free acid **6** as a white solid. Yield 0.195 g, 92 %.

^{1}H NMR (DMSO D_6): δ 10.2 (bs, 1H), 8.10-7.90 (d, 2H, H6), 7.60-7.25 (m, 5H), 7.05-6.90 (d, 1H, H5), 5.2 (s, 2H, benzyl CH_2), 4.5 (s, 2H, N-CH_2).

Methyl (2S) N1-(*tert*-Butoxycarbonyl) Proline carboxylate 9

L-Proline **7** (10 g, 87 mmol) was suspended in methanol (100 mL) and cooled to 0°C with stirring. To this was added thinoyl chloride (7 mL, 95.7 mmol, 1.1 eq.) dropwise over a period of 10 min. Stirring was continued at 0°C for 4 h. and then at ambient temperature until the completion of the reaction (12 h). Removal of methanol under vacuum gave an oily ester hydrochloride salt **8** (14.1 g, 97.9%). This oil was used for the next step without further purification.

Ester hydrochloride **8** (10 g, 60.4 mmol) was redissolved in 1:1 dioxane/water (100 mL). To this were added triethylamine (23 mL, 166 mmol, 2.75 eq.) and $BocN_3$ (11.2 g, 78.5 mmol, 1.3 eq.) and the mixture was stirred at 50° C for 24 h. under argon atmosphere. After the completion of the reaction, the mixture was concentrated to a paste by rotary evaporation, subjected to usual work up and extracted with ether (3 x 50 mL). Ether layer was washed with water, followed by brine and dried over sodium sulphate and concentrated to get an yellow oil. This was purified by column chromatography using 100-200 silica gel and 10% ethyl acetate-petroleum ether as eluent to get **9** as a pale yellow oil. Yield 12.1 g, 82.9%.

^{1}H NMR ($CDCl_3$): δ 4.35-4.10 (m, 1H, H2), 3.65 (s, 3H, -OCH_3), 3.65- 3.25 (m, 2H, H5), 2.30-1.65 (M, 4H, H3 & H4), 1.45-1.35 (d, 9H, -$(CH_3)_3$).

Methyl (2S)-N1-(*tert*-Butoxycarbonyl)-5-methoxy proline carboxylate 10

N-(*tert*-Butoxycarbonyl)-L-proline methyl ester **9** (8 g, 34.9 mmol) was dissolved in a 0.5 M solution of tetrabutylammonium tetrafluoroborate in methanol (100 mL). The reaction flask was cooled to 5° C in an ice bath. The stirred solution was oxidized at a carbon anode and cathode using a constant current (270 mA). After the completion of reaction (6 h.) solvent was evaporated under reduced pressure and the residue was treated with ether (3 x 75 mL) leaving the supporting electrolyte as a crystalline solid. The combined ether layers were concentrated under vacuum to get the crude product as an oil which was purified by flash chromatography on 230-400 silica gel by isocratic elution using 10% ethyl acetate/petroleumether as eluant to get the methoxylated product **10** (7.8 g, 86%).

^{1}H NMR ($CDCl_3$) δ: 5.35-5.10 (m, 1H, H2), 4.45-4.15 (m, 1H, H5), 3.80-3.70 (m, 3H, -OCH_3 of ester), 3.45-3.30 (m,3H, -OCH_3), 2.50-1.65 (m,4H, H3 & H4), 1.55-1.35 (d, 9H, Boc methyl).^{13}C NMR ($CDCl_3$) δ: 173.0, 172.8 (-C=O Boc); 153.9 (-C=O ester); 89.7, 88.4 (C5); 80.3 (tertiary carbon); 59.1, 58.7 (C2); 55.9, 55.6, 54.9; 51.64 (-OCH_3); 32.3, 30.9, 30.0, (C3); 28.0 (Boc methyl); 26.9 (C4).

Methyl (2S)-N1-(*tert*-butoxycarbonyl)-5-cyano proline carboxylate 11a & b

The C5-methoxy product **10** (2.46.0 g, 9.5 mmol) was dissloved in anhydrous dichloromethane (25 mL) and cooled to –35°C by a cryostat. To this was added 1% TMSTf (0.25 mL) followed by slow addition of TMSCN (1.46 mL, 10.9 mmol, 1.15 eq.) at –35 °C while stirring. After the completion of the reaction (10 minutes) methanol was added to the reaction mixture and the solvents were removed by rotary evaporation. The residue was purified by flash column chromatography using 10% ethylacetate-petether as eluant to get the diastereomeric nitriles **11a** (major isomer**,** lower spot on tlc

R_f 0.28 in 10% ethylacteate-petroleum ehter) 1.43 g & **11b** (minor isomer, top spot on tlc R_f 0.3 in 10% ethylacetate-petroleum ether) 0.69 g. (68:32).

Major isomer 11a

IR cm^{-1} (neat): 2246, 1755, 1747, 1713. ^1H NMR ($CDCl_3$): δ 4.70-4.50 (m, 1H, H2), 4.45-4.20 (m, 1H, H5), 3.75 (s, 3H, ester –OCH_3), 2.45-2.10 (m, 4H, H3 & H4), 1.55-1.35 (d, 9H, $(CH_3)_3$. ^{13}C NMR ($CDCl_3$): δ 171.7, 171.5 (Boc. carbonyl), 152.1 (ester carbonyl), 118.1 & 117.9 (cyano), 81.8 & 81.4 (C-$(CH_3)_3$) 59.1 & 58.5 (C2), 51.9 (C5), 47.3 (COOCH_3), 30.0, 29.5, 29.2 (C3), 28.3 (C4), 27.8 (Boc. $(CH_3)_3$). Mass (m/e): M^+ 254, 195 (100 %), 181 (35 %), 153 (38 %), 95 (100 %). $[\alpha]_D^{25\,o}$ +41.8° (C = 0.665, $CHCl_3$).

Minor isomer 11b

^1H NMR ($CDCl_3$): δ 4.80-4.55 (m, 1H, H2), 4.50-4.25 (m, 1H, H5), 3.70 (s, 3H, OCH_3), 2.60-2.00 (m, 4H, H3 & H4), 1.60-1.30 (d, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 171.9 & 171.7 (Boc. carbonyl), 152.3 (ester carbonyl), 118.4 (cyano), 81.7 & 81.3 (C$(CH_3)_3$), 58.6 & 58.2 (C2), 51.9 (-OCH_3), 47.4 & 47.2 (C5), 29.4 & 29.1 (C3), 28.5 & 28.1 (C4), 27.7 & 27.4 ((CH_3)$_3$).Mass (m/e): M^+ 254, 195 (25 %), 95 (100 %). $[\alpha]_D^{25\,o}$ –93.7° (C = 0.365, $CHCl_3$).

Methyl (2*S*)-N1-(*tert*-butoxycarbonyl)-(5-aminomethyl) proline carboxylate 12

The major isomer of the cyano compound **11a** (1 g, 3.9 mmol) was dissolved in methanol (5 mL) and hydrogenated at 50 psi H_2 pressure at room temperature using $Pd(OH)_2$ (0.2 g) as a catalyst. After the completion of the reaction (5 h) the catalyst was filtered off by passing through celite and the filtrate was concentrated under vacuum to get the amino ester **12**. Yield: 0.96 g, 96 %.

^{1}H NMR ($CDCl_3$): δ 4.40-4.10 (m, 1H, H2), 4.10-3.80 (m, 1H, H5), 3.70 (s, 3H, $\underline{CH_3}$), 3.05-2.70 (m, 2H, CH_2), 2.40-1.80 (m, 6H, 2 are exchangeable), 1.50-1.25 (s, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 173.4 ($\underline{CO}OC(CH_3)_3$), 153.8 & 153.4 ($\underline{CO}OMe$), 79.9 & 79.7, 76.9, 59.7 & 59.3, 57.7 & 57.4, 51.9 & 51.5, 44.3, 28.5, 27.8, 17.9.

Dimer 13

^{1}H NMR ($CDCl_3$): δ 7.80-7.70 (d, 2H), 7.70-7.50 (m, 2H), 7.50-7.30 (m, 4H), 4.90-4.10 (m, 6H), 3.90-2.75 (m, 6H), 2.40-2.15 (m, 2H), 2.15-1.75 (m, 3H), 1.70-1.50 (m, 1H), 1.50-1.10 (d, 18H). FAB Mass: 716 (M^++Na).

(2S -N1-(*tert*-butoxycarbonyl))-[5-(flourenylemthoxycarbonyl)aminomethyl] Proline 14[28]

The proline methyl ester **12** (0.4 g, 1.55 mmol) was dissolved in methanol (2 mL) and hydrolysed using 2N aq. NaOH. After 30 minutes excess of sodium hydroxide was neutralized using potassium bisulfate and the pH was adjusted to 7.0. Methanol was removed by rotary evaporation and the residue was redissolved in 10% Na_2CO_3 (2 mL) The reaction mixture was cooled to 0 ^{o}C in an ice-bath. To this was added 2 mL of dioxane (peroxide free) followed by the slow addition of Fmoc-Cl (0.44 g, 1.7 mmol, 1.1 eq.) in dioxane at 0^o C. Stirring was continued at 0^o C for 4 h. followed by room temperature stirring for 18 h. The reaction as monitored by TLC, after the completion of the reaction contents were poured in ice- water and extracted with ether (2 x 20 mL) to remove the unreacted chlroformate. The aqueous phase was chilled in ice and acidified by the addition of saturated $KHSO_4$. The pH of the solution was brought to 2.0 at which the compound started getting separated as foam. This was then extracted with ethyl acetate (3 x 10 mL) and dried over $MgSO_4$ and the solvent was removed under vacuum to get the crude product as a solid. This was purified by flash column chromatography[29]

on 230-400 silica gel using 4% MeOH/CH_2Cl_2 (0.3 R_f) as eluant to get 0.27 g, 37 % of the desired product **14** along with undesirable **13** as the major product 0.48 g, 45 %.

^{1}H NMR ($CDCl_3$): δ 7.85-7.15 (m, 8H, aromatic), 6.25-6.15 (s, 1H, -NH), 4.60-3.95 (m, 5H, -CH, -CH_2, -CH_2NH), 3.55-3.30 (m, 2H, H5 & H2), 2.45-1.70 (9m, 4H, H3 & H4), 1.40 (s, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 156.6, 143.3 & 143.1, 140.8 & 140.5, 126.8 & 126.2, 124.4 & 124.0, 119.1, 80.4, 66.2, 59.6, 57.5& 57.3, 46.4, 43.7, 42.8, 28.9 & 28.2, 27.4. FAB Mass: 489 (M^++Na), 467 (M^++1), 179 (100 %).

Methyl (2R)-N1-(*tert*-Butoxycarbonyl) proline carboxylate 17

D-proline (6 g, 52.2 mmol) was esterified using methanol (60 mL) and thionyl chloride (4.2 mL, 57.4 mmol, 1.1 eq.) as discussed in the case of compound **9**. Removal of methanol gave the ester **16** as a hydrochloride salt (8.30 g, 96 %). This was used as such for the next step without further purification. The ester **9** (5 g, 30.2 mmol) was dissolved in in 1:1 dioxane/water, treated with triethylamine (10.5 mL, 75.5 mmol, 2.5 eq.) and $BocN_3$ (5.2 mL, 36.2 mmol, 1.2 eq.) under argon atmosphere at 50 ^{o}C. Usual work up and purification as mentioned for the L-isomer gave the tert-butoxycarbonyl derivative **17**. Yield: 6.1 g, 88 %.

IR cm^{-1} (neat): 2977, 1747, 1699.^{1}H NMR ($CDCl_3$): δ 4.35-4.10 (m, 1H, H2), 3.72 (s, 3H, CH_3), 3.60-3.25 (m, 2H, H5A & H5B), 2.30-1.70 (m, 4H, H3 & H4), 1.38 (d, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 173.3 (Boc. carbonyl), 153.4 (ester carbonyl), 79.2 (C(CH_3)$_3$), 58.8 & 58.5 (C2), 51.5 (-OCH3), 46.1 (C5), 30.6 & 29.6 (C3), 27.9 ((CH_3)$_3$), 23.4 (C4). $[\alpha]_D^{25o}$ +61.3^o (C = 1.135, $CHCl_3$).

Methyl (2R)-N1-(*tert*-Butoxycarbonyl)-5-methoxy proline carboxylate 18

The ester **17** (4.5 g, 19.7 mmol) was oxidized electrochemically to get the 5-methoxy product **18** as a diastereomeric mixutre after the work up and purification as mentioned for the L-isomer **10**. Yield 4.4 g, 88%.

^{1}H NMR ($CDCl_3$): δ 5.35-5.05 (m, 1H, H2), 4.40-4.15 (m, 1H, H5), 3.80-3.60 (m, 3H, ester methyl), 3.50-3.25 (m, 3H, methoxy), 2.50-1.70 (m, 4H, H3 & H4), 1.60-1.30 (d, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 159.5 (Boc. carbonyl), 153.5 (ester carbonyl), 88.7, 88.0, 87.9, 80.1, 79.9, 59.1, 58.7,& 58.3, 55.5, 55.3 & 54.7, 51.7 & 51.4, 47.7, 32.4, 31.7, 30.6, 29.6, 27.6, 26.5.

Methyl (2R)-N1-(*tert*-Butoxycarbonyl)- 5-cyano proline carboxylate 19a & 19b

Treatment of methoxy compound **18** with TMSCN and TMSTf (1 %) in anhydrous dichloromethane at –35 °C gave the diastereomeric mixture of nitriles which were purified by flash chromatography to get the nitriles major isomer **19a** and minor isomer **19b** in 68:32 ratio.

Major isomer 19a

IR cm^{-1} (neat): 2934, 2243, 1747, 1715. ^{1}H NMR ($CDCl_3$): δ 4.73-4.45 (m, 1H, H2), 4.45-4.20 (m, 1H, H5), 3.75 (s, 3H, ester methyl), 2.50-2.05 (m, 4H, H3 & H4), 1.65-1.35 (d, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 171.3 & 171.1 ($COOC(CH_3)_3$), 151.7 ($COOCH_3$), 117.8 & 117.6 (C≡N), 80.8 & 80.4 ($C(CH_3)_3$), 58.6 & 58.3, 51.4, 46.8, 29.5, 28.9 & 28.8 (C3), 27.8 (C4), 27.1 ($(CH_3)_3$). $[\alpha]_D^{25^o}$ - 41.9° (C = 0.315, $CHCl_3$)

Minor isomer 19b

IR cm^{-1} (neat): 2243, 1747, 1716. ^{1}H NMR ($CDCl_3$): δ 4.80-4.55 (m, 1H, H2), 4.50-4.25 (m, 1H, H5), 3.70 (s, 3H, CH_3), 2.60-2.00 (m, 4H, H3 & H4), 1.60-1.25 (d, 9H, 3 x CH3). ^{13}C NMR ($CDCl_3$): δ 172.0 & 171.7 ($COO(CH_3)_3$), 152.4 (COOMe), 118.5 (C≡N), 81.6 & 81.2 ($C(CH_3)_3$), 58.7 & 58.3, 51.9, 47.5 & 47.3, 29.5 & 29.2 (C3), 28.6 & 28.2 (C2), 27.7 ($(CH_3)_3$). $[\alpha]_D^{25^o}$ + 101.5° (C = 0.476, $CHCl_3$).

Methyl (2*R*)-N1-(*tert*-butoxycarbonyl)-5-(aminomethyl) proline carboxylate 20

IR (neat): ^{1}H NMR ($CDCl_3$): δ 4.50-4.10 (m, 1H, H2), 4.10-3.80 (m, 1H, H5), 3.70 (S, 3H, CH_3), 3.10-2.60 (m, 4H, -CH_2, 2 are exchangeable), 2.30-1.85 (m, 4H, H3 & H4). 1.55-1.30 (d, 9H, 3 x CH_3).

(2*R*)- N1-(*tert*-butoxycarbonyl)-[5-(flourenylmethoxycarbonyl)aminomethyl] Proline 22

^{1}H NMR ($CDCl_3$): δ 7.80-7.62 (d, 2H, aromatic), 7.62-7.45 (d, 2H, aromatic), 7.45-7.15 (m, 4H, aromatic), 6.20-5.95 (s, 1H, -NH), 4.60-3.90 (m, 5H, -CH, -CH_2, CH_2), 3.60-3.25 (m, 2H), 2.45-1.65 (m, 4H, H3 & H4), 1.40 (s, 9H, 3 x CH_3). ^{13}C NMR ($CDCl_3$): δ 176.6, 157.5 & 157.2, 154.9, 154.5, 143.8, 141.1, 127.5, 126.9, 125.1, 119.8, 81.0, 66.9, 60.1, 58.1, 47.1, 43.9, 28.9, 28.2. Mass M^++Na 489 (26%), 367 (100%).

N1-(*tert*-Butoxycarbonyl)-1,2-diaminoethane 24

1,2-Ethylenediamine **23** (20g, 0.33 mmol) was taken in dioxane:water (1:1, 500ml), and cooled in an ice bath. Boc azide (5g, 35 mmol) in dioxane (50 mL) was slowly added with stirring and the pH was maintained at 10.0 by continuous addition of 4N NaOH. The mixture was stirred for 8 h. and the resulting solution was concentrated to 100 mL. The N^1,N^2-di-tert-butoxycarbonyl derivative not being soluble in water, precipitated and could be removed by filtration from the corresponding N1-mono-*tert*-butoxycarbonyl derivative. Removal of the solvent by rotaryevaporation yielded the mono Boc ethylenediamine **24** (3.45 g, 63%).

^{1}H NMR ($CDCl_3$) δ: 5.21 (brs, 1H, NH), 3.32 (t, 2H, J = 8 Hz,), 2.54 (t, 2H, J = 8 Hz), 1.42 (S, 9H).

Ethyl N-(2-*tert*-Butoxycarbonyl aminoethyl) glycinate 25

N1-mono-*tert*-Butoxycarbonyl-1,2-diaminoethane **24** (3.2 g, 20 mmol) was treated with bormoethyl acetate (2.25 mL, 20 mmol) in acetonitrile (100mL) in presence of K_2CO_3 (2.4 g, 20 mmol) and the mixture was stirred at an ambient temperature for 5 h. The solid that separated was removed by filtration and filtrate was evaporated to obtain **25**. (4.3 g, 83%) as a colorless oil.

^{1}H NMR, ($CDCl_3$) δ: 5.02 (br, 1H), 4.22 (q, 2H, J=8 Hz) 3.35 (s, 2H), 3.20 (t, 2H, J=6 Hz) 2.76 (t, 2H, 6 Hz) 1.46 (s,9H), 1.28 (t, 3H, J=8 Hz)

Ethyl N1-(*tert*-butoxycarbonyl aminoethyl)-N-(chloroacetyl) glycinate (26):

The above ester **25** (4.0 g, 14 mmol) was taken in 10% aq. Na_2CO_3 (75 mL) and dioxane (60 mL). Bromoacetyl chloride (6.5 mL, 0.75 mmol) was added in two portions with vigorous stirring.[30] After the completion of the reaction (5 min.) pH of the reaction mixture was brought to pH 8 by adding 10% aq. Na_2CO_3 and concentrated to remove dioxane. The product was extracted with dichloromethane and concentrated. The crude product was purified by column chromatography to obtain **26** as a colorless oil in good yield (4.2 g, 80%).

^{1}H NMR ($CDCl_3$) δ: 5.45 (br s, 1H), 4.14 (s, 2H), 4.00 (s, 2H), 3.53 (t, 2H), 3.28 (q, 2H, J=8 Hz), 1.46 (s, 9H), 1.23 (t, 3H, J=8 Hz). Mass (m/e): 380 (M^++1).

N-[(*tert*-Butoxycarbonyl) aminoethylglycyl] thymine ethyl ester 27

N-Chloroacetylglycinate **26** (4.0 g, 11.6 mmol) was stirred with anhydrous K_2CO_3 (1.56 g, 11.8 mmol) in DMF with thymine (1.4 g, 11.2 mmol) to obtain the desired thymine monomer **27**. DMF was removed under reduced pressure and the oil obtained was purified by column chromatography.

^{1}H NMR ($CDCl_3$) δ: 9.00 (br, 1H, NH), 7.05 (min) 6.98 (maj) (s, 1H, T-CH), 5.65 (maj), 5.05 (min) (br, 1H, NH), 4.58 (maj), 4.44 (min) (s, 1H, T-CH2), 4.20 (min), 4.05 (maj) (s, 1H, T-CH_2), 4.25 (m, 2H, OCH_2), 3.55 (m, 2H), 1.95 (s, 3H, T-CH_3), 1.48 (s, 9H), 1.29 (m, 3H).^{13}C NMR ($CDCl_3$) δ: 170.8, 169.3, 167.4, 164.3, 156.2, 151.2, 141.1, 110.2, 79.3, 61.8, 61.2, 48.5, 48.1, 47.7, 38.4, 28.1, 13.8, 12.2.

N-(*tert*-Butoxycarbonyl -aminoethylglycyl)-N^4-(benzyloxycarbonyl) cytosine ethyl ester 28

A mixture of NaH (.25 g, 6.2 mmol) and N-4-(benzyloxycarbonyl) cytosine **4** (1.24 g, 6.2 mmol) was taken in DMF and stirred at 75^{o}C till the effervescence ceased. The mixture was cooled and **26** (2.0 g, 6.1 mmol) was added to obtain the required cytosine monomer **28**. Yield 1.62 g, 50%.

^{1}H NMR ($CDCl_3$) δ: 7.65 (d, 1H, H6, J=8Hz), 7.35 (s, 5H, Ar), 7.25 (d, 1h, H5, J=8), 5.70 (br, 1H, NH), 5.20 (s, 2H, $ArCH_2$), 4.71 (ma) and 4.2f2 (br s, 2H), 4.15 (q, 2H), 4.05 (s, 2H), 3.56 (m, 2H), 3.32 (m,2H), 1.48, s,9H), 1.25 (t, 3H).

N-(*tert*-Butoxycarbonyl aminoethylglycyl) adenine ethyl ester 29

NaH (0.246 g, 6.1 mmol) was taken in DMF (15 mL) and adenine (0.8 g, 6.1 mmol) was added. This mixture was stirred at 75 ^{o}C till the effervescence ceased and the mixture was cooled before adding **26**. (2.0 g, 6.1 mmol). The reaction mixture was heated at 75 ^{o}C for 1h and DMF was removed. The thick oil was taken in water and extracted with ehtyl acetate and purified by column chromatography to obtain the product **29** *aeg*-A monomer.

^{1}H NMR ($CDCl_3$) δ: 8.32 (s, 1H), 7.95 (min), 7.90 (maj), (s, 1H), 5.93 (maj) and 5.80 (min), (br, 2H), 5.13 (maj) and 4.95 (min), 4.22 (min) and 4.05 (maj) (s, 2H), 4.20 (m, 2H), 3.65 (maj) and 3.55 (min), (m, 2H), 3.40 (maj) and 3.50 (min), (m, 2H), 1.42 (s, 9H), 1,25 (m,3H).

N-(tert-Butoxycarbonyl-aminoethylglycyl)-2-amino-6-chloropurine ethyl ester 30

A mixture of 2-amino-6-chloropurine (1.14 g, 6.8 mmol), K_2CO_3 (0.932 g, 7 mmol) and **26** (2.4 g, 7 mmol) were taken in DMF (20 mL) and stirred at room temperature for 4 h. K_2CO_3 was removed by filtration and DMF was removed under reduced pressure. The resulting residue was purified by column chromatography to obtain **30** in excellent yield (2.65 g, 98%).

^{1}H NMR ($CDCl_3$) δ: 7.89 (min) and 7.85 (maj) (s, 1H), 5.80 (br s, 1H), 5.18 (br s, 2H), 5.02 (maj) and 4.85 (min) (s, 2H), 4.18 (min) and 4.05 (maj) (s,2H), 3.65 (maj) and 3.16 (min) (m, 2H), 3.42 (maj) and 3.28 (min) (m, 2H), 1.50 (s, 9H), 1.26 (m,3H).

Hydrolysis of the Ethyl esters of PNA monomers (27-30): General Method

Ethyl esters of PNA monomers were hydrolysed using 2N aq. NaOH (5 mL) in methanol (5 mL) and the resulting sodium salts of the acids were neutralized with Dowex-50 H$^+$ till the solution showed neutral pH. Resin was then removed by filtration and the filtrate was concentrated to obtain the resulting Boc-protected acids (**31-34**) in good yield (>85%).

Functionalization of the Merrifield's Resin

N-*tert*-Boc β-alanine was dissolved in ethanol (10 mL) and neutralized with saturated aqueous solution of cesium carbonate.[31] Ethanol was evaporated under vacuum and co-evaporating with toluene as an azeotrope dried the residue. The cesium salt of N-(*tert*-butoxycarbonyl)-β-alanine and Merrifield resin (3 g, 0.7 m eq./g) were suspended in anhydrous DMF (3 mL) and slowly stirred at 60^o C in an oil bath. After 24 h. the resin was filtered, washed successively with DMF (20 mL), water (20 mL), DMF (20 mL), methanol (50 mL) followed by DCM (50 mL) and dried under vacuum.

Picric acid assay for the estimation of the amino acid loading[32]

The functionalized dry resin (5 mg) was taken in a sintered funnel and swelled in CH_2Cl_2 for 1 h. The solvent was drained off and the resin was treated with 50% TFA/DCM for 15 min (1 mL x 2) each time. The resin was thoroughly washed with CH_2Cl_2 and the TFA salt was neutralized with 5% diisopropyl ethylamine 2 min (1 mL x 3). The free amine was treated with 0.1 M picric acid in DCM 3 min (2 mL x 3) each time. The resin was thoroughly washed with CH_2Cl_2 to remove the unbound picric acid. The picrate bound to amino groups was eluted with 5% diisopropylethyl amine in CH_2Cl_2, followed by washing with CH_2Cl_2. The eluant was collected into a 10 mL vol. flask and made up to 10 mL using CH_2Cl_2. An aliquot (0.2 mL) of picrate eluant was diluted to 2 mL with ethanol and the optical density was measured at 358 nm, and the loading value of the resin (0.61meq/g) was calculated using the molar extinction coefficient of picric acid as ε_{358}=14,500 cm^{-1} M^{-1} at 358 nm. The loading value of the resin was reduced to 0.27 meq/g by partial capping with acetic anyhydride in pyridine and reestimating the loading value as mentioned earlier.

General Method for Solid Phase Peptide Synthesis (SPPS)

All PNA oligomers were assembled using SPPS.[33] Merrifields resin preloaded with β-alanine (0.27 meq/g) was used for the synthesis of the *aeg* PNA and *amp* PNA oligomers. 3 eq.of *aeg*/*amp* PNA monomers, 3 eq. of HOBt and 3 eq of DIPCDI and Thymine/Cytosine acetic acids were used for the coupling reaction. The *tert*-butoxycarbonyl protected amino acids were deprotected with 50%TFA-CH_2Cl_2.In case of *amp* PNA monomers Fmoc group was deprotected using 20% piperidine in DMF followed by TFMSA cleavage. Benzyloxy carbonyl group present in the side chains of cytosine, adenine and guanine were also deprotected in the end during TFMSA treatment.

Kaiser's test[34]

Kaiser's test was carried out for monitoring the completion of Boc/Fmoc deprotection and completion of the coupling steps. For this three reagents were used. (1) Ninhydrin (5.0 g) dissolved in ethanol (100 mL), (2) Phenol (80 g) dissolved in ethanol (20 mL) and (3) Cyanide, 2 mL of 0.01M KCN diluted to 100 mL with pyridine (33 mg KCN dissolved in 50 mL water, 2 mL of this diluted with 98 mL pyridine). The procedure consisted of adding 3 drops each of these reagents to a few beads of the resin to be tested in a test tube and heating to 100° C for 5 minutes. An intense blue color of the beads indicated the presence of free amine after deprotection and after coupling the test showed colorless beads and yellow solution.

Cleavage of the peptides from the solid support

The cleavage of the resin was carried out by taking the resin-bound oligomer (10mg) in a glass vial and stirring with thioanisole (20 μL) and 1,2-ethanedithiol (8 μL) in an ice-bath for 10 min. After the equilibration with TFA (120 μL) for 10 min. TFMSA (16 μL) was added and the stirring continued for another 2 h. The reaction mixture was filtered through a sintered funnel and the resin was washed with TFA (3 x 2 mL) and the combined filtrate and TFA washings were evaporated and coevaporated with ether without heating. The residue was precipitated using anhydrous ether and centrifuged to get a white precipitate free from scavengers. The precipitate was dissolved in methanol and re-precipitated with ether to get the crude PNA oligomer.

Gel filtration

The crude PNA oligomers were dissolved in 300 μL of water and desalted by passing through a column containing Sephadex G25 matrix having a void volume of 10 mL. Oligomers were eluted with water collecting 10 fractions of 1 mL each. The

absorbance in each fraction was measured at 260 nm to detect the presence of the oligomer in the fractions. The purity of oligomers in every fraction was determined by RP HPLC on a C18 column. The fractions of identical purity were combined and the impure fractions were subjected to purification by semi-preparative HPLC on RP C4 column (isocratic elution) or by FPLC semi-preparative RP C8 column (gradient elution). 8-11% acetonitrile buffer containing 0.1% TFA was used for the HPLC.

Fast Protein/polynucleotide Liquid Chromatography (FPLC)

Crude oliogmers were dissolved in water containing 0.1% TFA. The polypyrimidine sequences were purified using an ascending gradient of 0-50% buffer B in 30 min at a flow rate of 1.0 mL/min, where buffer A = water with 0.1% TFA and buffer B = 60% CH_3CN in water containing 0.1% TFA. The mixed sequence PNAs eluted faster and hence had to be purified using a gradient of 0 to 30% B in 30 min at a flow rate of 1.0 mL/min. The purity was rechecked by HPLC and found to be satisfactory.

High Performance Liquid Chromatography (HPLC)

HPLC was used for both checking the analytical purity as well as for the purification of the amp PNA oliogmers. Purity of the homopyrimidine PNA oligomers was ascertained on an analytical RP C18 column using a gradient of 5-80% CH_3CN in water containing 0.1% TFA at a flow rate of 1.5 ml/min. The oligomers were purified on a semi-preparative RP C4 column using 8-12% CH_3CN in water containing 0.1% TFA to obtain the optimum separation of the constituent peaks and the purified oligomers were rechecked for purity on RP C18 column.

MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry was used for the molecular weight determination of the PNA oligomers as reported in the literature.[35] For this analysis

several matrices have been used viz. sinapinic acid (3,5-dimethoxy-4-hyroxycinnamic acid), CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid). Of these, sinapinic acid was found to give the best signal to noise ratio with all the other matrices typically producing higher molecular ion signals.

5.8. REFERENCES

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- Compound **11b** ^{1}H NMR Page 253
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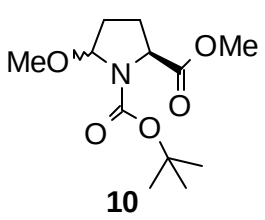
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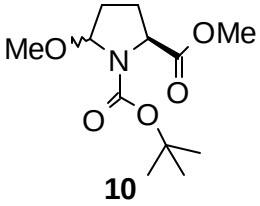

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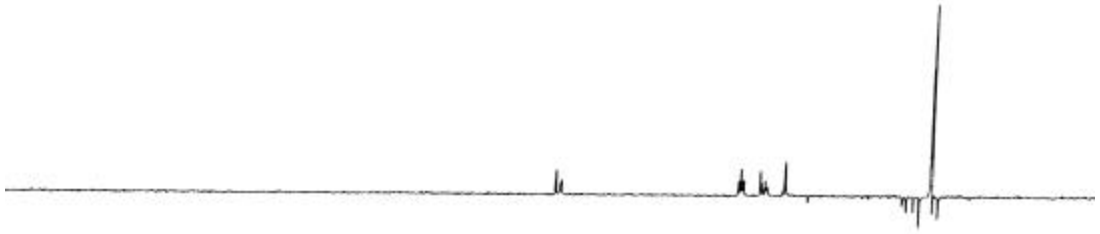

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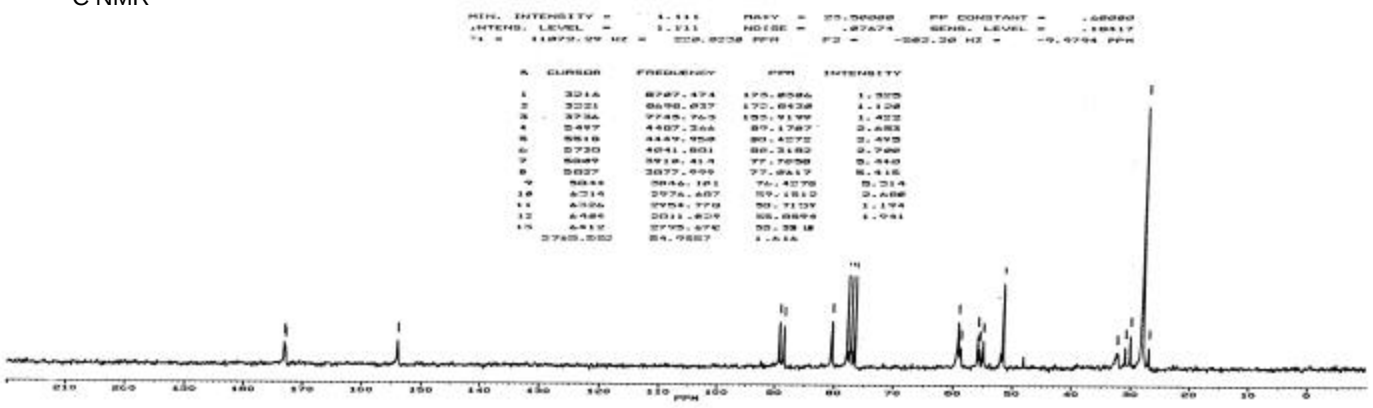

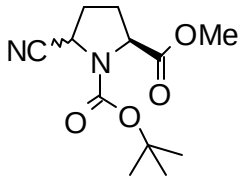
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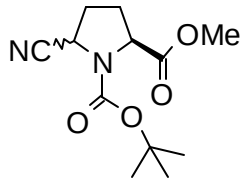

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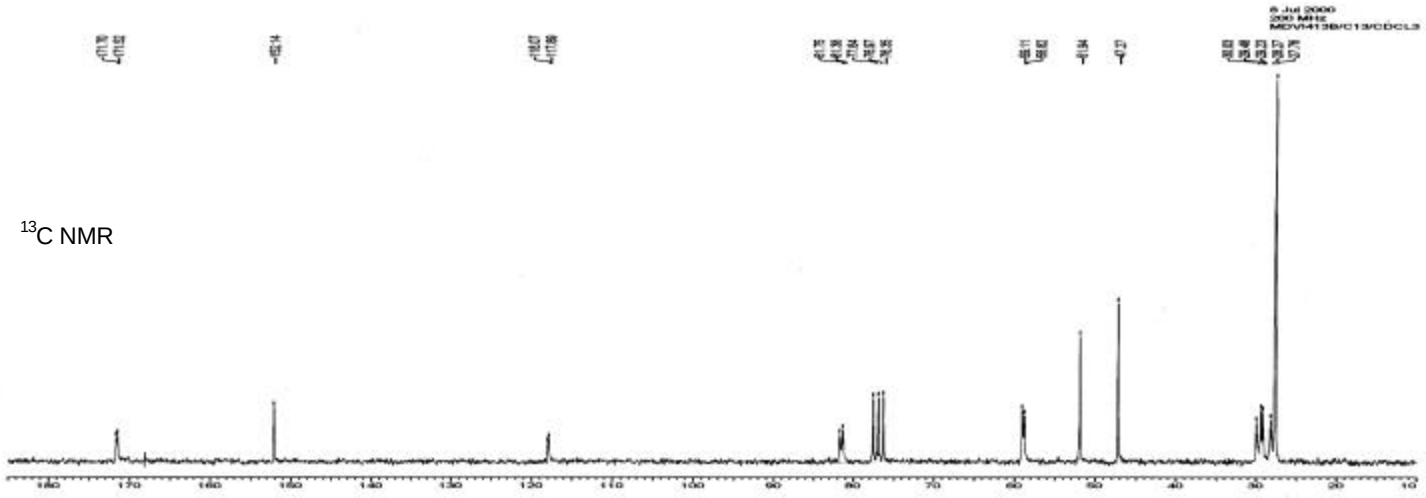

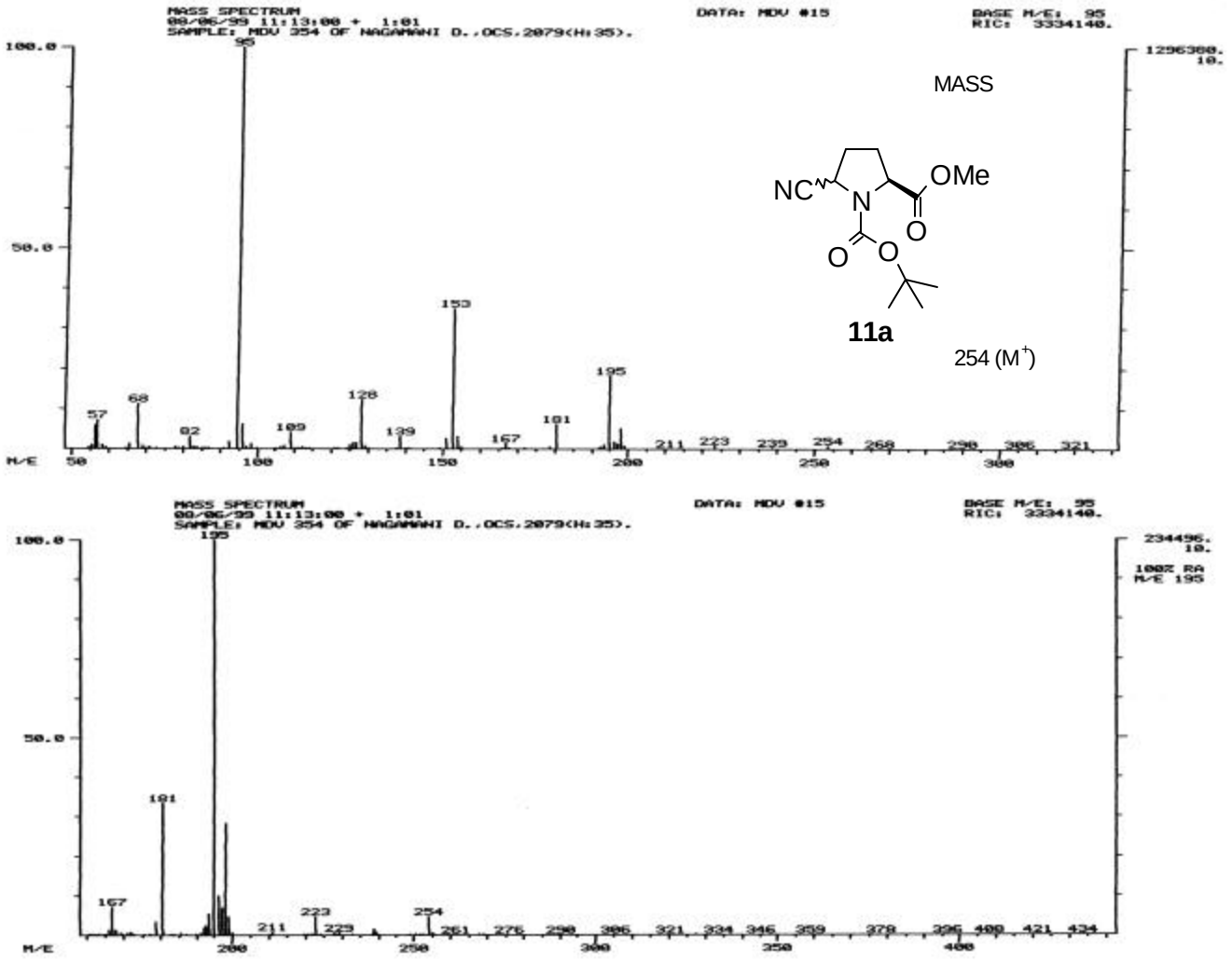
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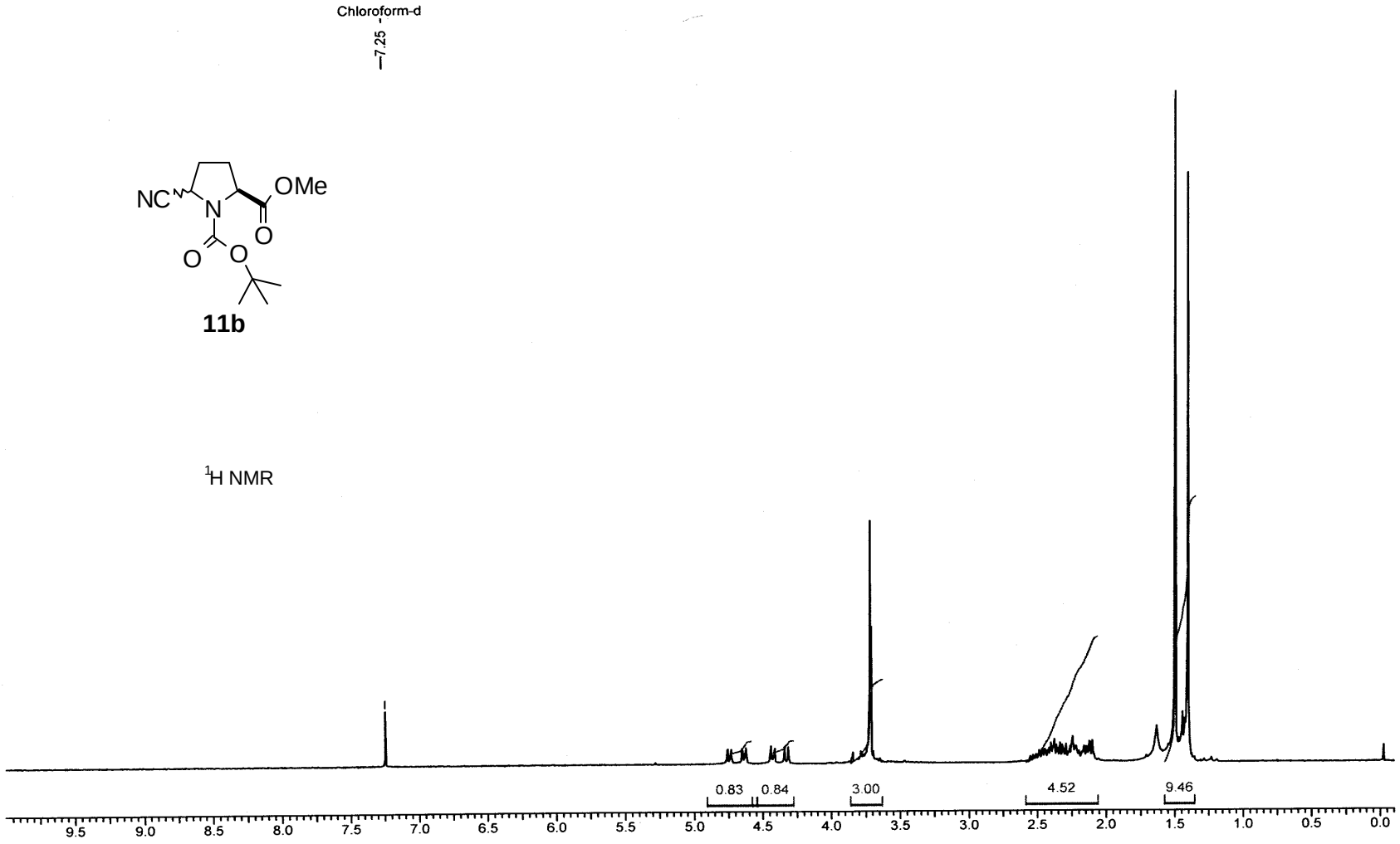
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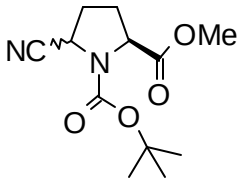
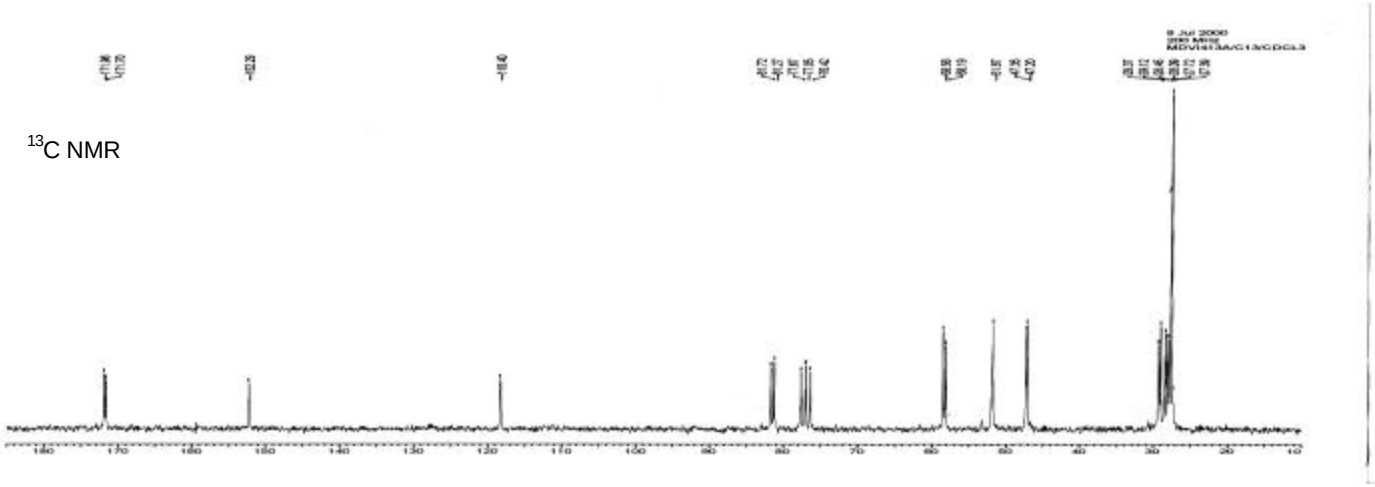
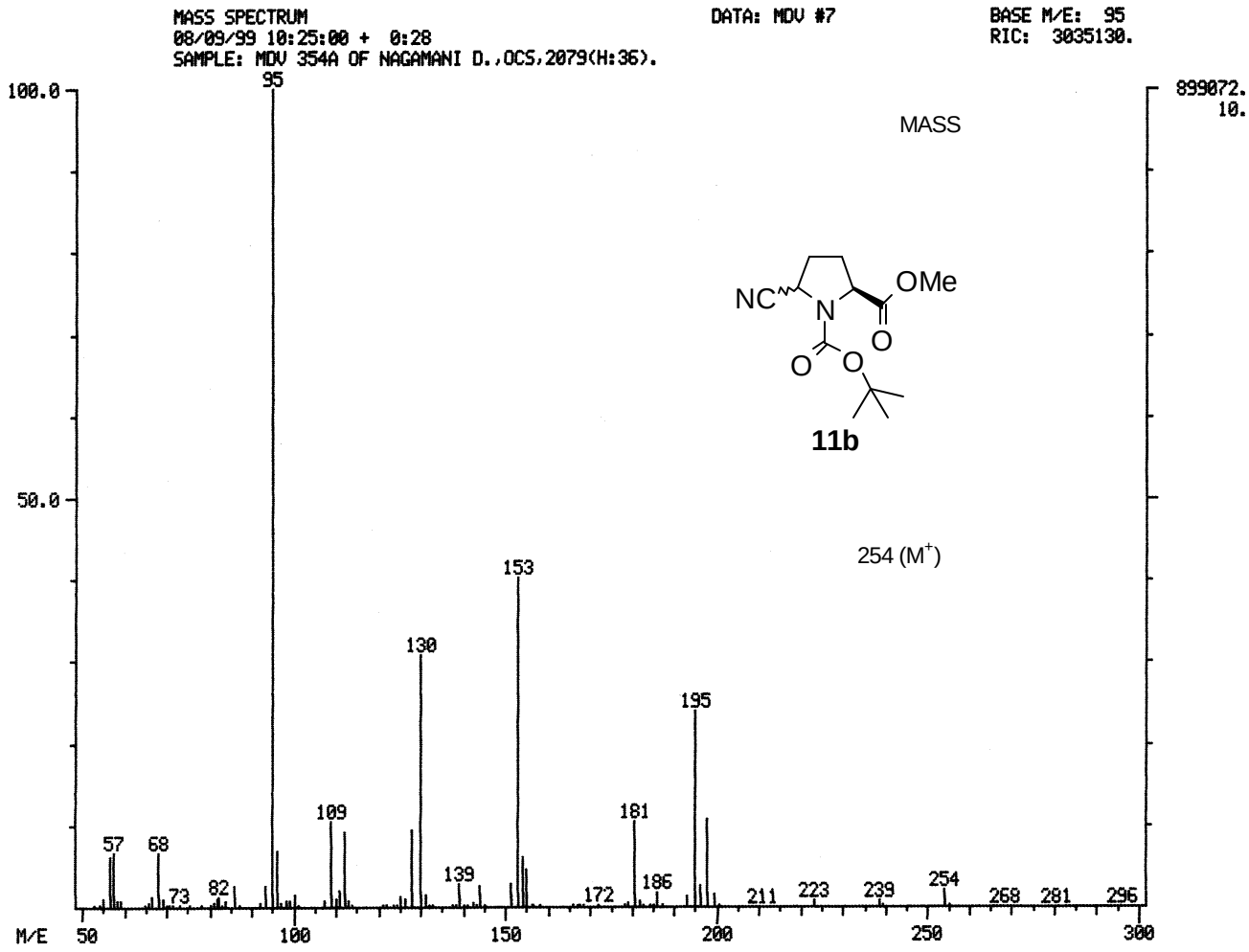


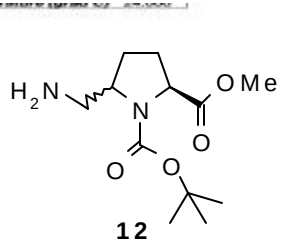
11b

DEPT

^{13}C NMR



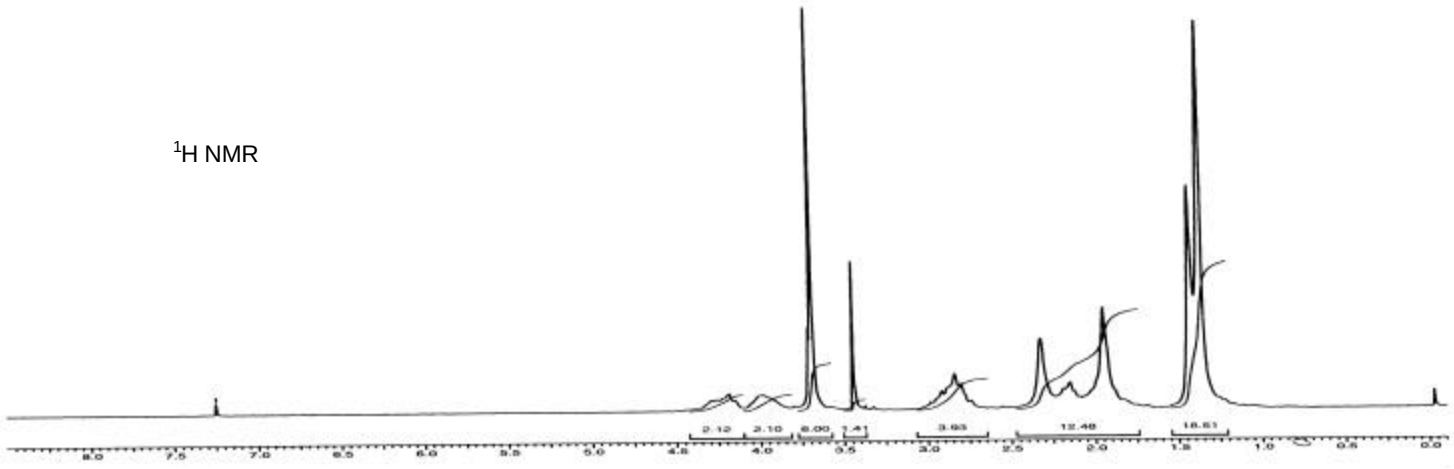

MASS SPECTRUM
08/09/99 10:25:00 + 0:28
SAMPLE: MDV 354A OF NAGAMANI D.,OCS,2079(H:36).
DATA: MDV #7
BASE M/E: 95
RIC: 3035130.
899072.
10.
100.0
50.0
M/E
MASS
NC
OMe
N
O
O
O
11b
254 (M+)
95
153
130
195
109
181
57
68
73
82
139
172
186
211
223
239
254
268
281
296
50
100
150
200
250
300

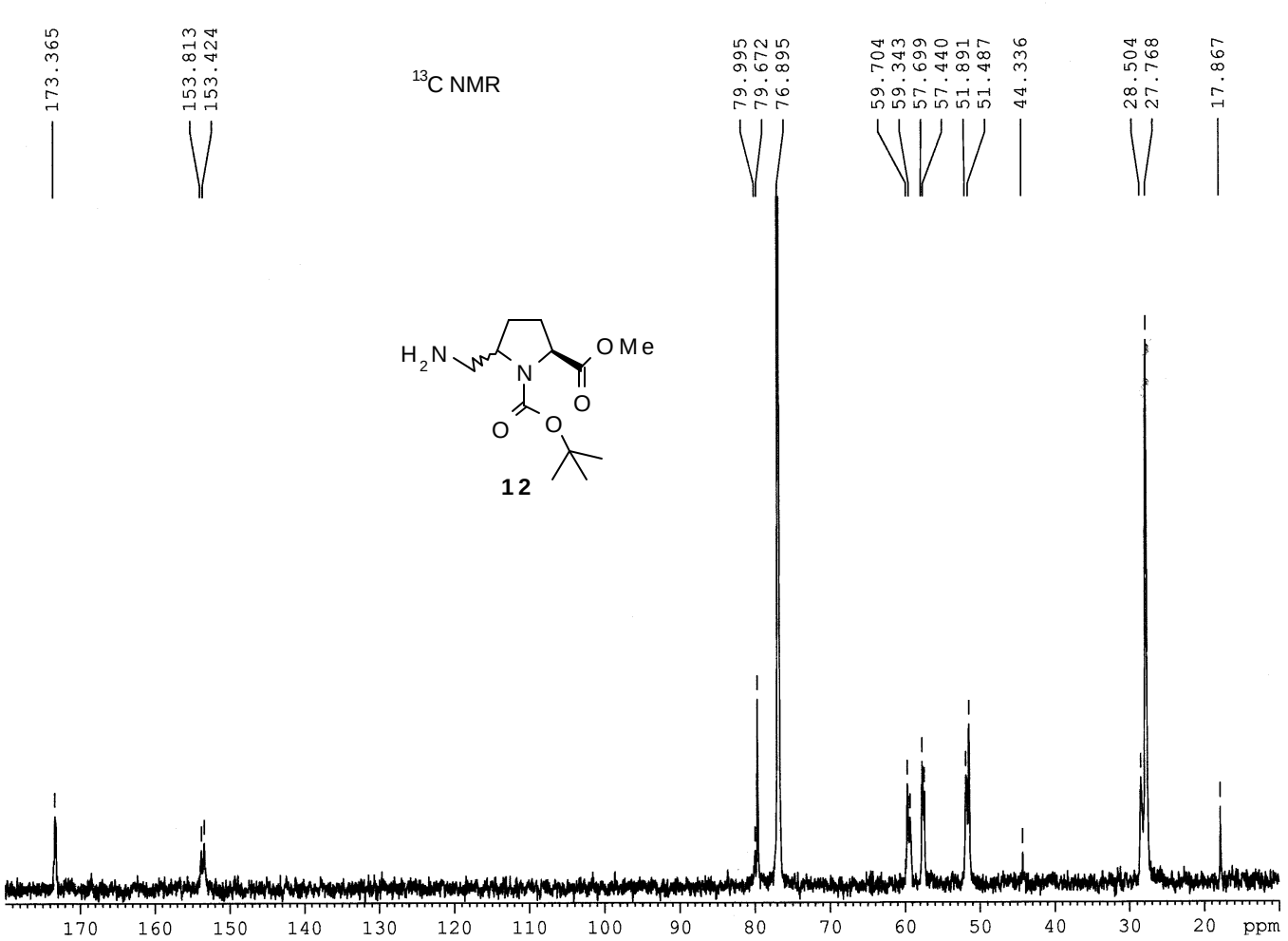


12

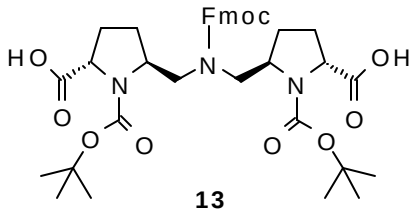
D_2O exchange

^1H NMR



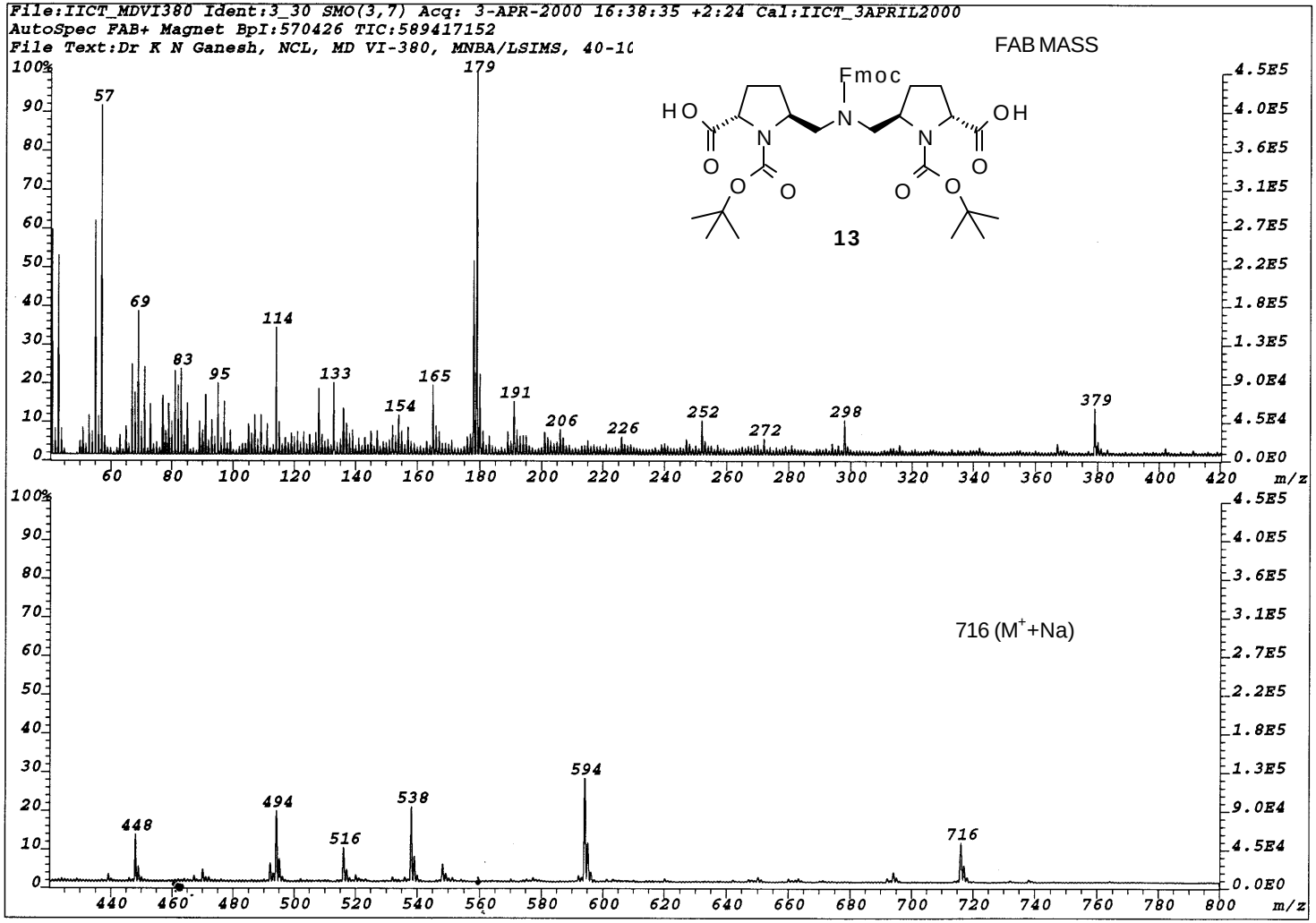

173.365
153.813
153.424
^{13}C NMR
79.995
79.672
76.895
59.704
59.343
57.699
57.440
51.891
51.487
44.336
28.504
27.768
17.867
H_2N
N
OMe
O
O
O
12
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

12 Dec 1999
MDVI380



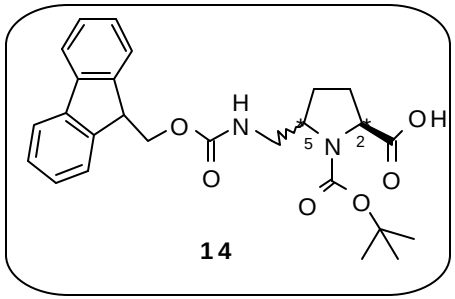
13

^{1}H NMR

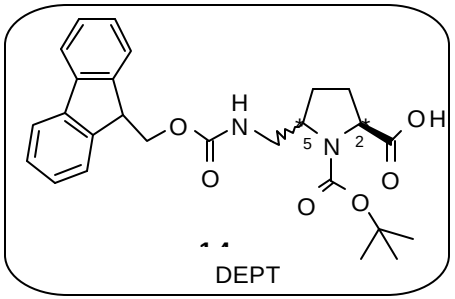

File:IICT_MDVI380 Ident:3_30 SMO(3,7) Acq: 3-APR-2000 16:38:35 +2:24 Cal:IICT_3APRIL2000
AutoSpec FAB+ Magnet BpI:570426 TIC:589417152
File Text:Dr K N Ganesh, NCL, MD VI-380, MNBA/LSIMS, 40-1(
FAB MASS
Fmoc
HO
OH
13
716 (M+Na)
m/z
m/z

22 Mar 2000
H1/CDCl3
mdvi-364a

^{1}H NMR

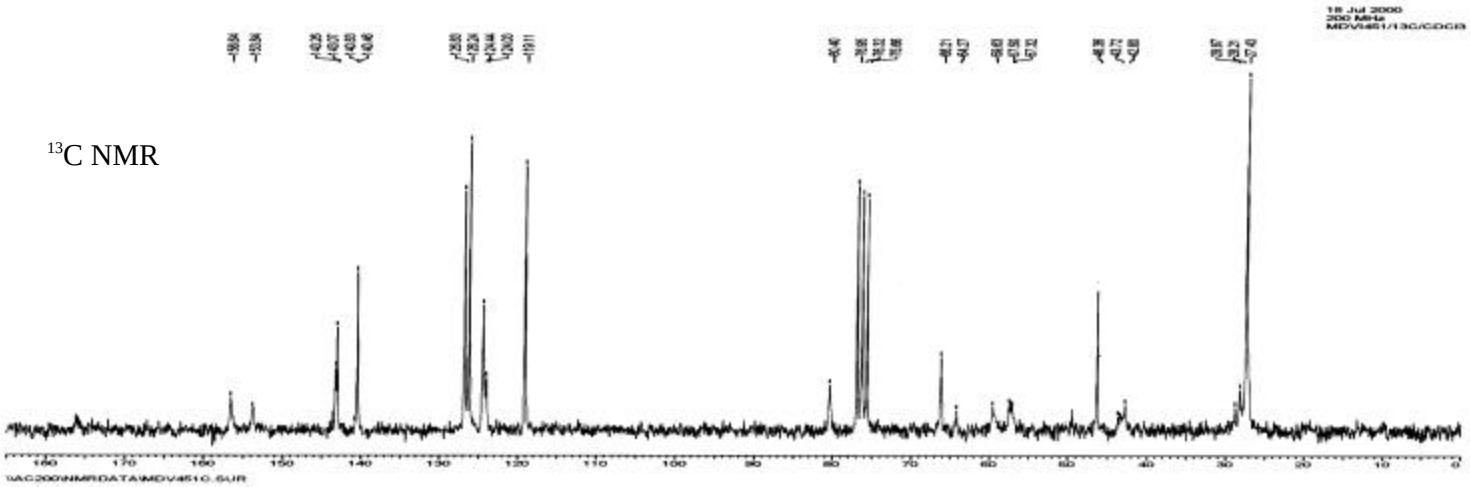


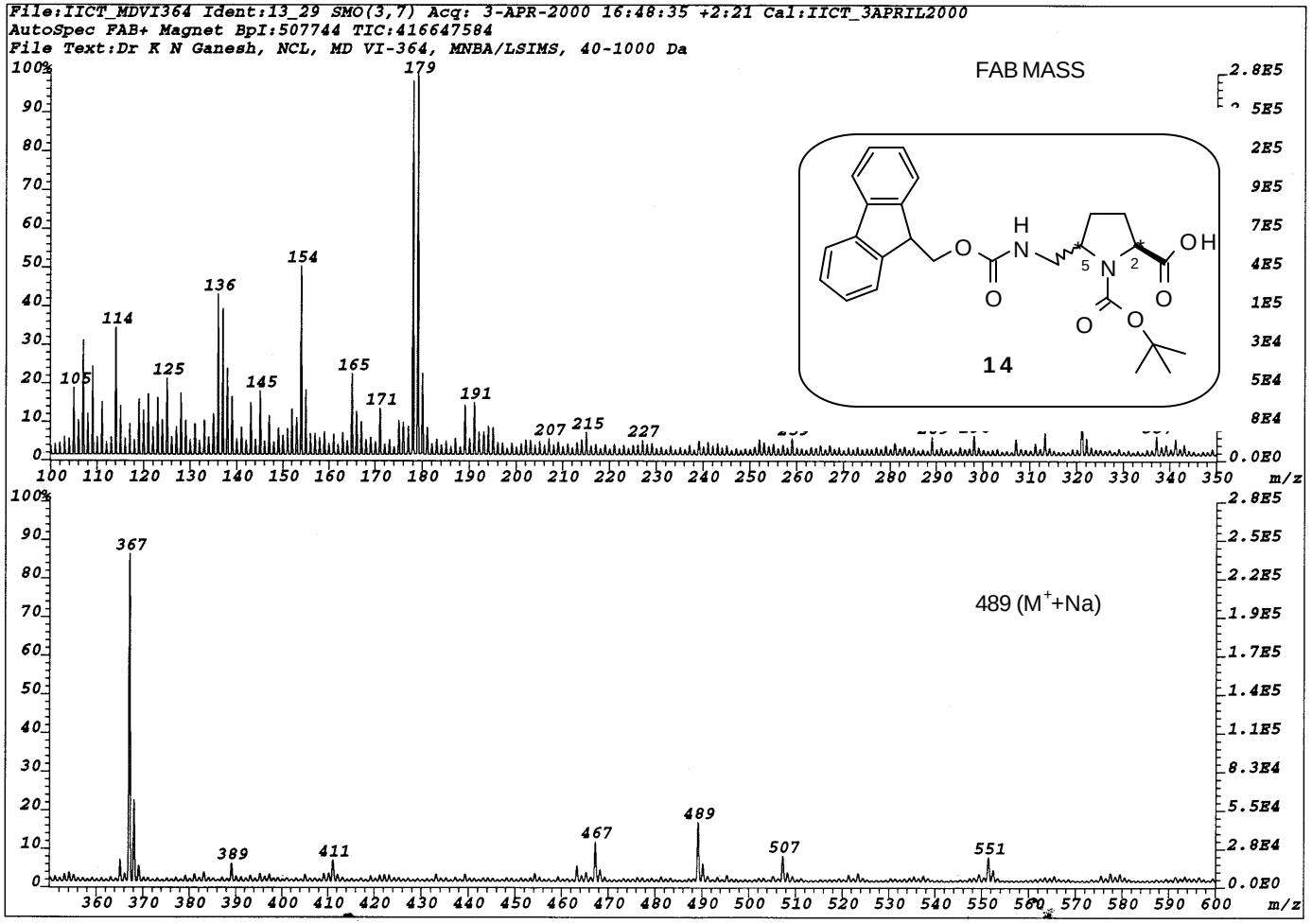
14

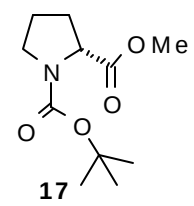


DEPT

^{13}C NMR



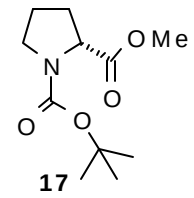

File:IICT_MDVI364 Ident:13_29 SMO(3,7) Acq: 3-APR-2000 16:48:35 +2:21 Cal:IICT_3APRIL2000
AutoSpec FAB+ Magnet BpI:507744 TIC:416647584
File Text:Dr K N Ganesh, NCL, MD VI-364, MNBA/LSIMS, 40-1000 Da
FAB MASS
14
489 (M+Na)
m/z
m/z


OMe
N
O
O
O
O

17

^{1}H NMR

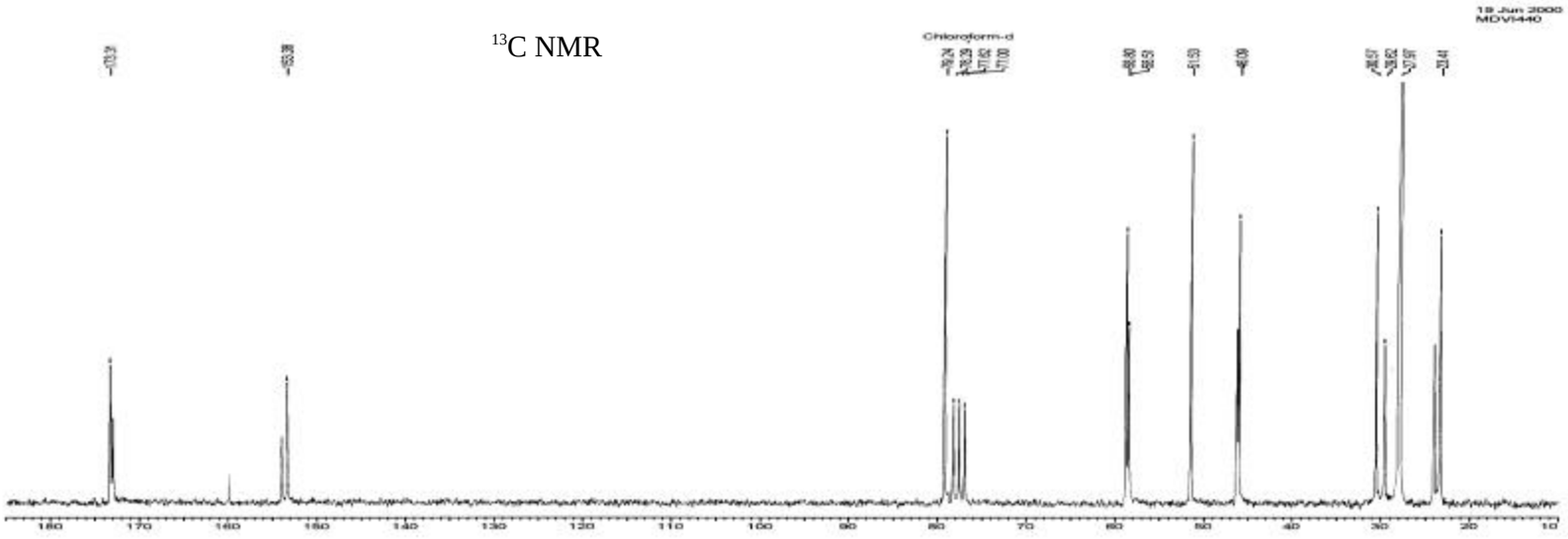
1.05
2.87
2.35
4.71
9.00
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0



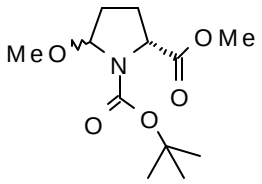
17

DEPT

^{13}C NMR

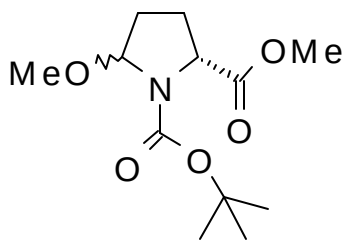


^{1}H NMR


MeO
N
OMe
O
O
O

18

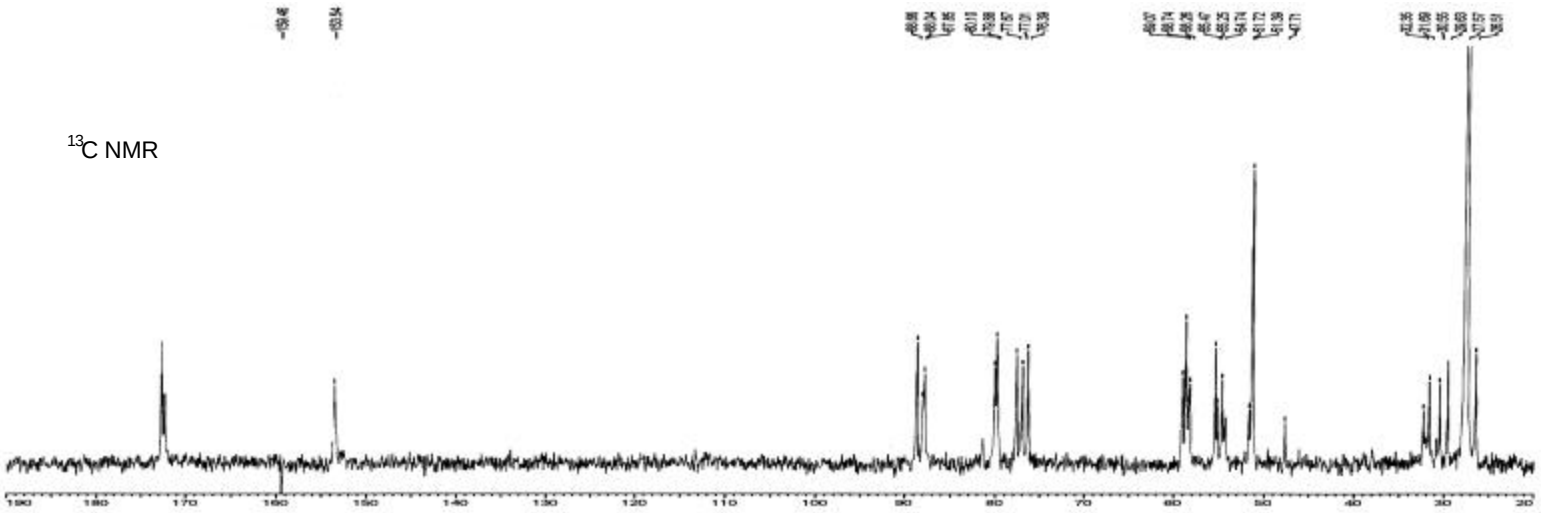
0.81
0.77
2.91
3.08
4.26
9.00
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

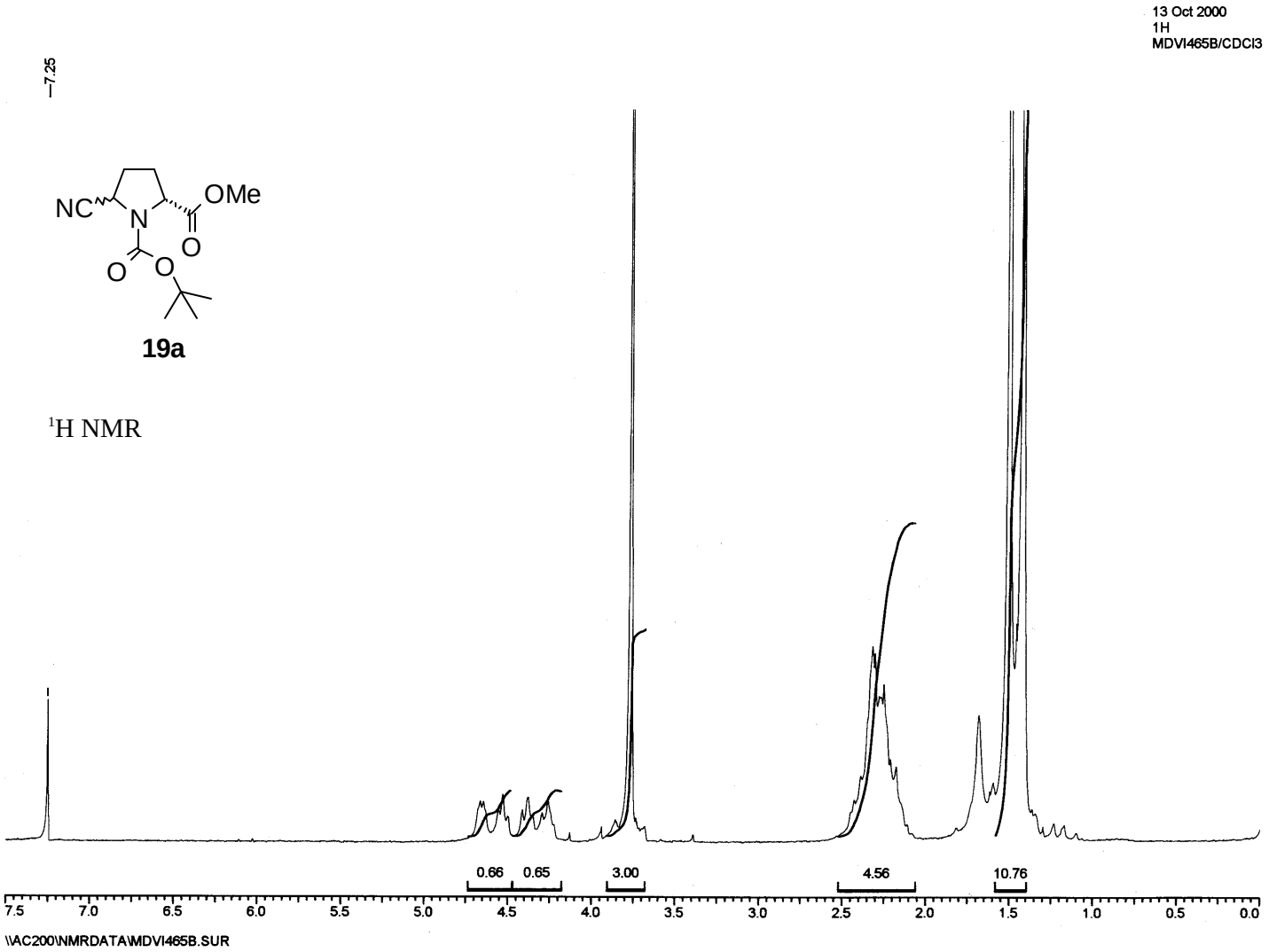


DEPT

18

^{13}C NMR




13 Oct 2000
1H
MDVI465B/CDCl3
7.25
NC
N
OMe
O
O
O
0.66
0.65
3.00
4.56
10.76
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
\\AC200\NMRDATA\MDVI465B.SUR

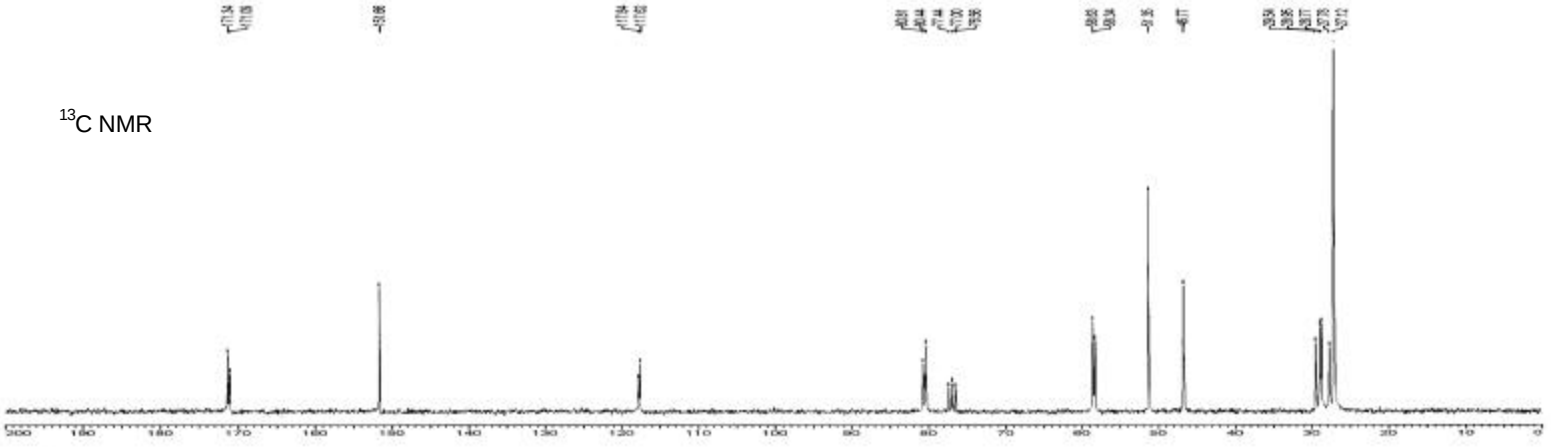
19a

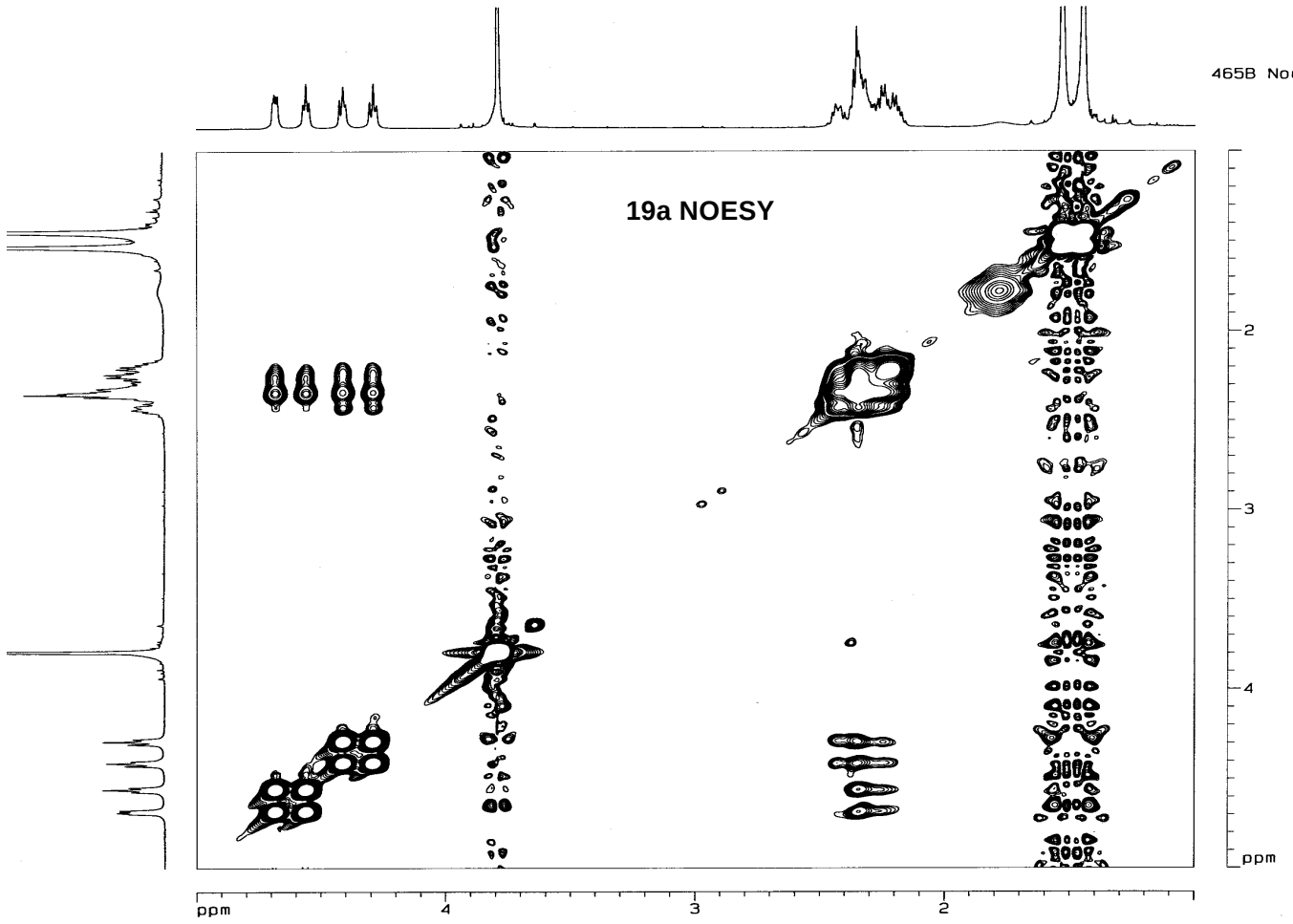
^{1}H NMR

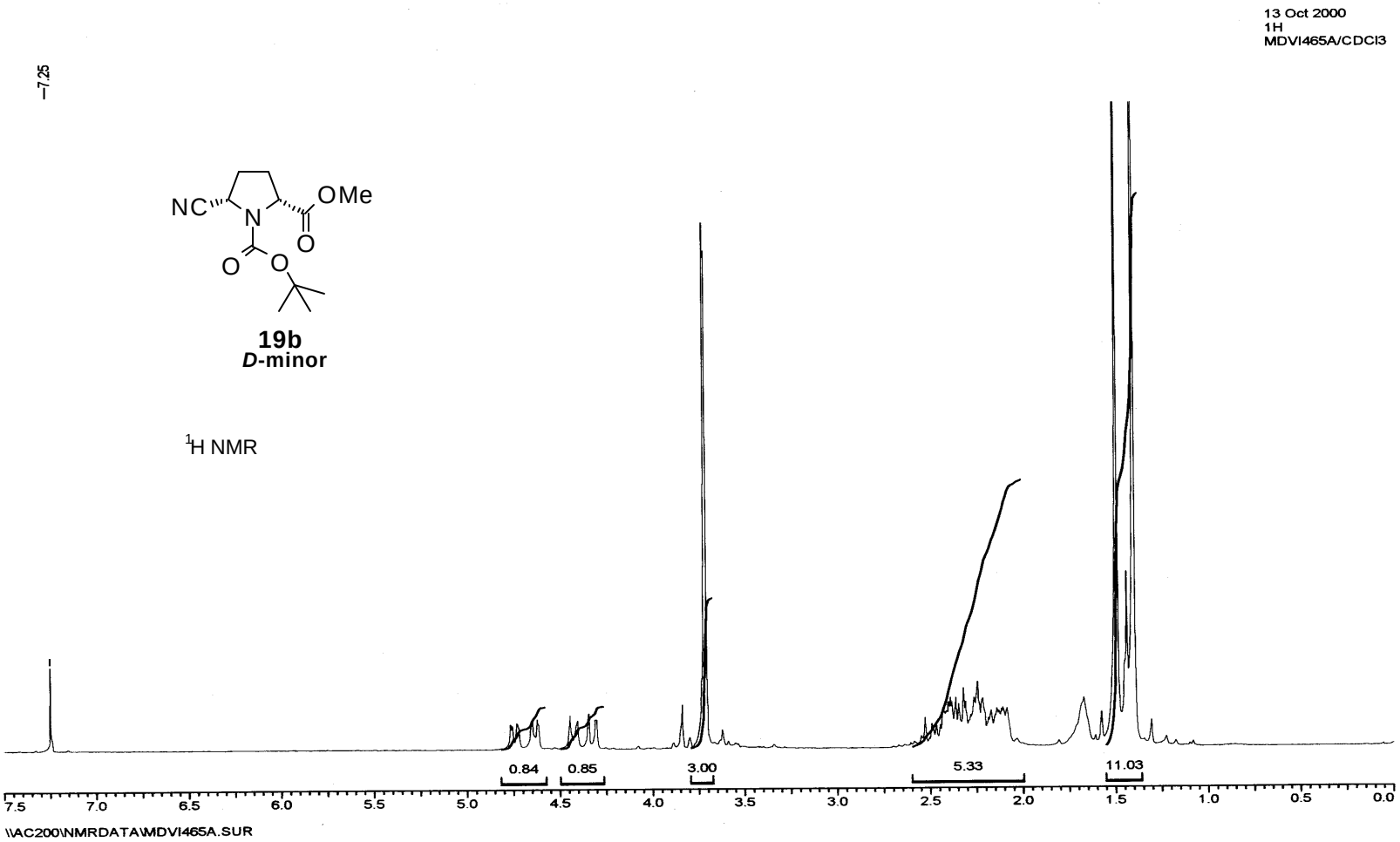
19a

DEPT

^{13}C NMR

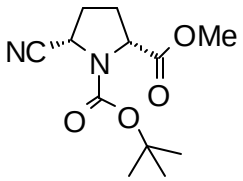



19a NOESY
465B Noesy
ppm
4
3
2
ppm
2
3
4
ppm



19b
***D*-minor**

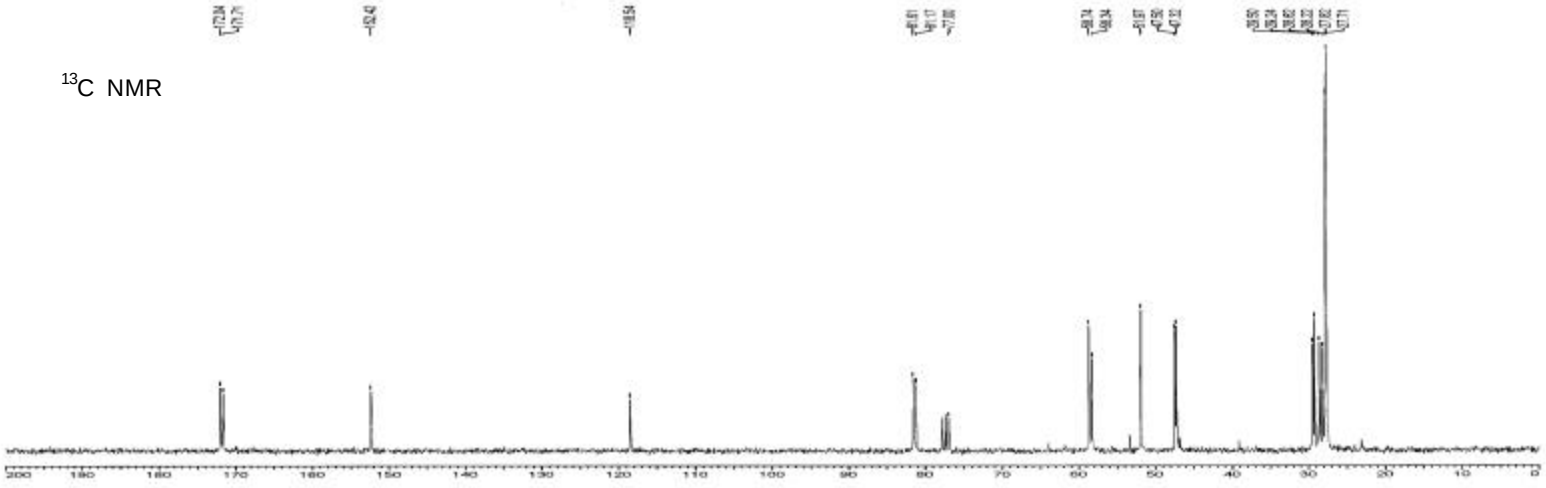
^{1}H NMR

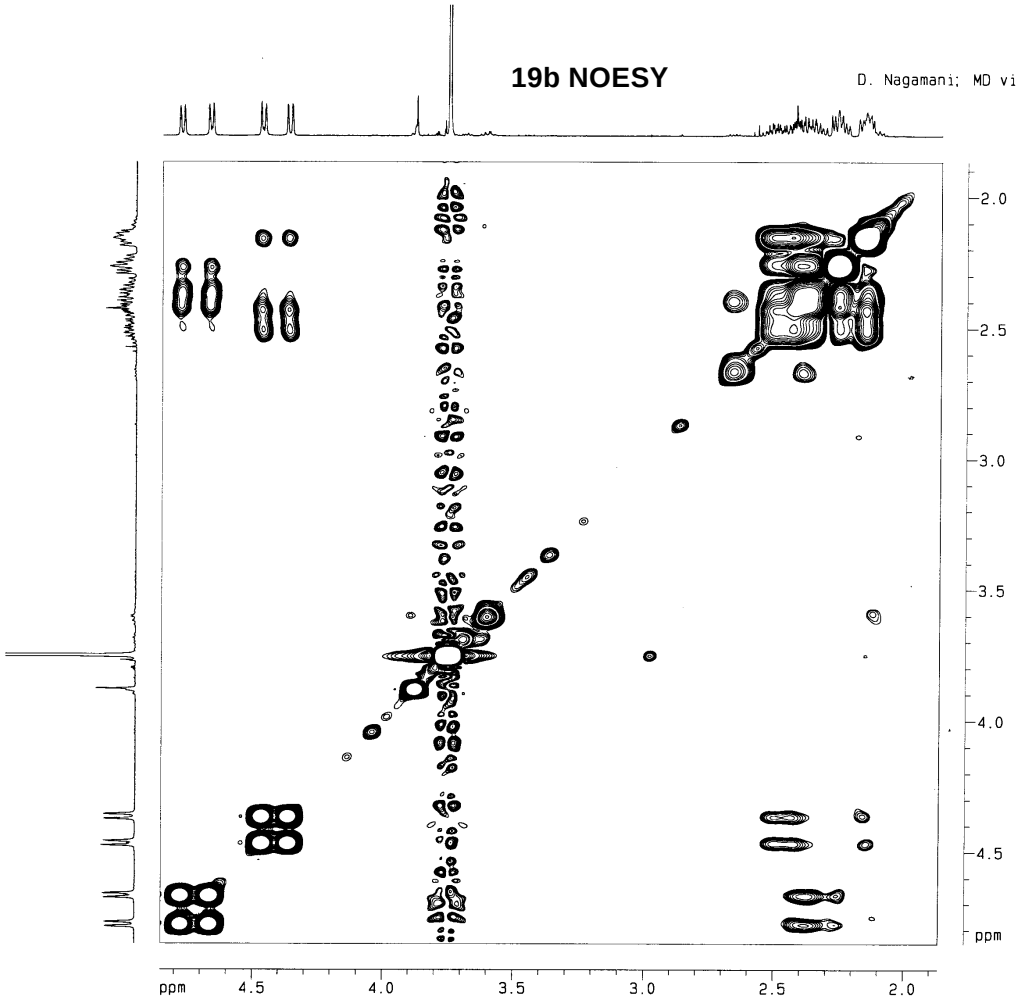


19b
***D*-minor**

DEPT

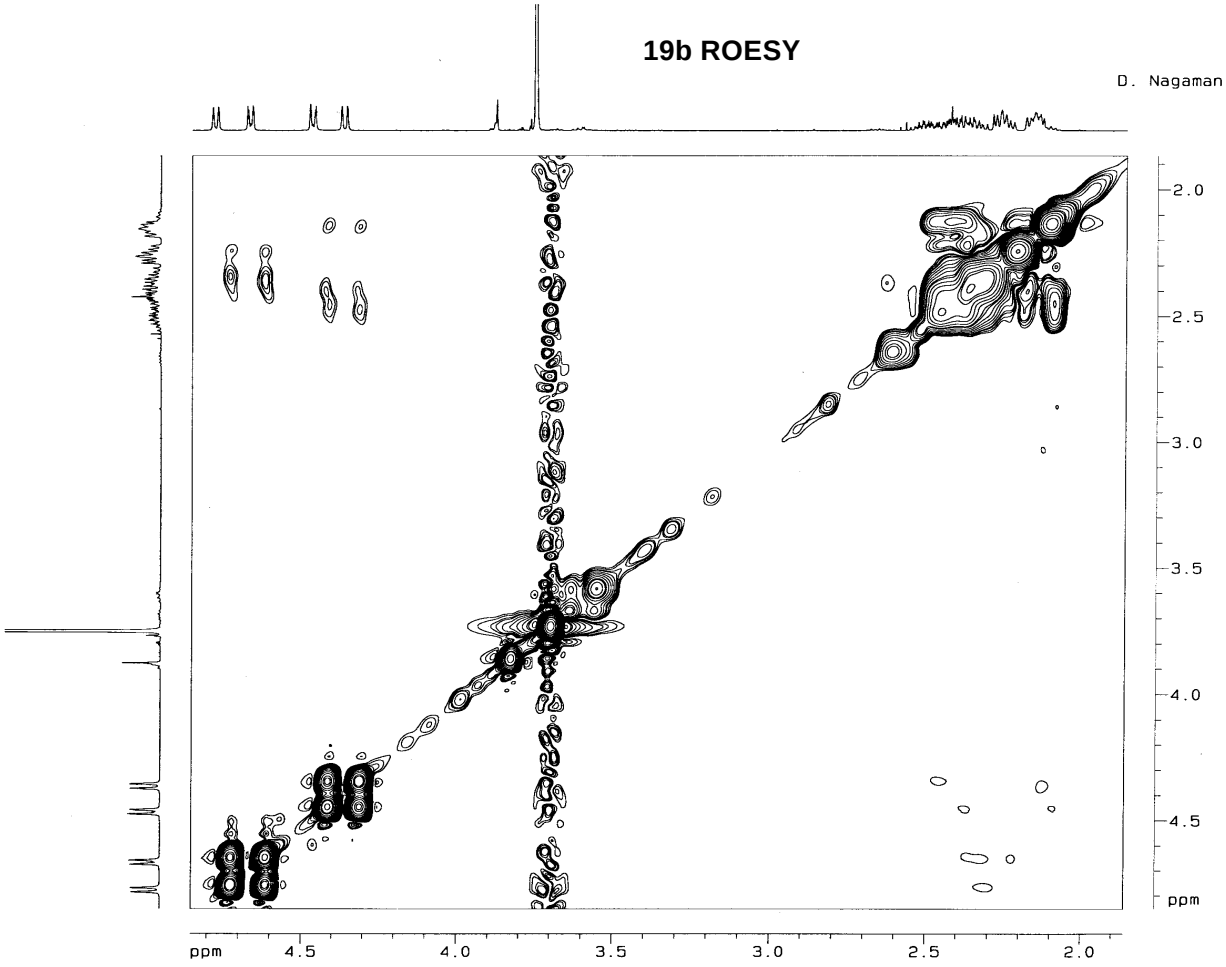
^{13}C NMR

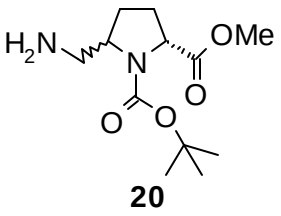



19b NOESY
D. Nagamani; MD vi
2.0
2.5
3.0
3.5
4.0
4.5
ppm
ppm
4.5
4.0
3.5
3.0
2.5
2.0

19b ROESY

D. Nagamani;

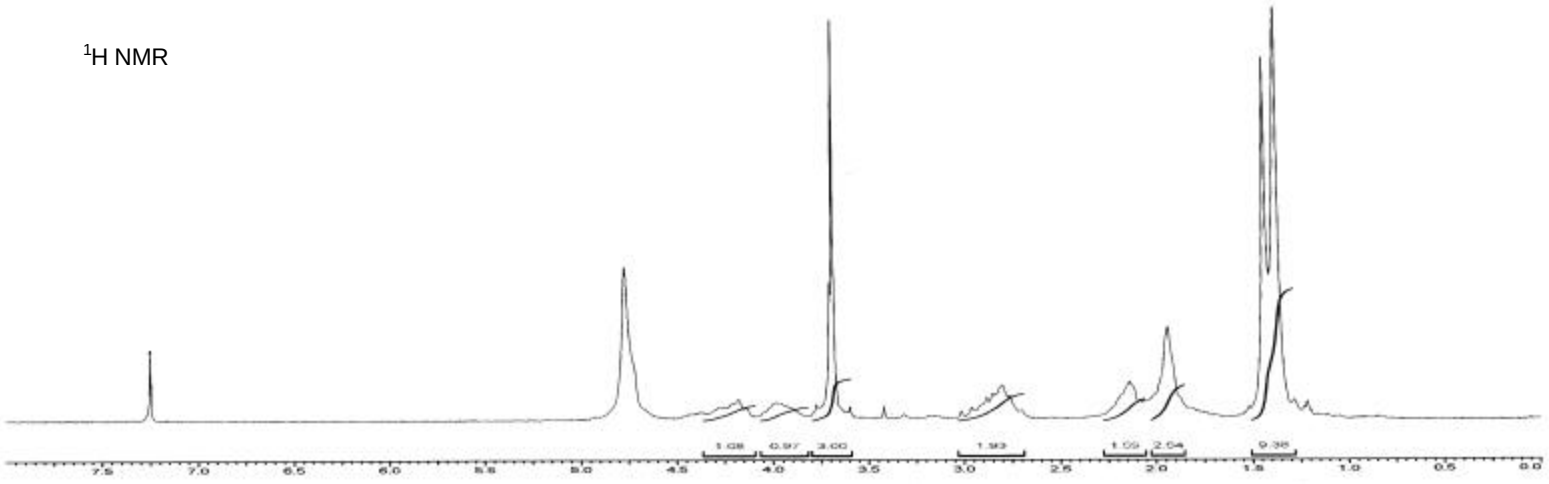




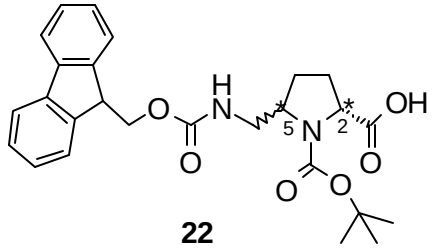
20

D_2O exchange

^1H NMR

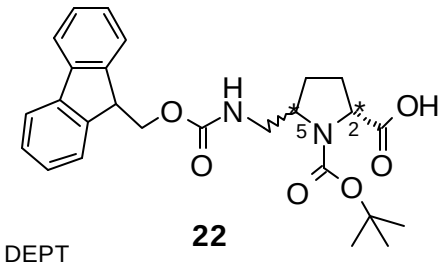


^{1}H NMR



22

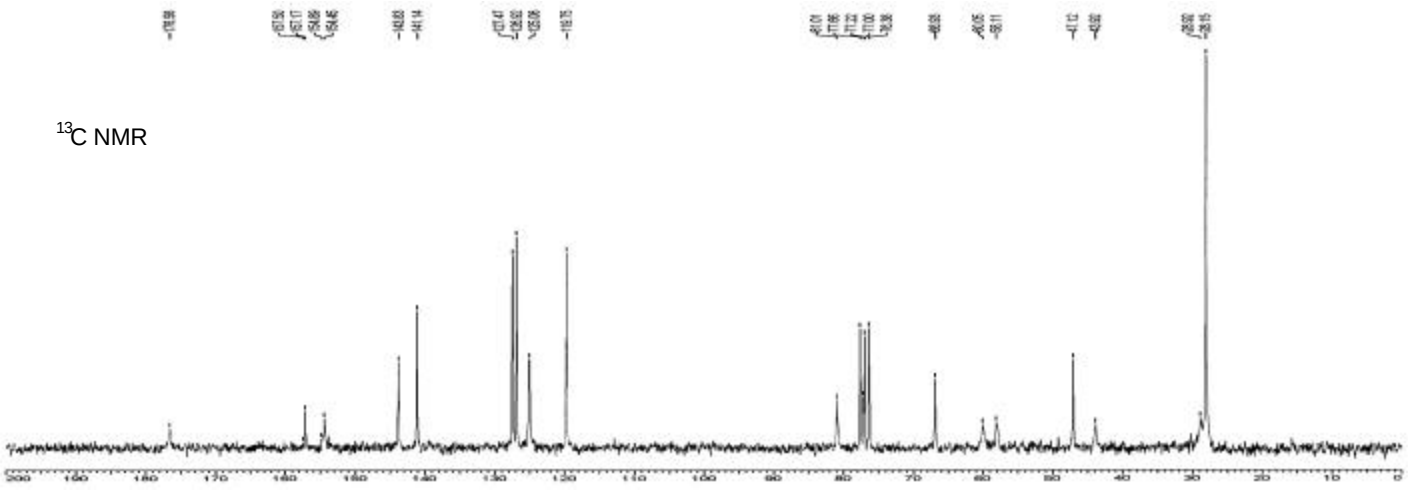
1.54
3.67
0.63
2.46
4.87
1.92
4.96
9.00
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
\\AC200\NMRDATA\MDVI472.SUR



22

DEPT

^{13}C NMR



DR K N GANESH
MDIV-472 MD V-472
Data: MD4720001.13 10 Nov 2000 17:41 Cal: 10 Nov 2000 17:42
Kratos PCKompact SEQ V1.2.2: + Linear High, Power: 80, P.Ext. @ 1000 (bin 56)
%Int. 100% = 65 mV[sum= 2223 mV] Profiles 1-34 Smooth Av 50