Synthesis and evaluation of charged peptide nucleic acid analogues/conjugates for improved DNA/RNA binding selectivity and better cell entry

A thesis submitted toward the partial fulfillment of
BS-MS Dual Degree Programme

by

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Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled "**Synthesis and evaluation of charged peptide nucleic acid analogues/conjugates for improved DNA/RNA binding selectivity and better cell entry**" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Pramod Kumar at IISER Pune under the supervision of Prof. K. N. Ganesh, Professor, Department of Chemistry, IISER Pune during the academic year 2013 - 2014.

Place: Pune
Date:

Supervisor & Head
(Department of Chemistry)
Prof. K. N. Ganesh,
Director, IISER Pune

Declaration

I hereby declare that the thesis entitled "**Synthesis and evaluation of charged peptide nucleic acid analogues/conjugates for improved DNA/RNA binding selectivity and better cell entry**" submitted for the partial fulfillment of the BS-MS dual degree programme at Indian Institute of Science Education of Research (IISER), Pune has not been submitted elsewhere for any other degree. This work was carried out by me at the Indian Institute of Science Education of Research (IISER), Pune, India under the supervision of Prof. K. N. Ganesh.

Place: Pune Pramod Kumar

Date:

" This thesis is dedicated to....

My father, Late Radha Krishna Gohiwar
whose blessings are always with me..
And
My mother, Smt. Sugandha Devi "

ACKNOWLEDGEMENTS

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Contents

Abstract

Peptide nucleic acid (PNA) is a promising class of oligonucleotide analog with great potential for gene therapeutic applications. PNA is a DNA analog in which the normal phosphodiester backbone is replaced by acyclic, achiral and neutral amino-ethyl-glycyl (aeg) backbone. PNA can bind to complementary DNA/RNA sequence with high affinity and sequence specificity. PNA and its analogs are resistant to proteases and nucleases. Besides these advantages, PNA suffers from some limitations like low aqueous solubility, poor cell permeability and ambiguity in binding orientation. To overcome these limitations, in the present work, I have designed and synthesized PNA analog which has substitution at γ-C on the backbone. The substituted chain carries terminal cationic amino/guanidino group to improve binding affinity towards complementary DNA. The stability of PNA:DNA duplex is determined by temperature dependent UV spectroscopy. The γ -C modified PNA is shown to stabilize duplexes with complementary DNA better than the control unmodified PNA. The CD studies showed that the modification does not alter the conformation of PNA:DNA duplexes.

1. Introduction:

One of the most remarkable events in the history of this planet is undoubtedly the origin of life and its evolution to the present form. One of the very famous hypotheses that helps to understand the origin of life on earth is the RNA world hypothesis. In the definition of this hypothesis, RNA (ribonucleic acid) molecules are considered as the first living forms. The world of free RNA molecules is thought to have come together to form the complex organization for the synthesis of proteins that sustained life. RNA was able to store information for the synthesis of proteins via enzymatic reactions imperative for the survival of an organism. However, during the evolution of life forms RNA was gradually replaced by a more stable DNA molecule. In this new scenario DNA was the primary source of information (as well as hereditary material) that was translated into RNA, which was further translated into essential proteins. Actually, RNA acts as a template for protein production. In this way the genetic information flows (Figure **1**).

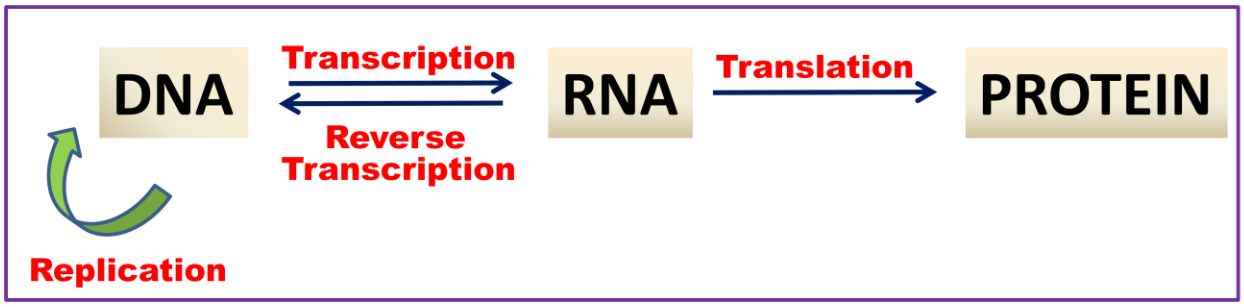


Figure1 : Central Dogma of Cell (Flow of genetic information)

This event of transition to a DNA molecule might have been driven by a combination of factors. Two crucial reasons could be that (a) The 2'O of the ribose is a very reactive atom that can attack the phosphodiester bond and (b) In DNA the deamination of cytosine into uracil (a common spontaneous chemical reaction) can be repaired.[1]

Having explained the evolutionary developments that surpassed by the life forms on earth, the molecules that had always served as the building blocks of life have been the **nucleic acids**. Not only, did nucleic acids existed in the prebiotic conditions and

gave structure and functionality to RNA, but also later rearranged to form the more stable DNA, which served as the most suitable form of hereditary material.

1.1. Nucleic acid: organization and structure

Nucleic acids are biopolymers made of nucleotides and were discovered by **Friedrich Miescher** in 1869.[2] Nucleic acids correspond to DNA and RNA.[3] The name is attributed to its initial discovery within the nucleus and acidic character is due to the presence of phosphoric acid moiety. Nucleic acids are essential for all known forms of life. Because of complementary hydrogen bonding between the nucleobases (Adenine:Thymine and Cytosine:Guanine) DNA forms duplex. The double-helical structure of DNA was proposed by James Dewey Watson and Francis Harry Compton Crick in 1953 (Figure**1.1.1**).[4]

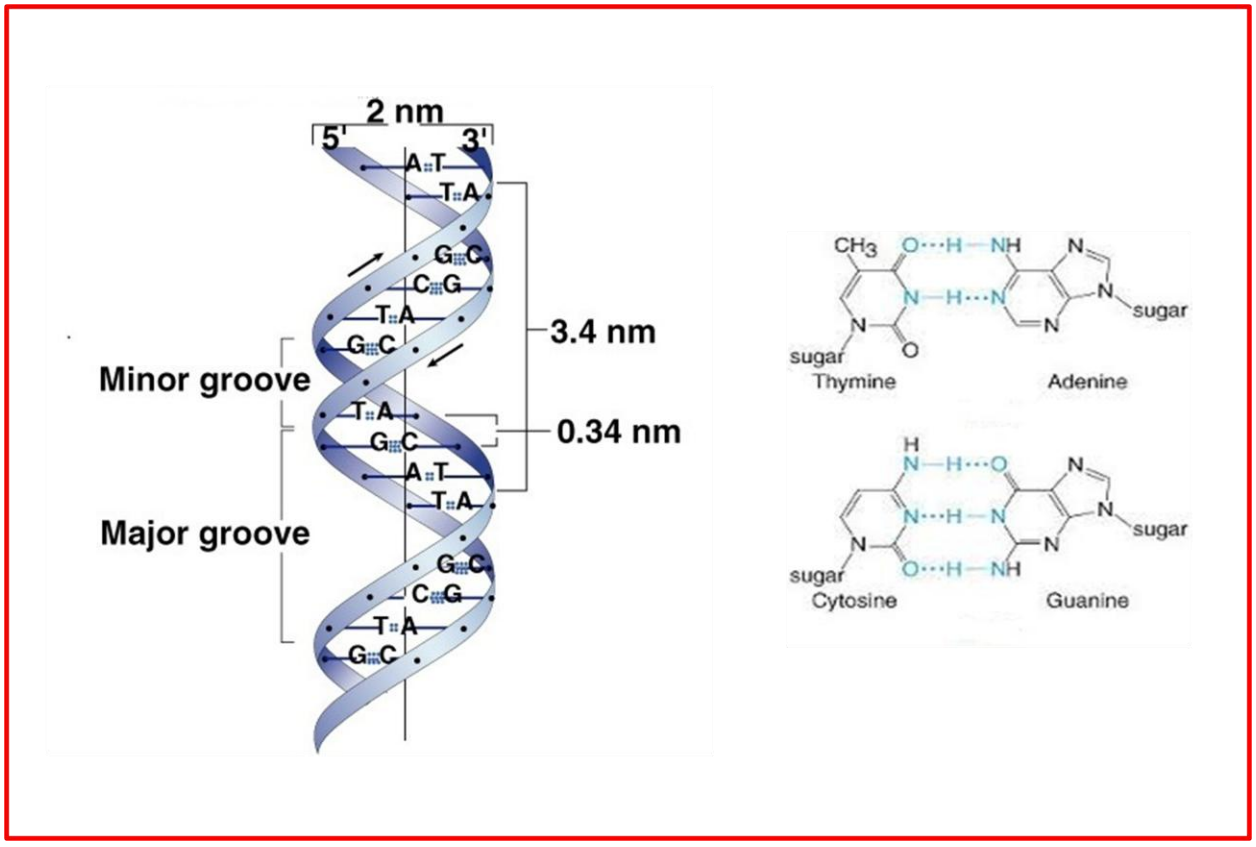


Figure 1.1.1: DNA double helical structure and H-bonding between A:T and G:C
(http://i179.photobucket.com/albums/w311/Mjhavok/Untitled-6.gif)

The nucleobases are capable of making H-bonds because of the donor:acceptor ability.[5] In RNA, the nucleobase thymine is replaced by uracil and sugar deoxyribose is replaced by ribose.

One of the DNA strands in the double helix keeps the genetic information, which is used for protein synthesis. This is called the **sense strand**. The complementary strand that binds to the sense strand is called the **anti-sense strand**.(Figure**1.1.2**).[5] The anti-sense strand serves as the template for the synthesis of RNA. The unwinding of the double strand of DNA starts at the site where the promoter binds, subsequently starting the process of transcription. The RNA thus formed is shifted to the ribosomes where the process of translation commences. In the process of translation the protein synthesizing machinery converts the RNA into proteins. This RNA is called mRNA because its serves as a messenger in carrying information from the DNA to the proteins.

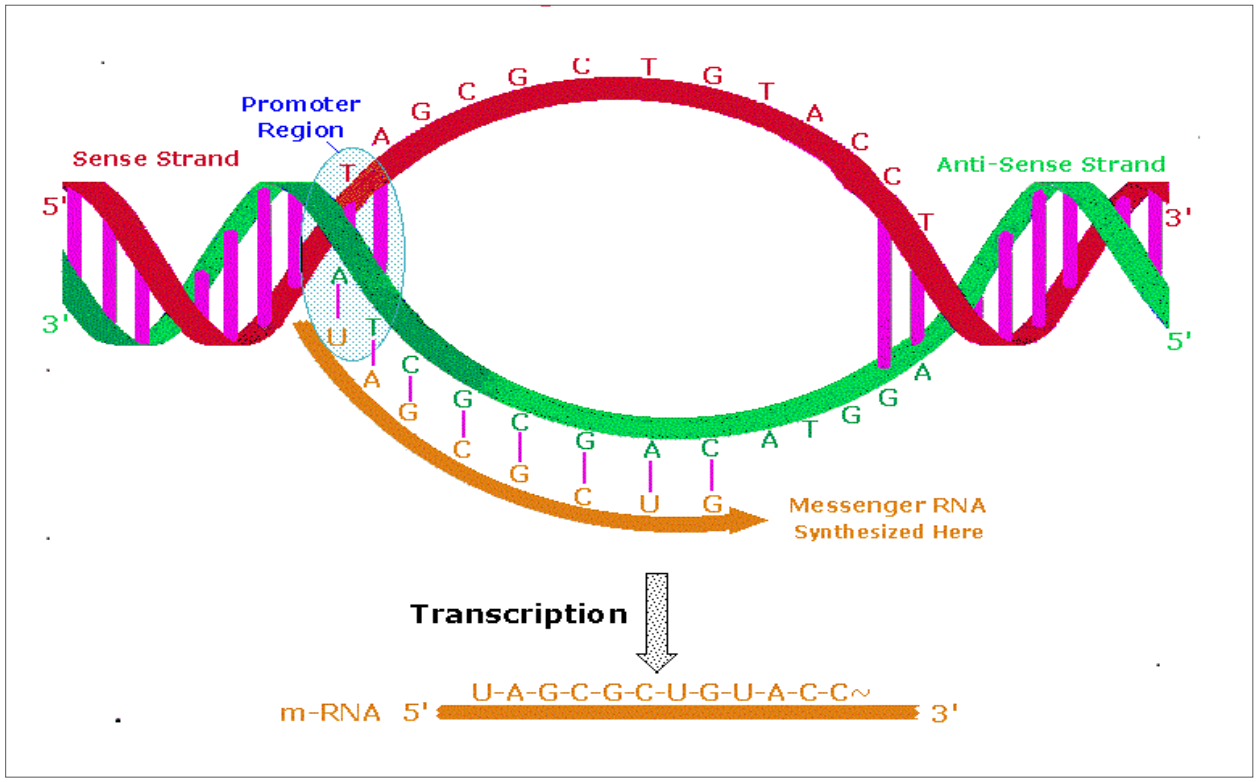


Figure 1.1.2. Sense and anti-sense stand of DNA.

The protein production can be inhibited if the RNA is blocked from undergoing the process of translation. Thus introducing an anti-sense strand inside the cell can inactivate the mRNA. The RNA interaction with the PNA is observed to be stronger than the same interaction between RNA/RNA or DNA/DNA.

1.2. Peptide Nucleic Acid

Peptide nucleic acid (PNA) was discovered in 1991 by the combined efforts of biochemist Peter Nielsen and organic chemist Ole Buchardt. PNA was originally designed and developed as a mimic of a DNA-recognizing, major groove binding, triplex-forming oligonucleotide. PNA mimics the behavior of DNA and binds complementary nucleic acid strands.[6]

PNAs are analogous to DNA in which the normal phosphodiester backbone is replaced by a reduced pseudodipeptide backbone (Figure **1.2.1**).[7] PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine (aeg) units linked by amide bonds. PNA is an uncharged oligomer as well as more flexible and so it binds with higher affinity to complementary DNA/RNA than their natural counterparts obeying Watson-Crick base pairing rule. PNAs seem to be non-toxic, and they are uncharged oligomers. They have higher mismatch discrimination and therefore form strong, selective duplexes upon binding to complementary DNA/RNA sequences.

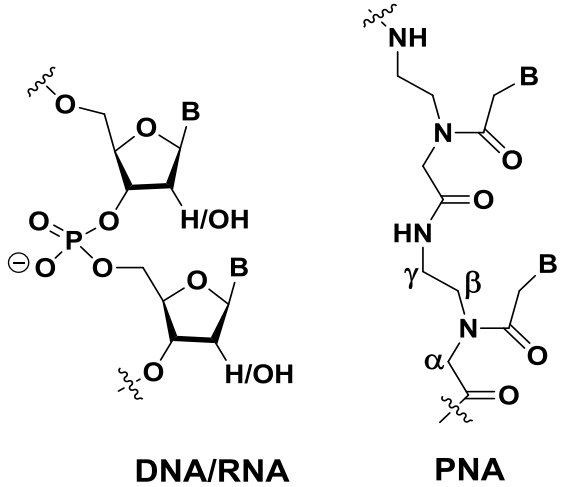


Figure 1.2.1 Structure of DNA/RNA and PNA

PNA can hybridize to complementary DNA/RNA sequence with better thermal stability. Since PNA can bind more tightly to the complementary DNA/RNA, it can act as an antigene as well as antisense inhibitor.[8,9] PNA can act as an antigene inhibitor when it binds to the complementary DNA by stopping the formation of RNA (no transcription). Similarly it can act as an antisense inhibitor by preventing the formation of protein if it binds to complementary RNA preventing translation.(Figure**1.2.2**) PNAs are resistant to degrading enzymes like proteases and nucleases.

Peptide nucleic acids have a few drawbacks like poor water solubility, lack of efficient cell permeability and ambiguity in binding orientation. To overcome these limitations, various modifications have been done which have improved PNA properties to different extents.[10]

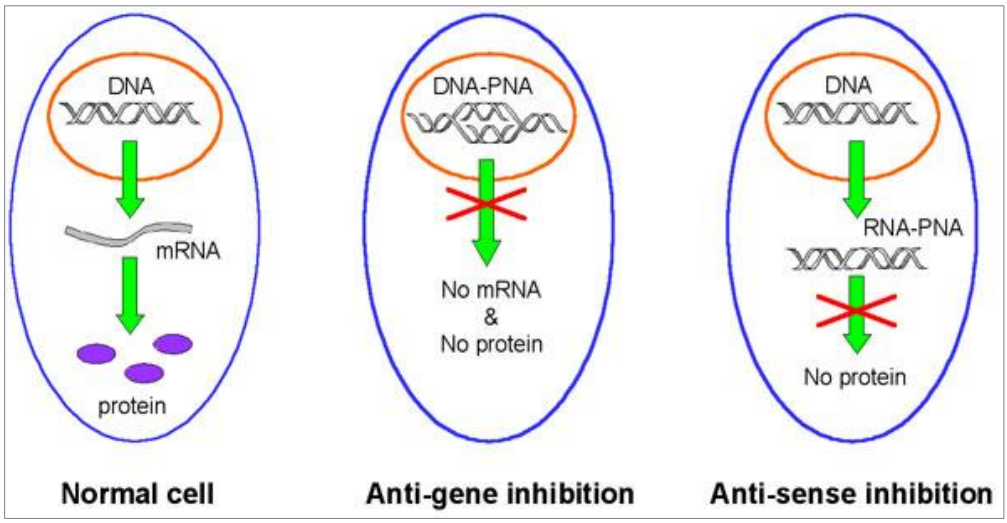


Figure 1.2.2. Binding of PNA with DNA/RNA
(http://monash.edu/science/about/schools/chemistry/assets/images/staff/spiccia/hiv-1.gif)

1.3. Aim of the work

- Introduce side chain carrying cationic amine functional group at γ-position through a three carbon spacer.
- To synthesize PNA oligomers by solid phase peptide synthesis.
- Biophysical evaluation of synthesized PNA oligomers by CD and UV- T_m studies.

1.4. Rationale of the work

Modifications in the PNA backbone are possible in many ways. Various PNA modifications incorporating cationic (amine/guanidine) functional groups in the side chain through various spacer chains at either α- or γ-positions have been reported.[11,12] These studies showed that modifications at γ-carbon has higher binding affinity towards complementary DNA/RNA than that of α-carbon. Recent reports have indicated that L-lysine[13] and L-serine[14] derived gamma substituted PNA units within unmodified PNA oligomers act as a helical director due to conformational pre-organization (spacer is 4xCH_2 groups). It has also been reported that cationic guanidinium groups grafted on PNAs (GPNA)[15] enhance the cell uptake of the substrates. Recently, Ganesh *et al*. have shown that PNAs grafted with (α/γ, *R/S*)-aminomethylene pendants (spacer is 1xCH_2 group) show regio and stereospecific effects on DNA binding and improve the cell permeation (Figure**1.4.1**).[16] Synthesis and characterization of γ-modified PNA incorporating aminoethylene (spacer is 2xCH_2 groups) group in the *aeg* PNA backbone has been done in our lab.[20]

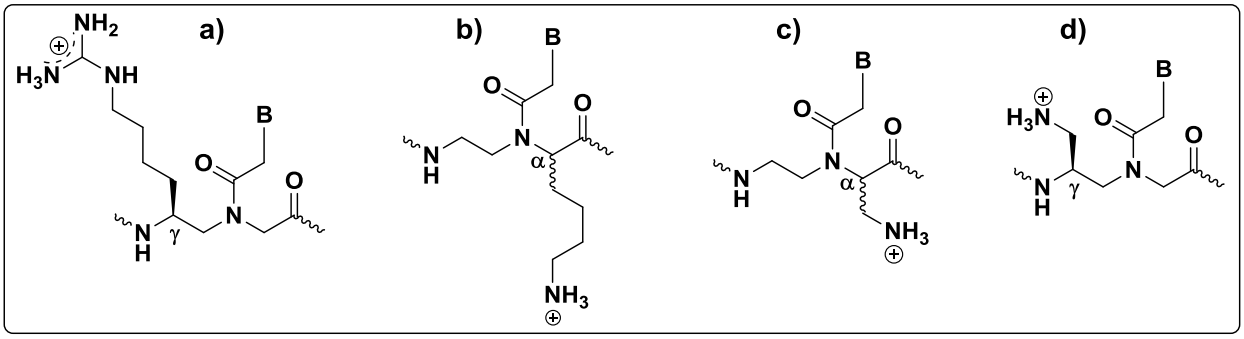


Figure 1.4.1 (a) γ-GPNA (b) replacing glycine unit with chiral L/D-Lysine (c) PNAs grafted with (α-*R/S*)-aminomethylene pendants (d) PNAs grafted with (γ-*S*)-aminomethylene pendants [B = Thymine nucleobase]

Although it is established that cationic modification at the γ-position introduces favourable cell penetrating properties to PNA, the optimal spacer length is required for an effective binding to complementary DNA/RNA and an efficient cellular uptake is yet to be identified. This inspired the design and synthesis of γ-modified PNA (Figure **1.4.2**) incorporating aminopropylene (spacer is 3xCH_2 groups) group in the *aeg* PNA backbone

in order to investigate its effective conjugation point and it is expected that its binding ability to DNA and RNA would be stronger than the duplex DNA-DNA and RNA-RNA. The incorporation of amine in the backbone will give a chiral PNA with a cationic functional group which can be directly correlated with an unmodified aeg PNA that is neutral. The studies of designed novel PNA would give insight on the structure activity relationships of the γ-modified cationic PNAs.

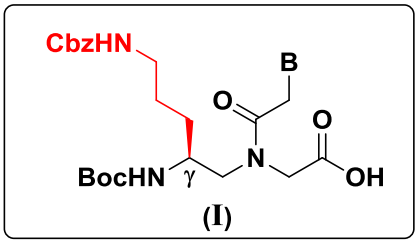


Figure 1.4.2. Target PNA monomer

1.5. Solid Phase Peptide Synthesis

Solid Phase Peptide Synthesis (SPPS) protocol was developed in 1963 by Robert Bruce Merrifield in order to synthesize peptide chains.[17] Now a days, SPPS method is being used to synthesize several types of peptides and oligomeric PNAs.[18]

In SPPS method, peptides are synthesized from the carbonyl group side (C-terminus) to amino group side (N-terminus) of the amino acid chain although the direction of synthesis of peptides is opposite in cells (N-terminus to C-terminus). In order to synthesize peptides through SPPS method, protecting groups are necessary to avoid unintentional side chain reactions. There are two strategies to use SPPS method. (Figure**1.5**). In the rudimentary SPPS, building blocks exhibiting two functional groups are generally used. One of the functional groups is protected and the other is made to bind with the bead and washed. SPPS is better than solution phase peptide synthesis because-

(a) no loss of material during workup as the peptide is not taken out of the reaction vessel.

(b) purification of the intermediates is not required in SPPS method.

(c) it is faster than solution phase peptide synthesis.

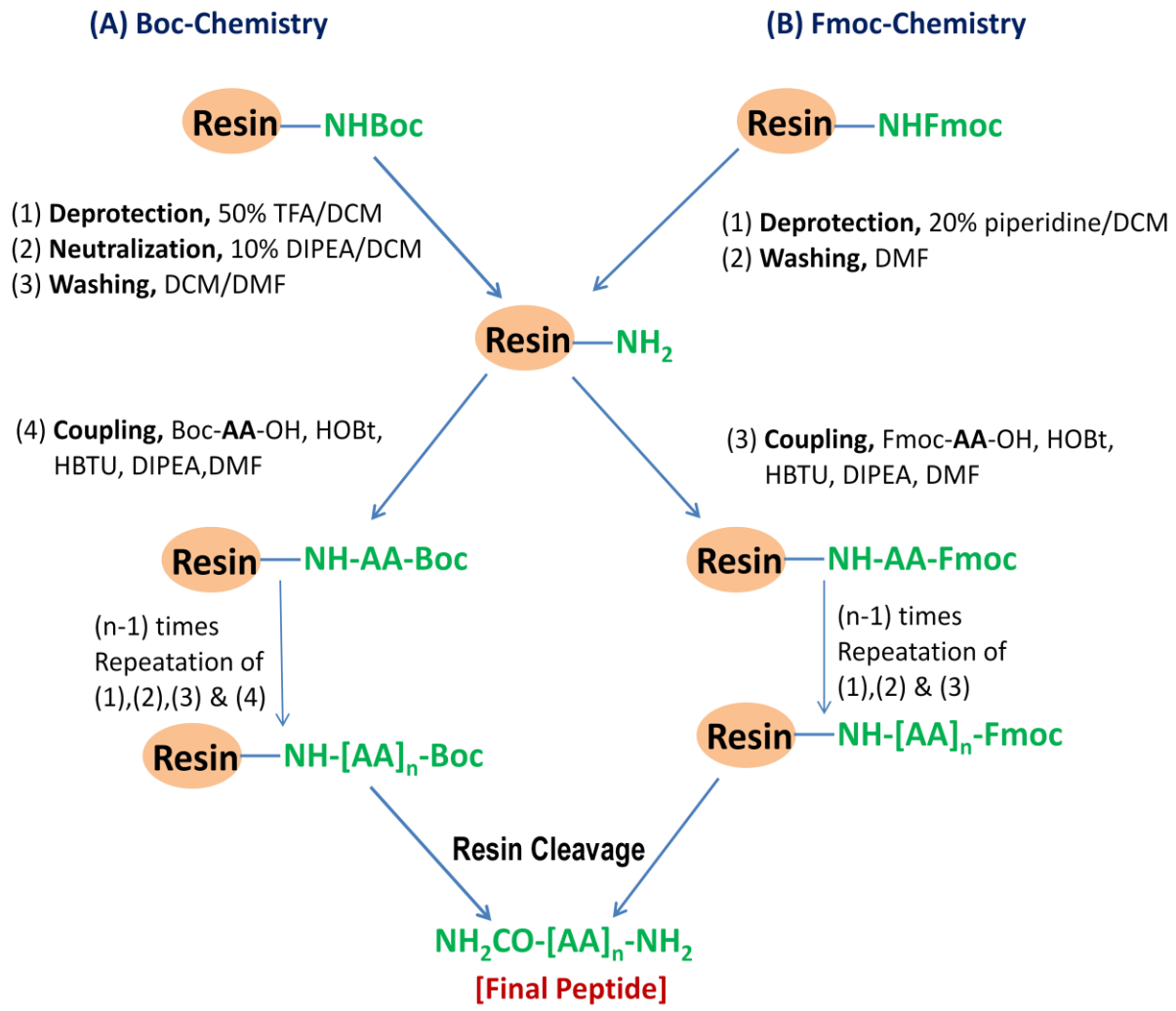


Figure.1.5: General protocol for SPPS via (A) Boc-chemistry and (B) Fmoc-chemistry.

1.6. High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a form of column chromatography to separate compounds that are present in solution. It is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres, which makes it much faster. It can identify and separate the

compounds that are present in a sample dissolved in a liquid. The compound separated by HPLC technique is highly pure.

1.7. Melting temperature (T_m):

Upon heating the duplex DNA hybrids separate into two single strands. The Melting temperature (T_m) is defined as the temperature at which 50% of all the molecules of a given DNA sequence are hybridized into a double strand, and 50% are present as single strands**.**(Figure**1.7**) T_m depends on the length of the DNA molecules and its specific nucleotide sequence. The main factors affecting T_m are salt concentration (most notably Mg^+ and K^+), concentration of DNA and length of DNA. The absorbance increases upon melting since in the duplex, base stacking causes hyperchromicity which is removed when the two strands separate out. Similarly, absorbance decreases upon cooling as single strands start forming duplex. T_m is calculated by using derivative plots of the absorbance measured and it corresponds to the temperature at which derivative is maximum.

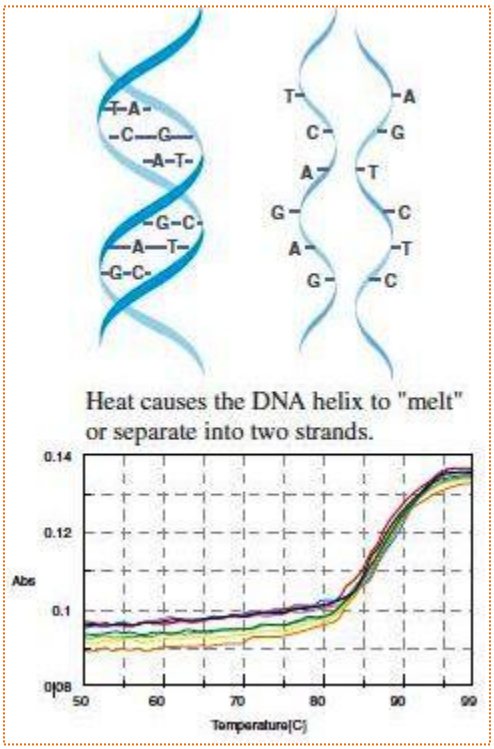
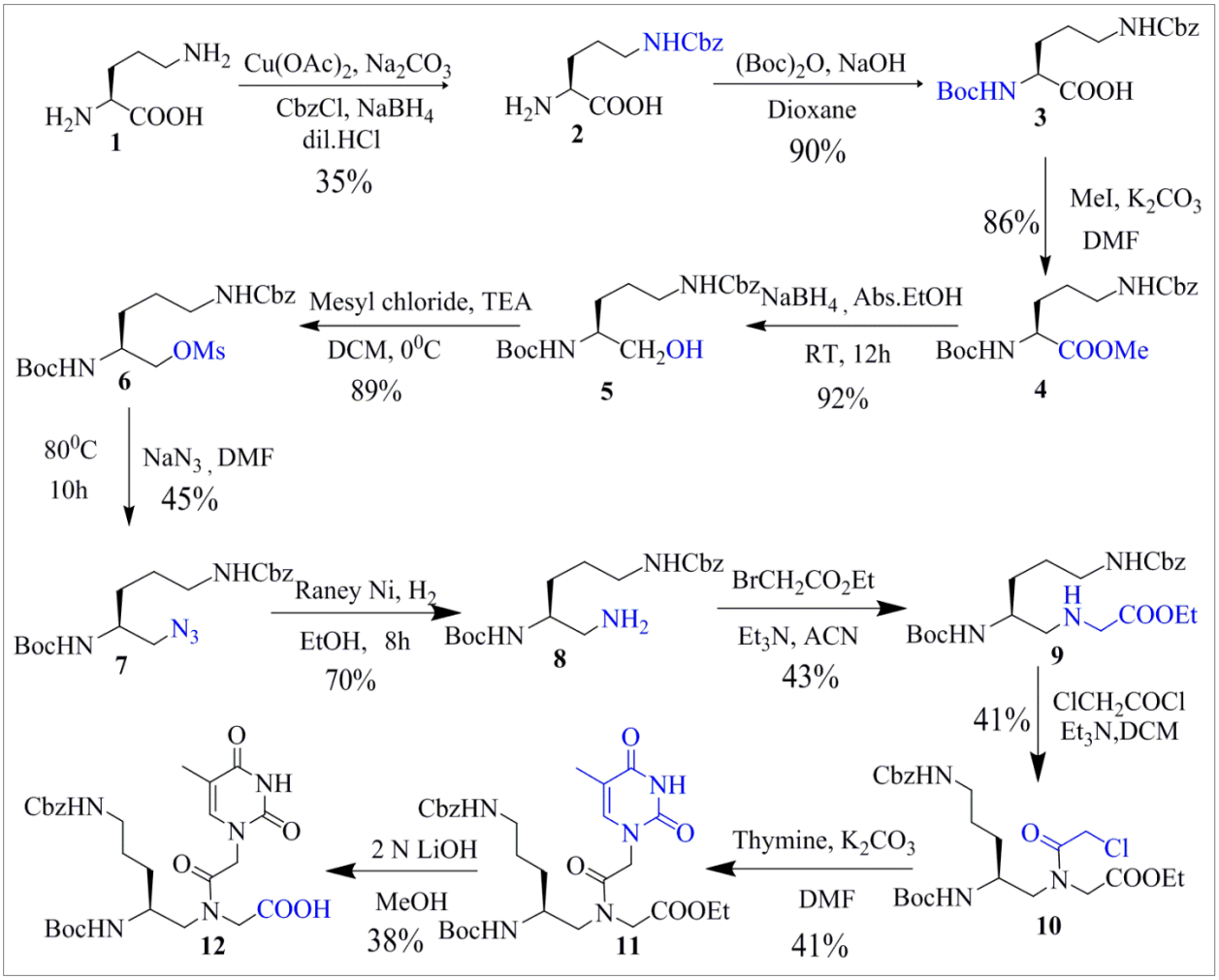


Figure.1.7. DNA Duplex melting

(http://www.jascoinc.com/Images/Product/jasco-uv-vis-spec-dna-melting.jpg)

2. Results and Discussion :

2.1. Scheme :



2.1.1. Synthesis of target monomer(12):

To synthesize PNA monomer **12**, commercially available L-ornithine **1** was reacted with Cbz-chloride in presence of $Cu(OAc)_2$ and Na_2CO_3 to get compound **2**. It was reacted with Boc-anhydride to get orthogonally protected compound **3**. Compound **3** was then esterified using methyl iodide to get ester derivative **4**. It was followed by the reduction of ester using sodium borohydride to get primary alcohol **5**. This was then reacted with mesyl chloride in presence of triethylamine to get mesyl derivative **6**.

Compound **6** was reacted with sodium azide to get azido derivative **7**. It was then reduced by using H_2 in presence of Raney Ni to get amino derivative **8** which was then immediately alkylated by using ethyl bromoacetate with triethyl amine in acetonitrile to get compound **9**. The compound **9** was acylated using chloroacetyl chloride in presence of triethyl amine to get chloro derivative compound **10** . The chloro derivative compound **10** was reacted with thymine in DMF in presence of activated K_2CO_3 to give compound **11**. Compound **11** was hydrolysed using aq. LiOH to get the final compound γ-(*S*-aminopropylene)aminoethylglycine PNA monomer.

2.2. Synthesis of PNA oligomers:

The synthesis of PNA oligomers were done using solid phase peptide synthesis protocols by incorporating the modified monomers at desired sites into unmodified *aeg* PNA strand. PNA synthesis was done from the *C*-terminus to the *N*-terminus using monomeric units with protected amino and carboxylic acid functions. MBHA resin (4-methyl–Benzhydryl amine resin) was chosen as the solid support on which the oligomers were built and the monomers were coupled by *in situ* activation with HBTU/ HOBt. In the synthesis of all oligomers, orthogonally protected (Boc/Cl-Cbz) L-lysine was selected as the *C*-terminal spacer-amino acid and it is linked to the resin through amide bond. The amine content on the resin was determined by the picrate assay and was found to be 2.00 mmol/g. So, the loading was suitably lowered to approximately 0.25 mmol/g by partial acetylation of amine content using calculated amount of acetic anhydride.[19] Free -NH_2 group on the resin available for coupling was again estimated before starting synthesis.

For the purpose of the study of DNA and RNA recognition, PNA oligomers were synthesized following the solid phase protocol using Boc-strategy as shown in Figure **1.5**. The unmodified *aeg* PNA homo oligomer (Table **1**, S.No.**1**, *aeg* PNA-**1**) was synthesized. This was used as the control sequence for the comparision of the hybridization and sequence discrimination property of modified PNA oligomer. The modified γ-(*S*-aminopropylene)aminoethylglycine PNA monomer has thymine as nucleobase. The modified PNA decamer [γ-(*S*-amp) aeg PNA] incorporating one modification at the N-terminus by the PNA monomer (12) was synthesized following the

t-Boc chemistry protocol of SPPS in order to study the effect of aminopropylene functionality on its triplex-forming ability and investigate the stability with DNA/RNA. (Table **1**, S.No.**2**, Mod (N)-PNA-**1**).

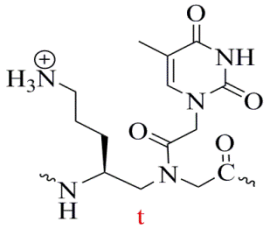
S. No.	Sequence Code	PNA Sequence	Monomer Used
1	aeg-PNA-1	H-TTACCTCAGT-Lys NH_2	A/T/G/C
2	Mod(N)-PNA-1	H-T*t*ACCTCAGT-Lys NH_2	

TABLE 1. PNA oligomers with modified/unmodified monomers

S. No.	Sequence Code	HPLC R.Time(min)	Mol. Formula	M(Calcd)/ M(Obsd)
1	aeg-PNA-1	12.0	$C_{113}H_{149}N_{55}O_{33}$	2804.1672/2805.8247$[M+1]^+$
2	Mod(N)-PNA-1	13.3	$C_{116}H_{156}N_{56}O_{33}$	2861.2250/2862.5986$[M+1]^+$

TABLE 2: HPLC retention time and MALDI-TOF mass spectral analysis of the PNAs:

2.3. Cleavage of the PNA oligomers from the solid support:

The oligomers were cleaved from the solid support (L-lysine derivatized MBHA resin), using trifluoromethane sulphonic acid (TFMSA) in the presence of TFA (Low, High TFMSA-TFA method) which yielded PNA oligomers having L-lysine amide at their *C*-termini. A cleavage time of 2 h at room temperature was found to be optimum for the oligomers and the side chain protecting groups of lysine as well as for the monomers were also removed during this process.

2.4. Purification of the PNA oligomers :

The purity of the obtained PNA oligomers were checked on analytical RP-HPLC (C18 column, acetonitrile: water system) and was found to be more than 80 % purity. These were subsequently purified by RP-HPLC on a semi-preparative C18 column to

give oligomers in 93-99% purity as determined by analytical RP-HPLC. HPLC profile of both the PNA sequences are shown below (Figure **2.4.1** and **2.4.2**).

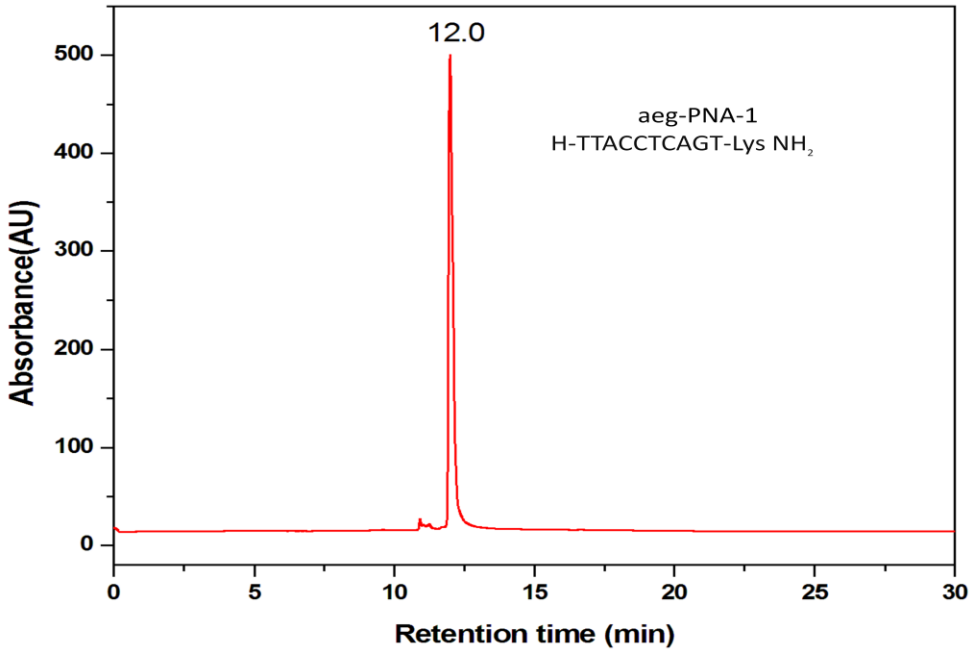


Figure 2.4.1 HPLC profiles for aeg-PNA-1 (10% CH_3CN in 0.1% TFA/H_2O, flow rate = 2 mL/min; λ = 260 nm)

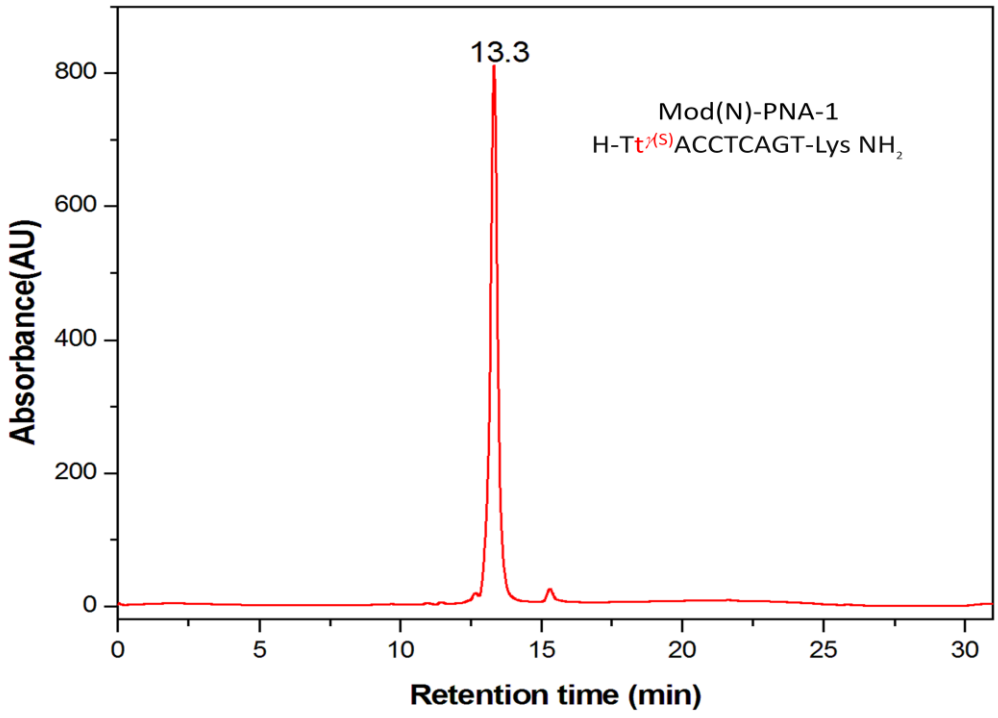


Figure 2.4.2 HPLC profiles for Mod(N)-PNA-1 (10% CH_3CN in 0.1% TFA/H_2O, flow rate = 2 mL/min; λ = 260 nm)

2.5. Circular dichroism :

Circular dichroism (CD) technique measures the interaction of circularly polarised light with molecules. CD is the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL) and occurs when a molecule contains one or more chiral chromophores. (Figure **2.5**).

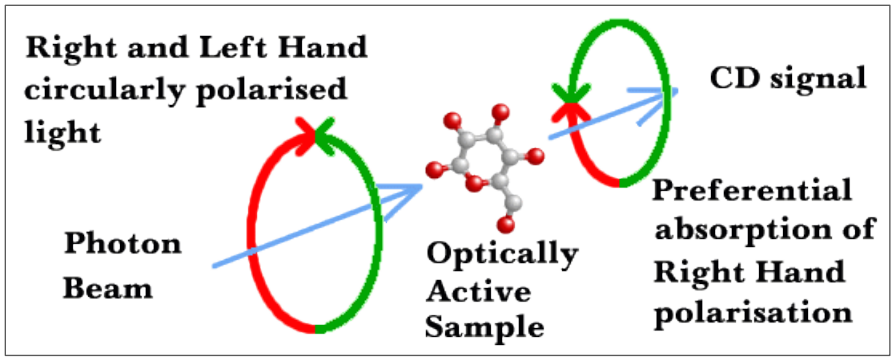


Figure 2.5 : Interaction of circularly polarised light with optically active molecule
(http://web.nmsu.edu/~kburke/Instrumentation/CD_trans.gif)

CD is used to study chiral molecules, but it is used mainly for the study of large biological molecules. CD has an important role in the structural determinants of peptides, proteins or DNA/RNA. A primary use is in analysing the secondary structure or conformation of macromolecules. It is being used to observe how secondary structure changes with environmental conditions upon interaction with other molecules. If the molecule contains chiral chromophores then one CPL (circularly polarized light) state will be absorbed to a greater extent than the other and the CD signal will be positive or negative, depending on whether L-CPL is absorbed to a greater extent than R-CPL (+ve) or to a lesser extent (-ve).

From the CD spectroscopic studies, it has been observed that the formation of PNA:DNA duplexes result in two positive bands with a maxima in the region of 265 to 275 nm (higher intensity) and another maxima in the region of 220 to 225 nm (lower intensity). PNA:DNA duplexes also showed a minima in the region of 238 to 250 nm (Figure **2.5.1**).

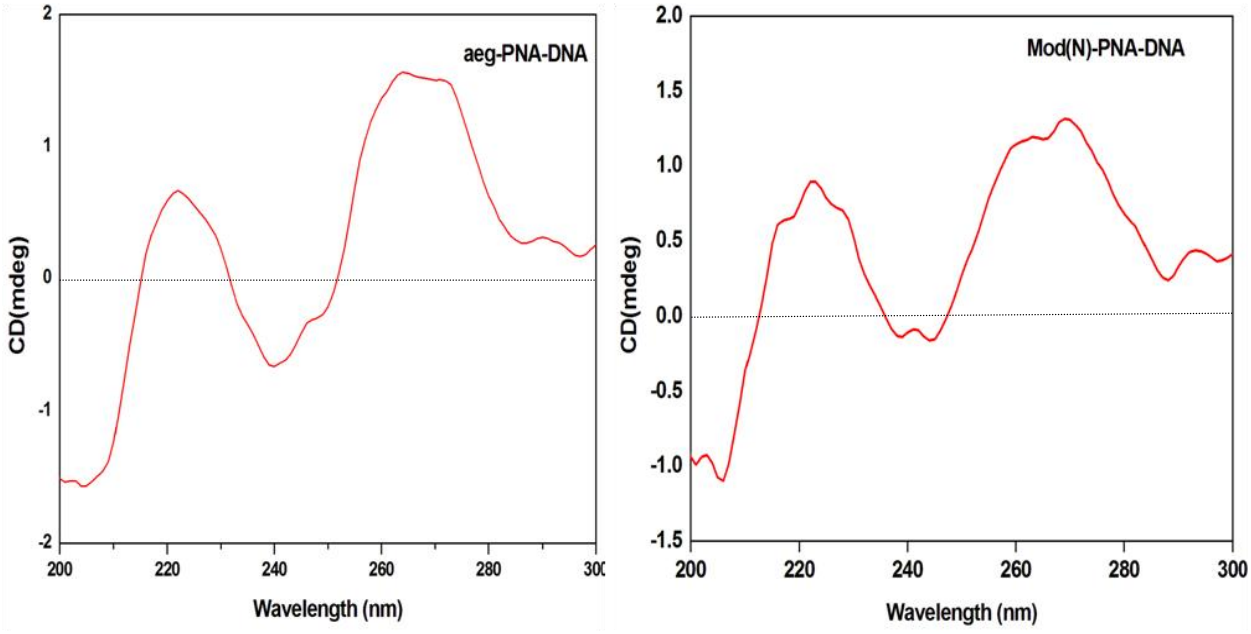


Figure 2.5.1: CD spectra of Duplexes of *aeg*-PNA and Mod(N)-PNA with complementary DNA 1
(DNA 1: 5' ACTGAGGTAA 3'; Buffer: 10 mM sodium phosphate, pH 7.2, NaCl 10 mM)

CD spectroscopic studies show that the modification at N-terminal does not alter the conformation of PNA:DNA duplexes.

2.6. Comparative study of UV- T_m value of modified PNA :

The temperature dependent UV-absorbance data shows that stability of modified PNA:DNA duplex is better than unmodified duplex. (Figure **2.6**) (Table **3**). Incorporation of amino functionality in the *aeg*-PNA backbone at γ-position gives better stabilizing effect when connected with a longer chain (3 X CH_2 spacer) to the backbone as compared to the shorter distance (2 X CH_2 spacer).

S. No.	Sequence Code	PNA Sequence	UV- T_m (°C)	ΔT_m
1	aeg-PNA-1	H-TTACCTCAGT-Lys NH_2	45.02 ± 1	-
2	Mod(N)-PNA-1	H-T*t*ACCTCAGT-Lys NH_2	49.72 ± 1	4.7

TABLE 3. PNA oligomers (3 X CH_2 spacer) with modified/unmodified monomers

Temperature dependent UV absorbance curves show that at any temperature between 20 to 90 ^oC absorption is higher for aeg-PNA:DNA duplex than Mod(N)-

PNA:DNA duplex but at 20 and 90 ^{o}C, both curves touch each other but not in between. This is because at the initial stage (20 ^{o}C) both are completely in duplex form and concentration taken is same for both PNA:DNA duplex.

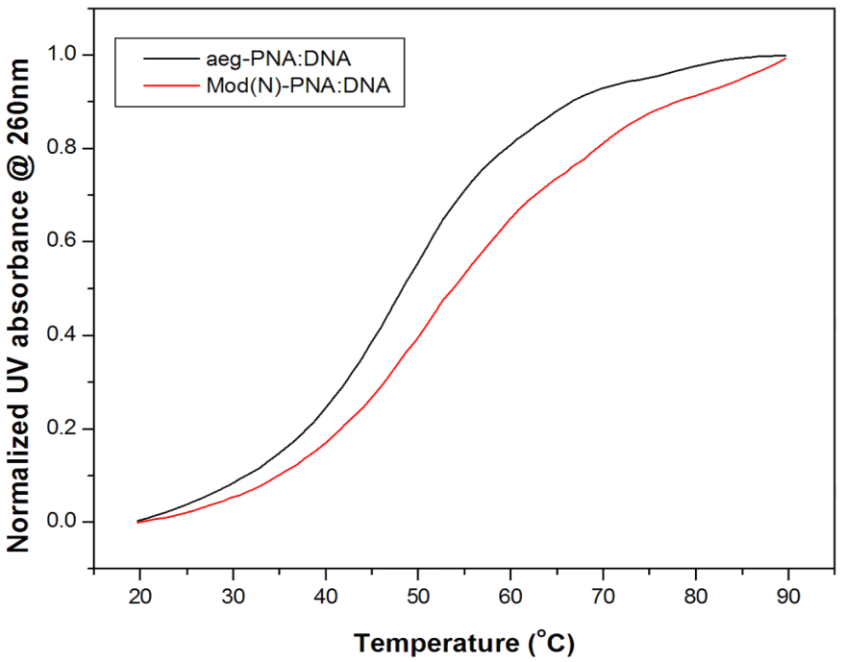


Figure 2.6 :Temperature dependent UV absorbance curves for complementary aeg-PNA and Mod(N)-PNA: DNA 1 duplexes (DNA 1 = 5' ACTGAGGTAA 3'; Buffer: 10 mM sodium phosphate, pH 7.2, NaCl 10 mM)

Now, as the temperature increases H-bonds starts cleaving and duplex strand converted in to the single strand and so the relative concentrations change. But the rate of conversion from duplex to single strand is different for both and so the concentration changes as the temperature increases after 20 ^{o}C. At 90 ^{o}C, all the duplex form converted in to single strands.

S. No.	Sequence Code	PNA Sequence	UV- T_m (^{o}C)	ΔT_m
1	aeg-PNA-1	H-TTACCTCAGT-Lys NH_2	43.4 ± 1	-
2	eam-t_2PNA-1	H-T*t*ACCTCAGT-Lys NH_2	47.3 ± 1	3.9

TABLE 4. PNA oligomers (2X CH_2 spacer) with modified/unmodified monomers

The ΔT_m value obtained for PNA:DNA duplexes formed by γ-substituted amino PNA with 3 carbon spacers is found to be greater than that formed by its analog with 2 carbon spacer (Table 4)[20] and 4 carbon spacer[21] but lesser than 1 carbon spacer[16] (Table 5).

However, further studies will be required to establish the contribution of the cationic group with 3 carbon spacer to the thermal stability of the duplex because experimental as well as instrumental error can't be ignored.

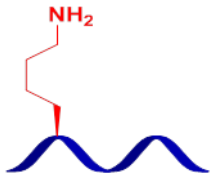
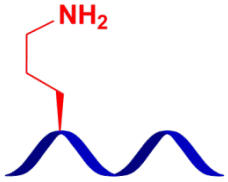
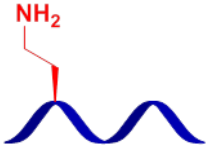
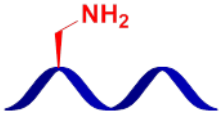
Carbon Chain Length	Amino Modification Incorporated	ΔT_m (°C) per modification	Reference
4		1.7	21
3		4.7	**Present Work**
2		3	20
1		6.5	16

TABLE 5. Comparative thermal stabilities of cationic γ-C-Substituted PNAs

Nevertheless, from the observation it is apparent that length of the spacer at γ-position has a strong influence in determining the thermal stability of the DNA:PNA duplex of these cationic PNA analogs.

3. Conclusion :

- The modified PNA (3 X CH_2 spacer) at γ-carbon show higher binding affinity for complementary DNA than the unmodified PNA.
- The effects of number of carbon chain spacer at γ-position indicate the role of steric interactions.
- CD study showed that incorporation of modified units in the aeg PNA does not alter the structure of PNA:DNA duplex which forms right handed helix when S-stereochemistry is used at γ-position.
- UV- T_m data shows that stability of modified PNA:DNA duplex with 3 carbon spacer is higher than that of 2 and 4 carbon spacer but lower than 1 carbon spacer.
- More studies are needed in order to define the stability of modified PNA with DNA.

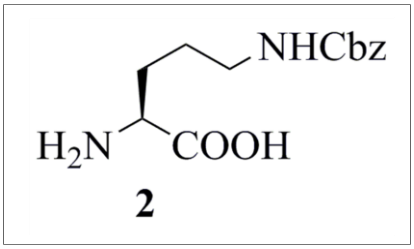
4.Experimental Section:

4.1.General:

The chemicals used were of laboratory or analytical grade. The purified solvents were used. Ethyl bromoacetate, HBTU, HOBt, EtOAc, NMP, Pet-ether were obtained from Spectrochem and used without further purification. Reactions were checked using thin layer chromatography (TLC). Column chromatographic separations were performed using silica gel 100-200 mesh (Merck). The 1H spectra were recorded on Bruker 500 MHz (or 125 MHz for 13C) and Jeol 400 MHz (or 100 MHz for 13C) using residual solvents signals as an internal reference [($CDCl_3$ δH, 7.26 ppm, δC 77.0 ppm) and CD_3OH δC 49.3 ppm]. The chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. MALDI-TOF/TOF mass spectra were obtained on Model 4800 (Applied Biosystems) instrument.

4.2. Procedures:

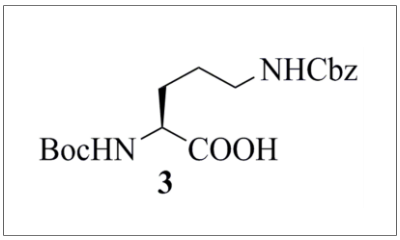
2: (S)-2-amino-5-[{(benzyloxy)carbonyl}amino] pentanoic acid



2

L-Ornithine hydrochloride (20 g, 118.6 mmol) and copper (II) acetate (11.8 g, 59.3 mmol) were dissolved in 100mL of 10% aq. sodium carbonate and the solution was vigorously stirred for approximately 35 minutes. To the stirred solution 200 mL of water and 200 mL of 1,4-dioxane were added, followed by slow addition of CbzCl solution (18.5 mL, 130 mmol) in 100 mL of 1,4-dioxane. After 6 hr, $NaBH_4$ (5.3 g, 142.3 mmol) was added slowely. After 35 min, the copper (I) oxide precipitate formed was filtered. The clear, colorless filtrate was neutralized with dil. HCl in cold condition. L- Nδ-Cbz ornithine was precipitated which was filtered. (35 % yield). MP= 240-250 °C. $[\alpha]^{25}_{D}$+ 21.0 $\pm$ 2.5 °C (c=2.9, 50% Acetone +1N HCl= 1:1)

3:(S)-5-[{(benzyloxy)carbonyl}amino]-2-[(tert-butoxycarbonyl)amino] pentanoic acid

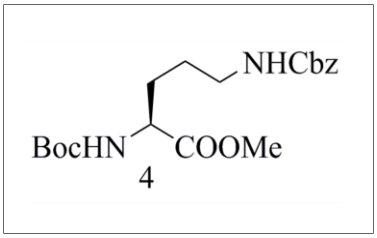


3

A solution of di-tert-butyl dicarbonate [$(Boc)_2O$] (3.2 g, 0.015 mol) in dioxane (50 mL) was added to an ice-cold stirred solution of compound **2** (3.3 g, 0.012 mol) in 1N aq. NaOH (50 mL) by means of an addition funnel. The bi-phasic mixture was stirred at 4 °C for half an hour and then allowed to warm to room temperature while stirring over 6 h. TLC analysis showed that reaction was completed. The reaction mixture was concentrated to half its original volume; cooled in ice water and acidified to pH 2-3 by

slow addition of 1 N $KHSO_4$ and then extracted 3 times with ethyl acetate. The organic layer were dried over anhydrous Na_2SO_4, filtered and concentrated to get the compound **(3)** which was used for next reaction without further purification (90 % yield). MALDI-TOF *m/z* calcd $C_{18}H_{26}N_2O_6$, 405.1428; obs. is 405.1447.

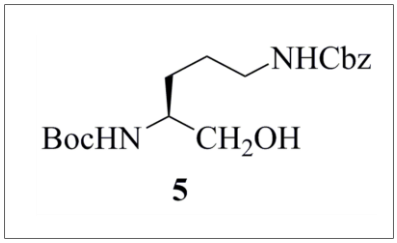
4:Methyl(S)-5-[{(benzyloxy)carbonyl}amino]-2-(tert-butoxycarbonyl)amino] pentanoate



4

To a cold solution of compound **(3)** (4.1 g, 0.011 mol) in DMF (40 mL) activated K_2CO_3 (1.73 g, 0.012 mol) was added. After stirring for 20 minutes at 0 °C, MeI (2.5 mL, 0.041 mol) was added to the white suspension and the stirring was continued at room temperature for 7 h. TLC showed the complete formation of methyl ester. The solvent was removed from the reaction mixture and the residue was extracted 3 times with ethyl acetate and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on silica gel using pet-ether and ethyl acetate to get compound **(4)** as pale amber oil which solidifies into shiny white compound after cooling (86% yield). MP = 65-72 °C; R_f = 0.5 petroleum ether/EtOAc (70:30). ^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.40 – 7.34 (m, 5H), 5.09 (s, 2H), 4.91 (s, 1H), 4.30 (s, 1H), 3.74 (s, 3H), 3.22 (d, 2H), 1.74 (m, 2H), 1.59 (m, 2H), 1.44 (s 9H). MALDI-TOF *m/z* calcd $C_{19}H_{28}N_2O_6$, 419.1584 and obs. is 419.0710 $[M+K]^+$.

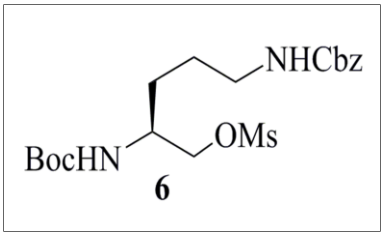
5: Benzyl tert-butyl (5-hydroxypentane-1,4-diyl)(S)-dicarbamate



5

To a stirred solution of compound **4** (3.7 g, 9.6 mmol) in absolute ethanol (40 mL) was added $NaBH_4$ (1.1 g, 28.9 mmol) and reaction mixture was stirred for 7 h under nitrogen atmosphere at RT. Completion of the reaction was monitored by TLC. After completion of reaction, absolute ethanol was removed under reduced pressure. Water (80 mL) was added to the concentrate which was extracted with ethyl acetate (3 x 50 mL). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was then purified on silica gel by using petroleum ether and ethyl acetate to give compound **5** as white solid (92% yield). MP: 80-85 °C; R_f = 0.4 pet-ether/EtOAc (50:50); ^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.36 – 7.34 (m, 5H), 5.09 (s, 2H), 4.98 (s, 1H), 4.77 (s, 1H), 3.63 (s, 3H), 3.21 (d, 2H), 1.80 (m, 1H), 1.57 (m, 3H), 1.44 (s 9H). MALDI-TOF *m/z* calcd $C_{18}H_{28}N_2O_5$, 391.1635 and obs. is 391.0913 $[M+K]^+$.

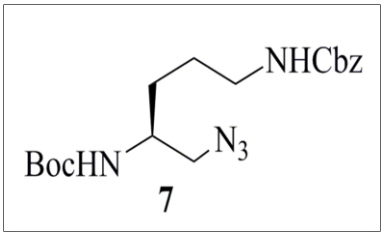
6: (S)-5-[{ (benzyloxy)carbonyl}amino]-2-[(tert-butoxycarbonyl)amino]pentyl methanesulfonate



6

The alcohol derivative compound **(5)** (2.7 g, 7.5 mmol) was dissolved in dry DCM and stirred at 0 °C for 5 mins. To this, TEA was added drop wise and stirred for next 10 mins. To this ice cooled solution was added distilled mesyl chloride (0.8 mL, 8.6 mmol) from a dropping funnel over a period of 10 mins under nitrogen atmosphere. The reaction mixture was stirred for 20 mins. DCM (30 mL) was added to reaction mixture and then washed with water (30 mL) and brine (20 mL). The organic layer was dried over an. Na_2SO_4, filtered and concentrated to give solid compound **6** (89% yeild) which was immediately used in next step without further purification. R_f = 0.58 pet-ether/EtOAc (50:50).

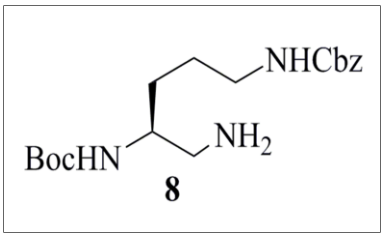
7: Benzyl tert-butyl (5-azidopentane-1,4-diyl)(S)-dicarbamate



7

To the mesyl derivative compound **(7)** (2.5 g, 5.8 mmol) in dry DMF, NaN_3 was added. The reaction mixture was heated at 75 °C for 9 h. TLC analysis showed that reaction was completed. To the reaction mixture water (70 mL) was added which was extracted thrice with ethyl acetate (50 mL). The organic layer was washed with water (30 mL) and brine (20 mL). The organic layer was dried over anh. Na_2SO_4, filtered and concentrated which was further purified on silica gel by using petroleum ether and ethyl acetate to give compound **7** as sticky yellowish oil (45% yield). R_f = 0.5, pet-ether/EtOAc(60:40).**IR:** 1168.29,1257.39,1438.35, 1458.52,1537.45,1709.01, 2100.01, 2855.06, 2926.52, 2926.52, 2965.77, 3019.70, 3306.64(cm^{-1}). ^1H NMR (400 MHz, CHLOROFORM-D) δ 7.35 – 7.33 (m, 5H), 5.08 (s, 2H), 4.85 (s, 1H), 4.58 (s, 1H), 3.70 (s, 1H), 3.37 (d, *J* = 15.2 Hz, 2H), 3.20 (d, *J* = 6.2 Hz, 2H), 1.56 – 1.38 (m, 13H).MALDI-TOF *m/z* calcd $C_{18}H_{27}N_5O_4$, 416.1700 and obs. is 416.1081 $[M+K]^+$.

8: Benzyl tert-butyl (5-aminopentane-1,4-diyl)(S)-dicarbamate

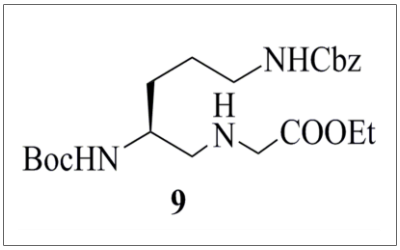


8

To a solution of the azide **(21)** (1.8 g) in abs. ethanol (25 mL) taken in hydrogenation flask, activated Raney Nickel (5 mL) was added. The reaction mixture was hydrogenated in a Parr apparatus for 7 h at RT and H_2 pressure of 40-50 psi. The catalyst from reaction mixture was filtered off and the solvent was removed under reduced pressure to yield a residue of the amine compound (**8**) as yellowish oil (70%

yield) which was used in next step without any further purification. MALDI-TOF *m/z* calcd $C_{18}H_{29}N_3O_4$, 390.1795 and obs. is 390.1346 $[M+K]^+$.

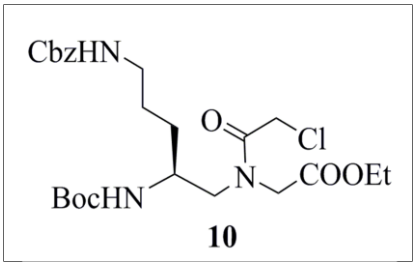
9:Ethyl(S)-(5-[{(benzyloxy)carbonyl}amino]-2-{(tert-butoxycarbonyl)amino} pentyl)glycinate)



9

The amine compound **(8)** (1.3 g, 3.7 mmol) was dissolved in acetonitrile (20 mL). To this TEA (1.5 mL, 11.1 mmoL) and the mixture was stirred at 0 °C for 10 mins. After that ethylbromoacetate (0.4 mL, 3.7 mmol) was added dropwise at 0 °C and reaction mixture was stirred for 9 h. TLC analysis showed that reaction was completed. Acetonitrile was evaporated completely and water (90 mL) was added to the residue. The aq. layer was extracted thrice with ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4, filtered and concentrated on rota evaporator. The residue obtained was purified on silica gel using petroleum ether and ethyl acetate to give compound (**9)** as yellowish oil (43% yield). R_f = 0.4, pet-ether/EtOAc(30:70). ^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.34 – 7.29 (m, 5H), 5.07 (s, 2H),4.16 (q, *J* = 7.1 Hz, 2H), 3.63 (s, 1H), 3.35 (t, *J* = 17.5 Hz, 2H), 3.20 (d, *J* = 6.2 Hz, 2H), 2.63 (s, 2H), 2.41 (s, 2H), 1.50 – 1.56 (m, 13H), 1.25 (t, *J* = 7.1 Hz, 3H). MALDI-TOF *m/z* calcd $C_{22}H_{35}N_3O_6$, 476.2163 $[M+K]^+$and obs. is 476.1411.

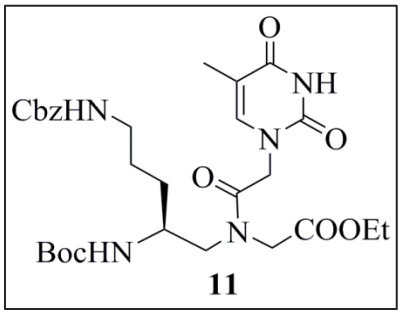
10:Ethyl(S)-N-(5-[{(benzyloxy)carbonyl}amino]-2-{(tert-butoxycarbonyl)amino}pentyl)-N-(2-chloroacetyl)glycinate



10

Compound **(9)** (630 mg, 1.4 mmol) was dissolved in DCM (8 mL) under ice cooled condition and TEA (0.8 mL, 5.8 mmol) was slowly added to it. The reaction mixture was stirred for 10 minutes and then chloroacetyl chloride (0.1 mL, 2.0 mmol) was added drop wise with vigorous stirring. The reaction mixture was stirred for 3 h. TLC analysis showed that reaction was completed. The compound was partitioned between organic layer and aqueous phase and the product was extracted into the organic phase. It was then purified on silica gel using column chromatography technique to obtain compound**(10)** as colourless sticky oil (41%yield). R_f=0.68, pet-ether/EtOAc(30:70). MALDI-TOF *m/z* calcd $C_{24}H_{36}N_3O_7Cl$, 552.1879 $[M+K]^+$ and obs. is 552.1608.

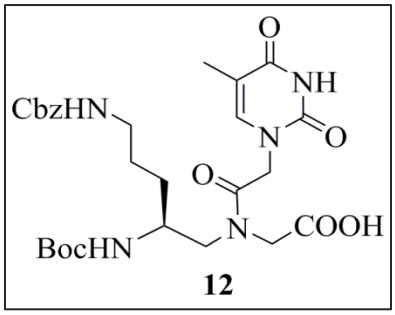
11:Ethyl(S)-N-(5-[{(benzyloxy)carbonyl}amino]-2-{(tert-butoxycarbonyl)amino}pentyl)-N-(2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetyl)glycinate



11

To the mixture of chloro compound **(10)** (370 mg, 0.72 mol) and anhydrous K_2CO_3 (128 mg, 0.93 mmol) in dry DMF (10 mL) thymine (286 mg, 0.0026 mol) was added slowly under nitrogen atmosphere and was heated with stirring at 60 °C for 7 h. TLC analysis showed that reaction was completed. After cooling, the solvent was removed under reduced pressure to leave a residue, which was extracted into DCM (3 × 12 mL) and dried over $NaSO_4$. The solvent was evaporated and the crude compound was purified by column chromatography (MeOH/DCM) to get compound **11** as white solid. (41% yield). HR-MS (*m/z)* calcd $C_{29}H_{41}N_5O_9$, 626.2801. $[M+Na]^+$, obs is 626.2799.

12:(S)-N-(5-[{(benzyloxy)carbonyl}amino]-2-{(tert-butoxycarbonyl)amino}pentyl)-N-(2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetyl)glycine



12

To the monomer ester **(11)**, THF and MeOH was added. After dissolution it was stirred for 5 mins at 0 °C and then aq. solution of 0.5 M LiOH was added slowly for a period of 15 mins. The mixture was stirred for 1.5 h. TLC showed the complete conversion of ester monomer to acid compound(in 100% EtOAc) .THF was removed from the reaction mixture. Water was added to it and it was acidified to PH 3 by using dil. HCl. Then it was extracted thrice with Ethyl acetate. The ethyl acetate layer was dried over anh. Na_2SO_4, filtered and concentrated to give white solid product. MP= 180-190 °C. ^{1}H NMR (500 MHz, CHLOROFORM-D) δ 7.34 – 7.33 (m, 5H), 5.06 (s, 2H), 4.67 (s, 2H), 4.29 (m, 3H), 3.85 (m, 2H), 3.31 (t, 2H), 1.87 (s, 3H), 1.45 (s 9H). HR-MS (*m/z)* calcd $C_{27}H_{37}N_5O_9$, 598.2489 $[M+Na]^+$, obs. is 598.2460.

4.3 Cleavage of the PNA oligomers from the resin:

The MBHA-resin (10 mg) with oligomers attached to it was stirred with thioanisole (20μL) and 1, 2-ethanedithiol (8 μL) in an ice bath for 10 min. TFA (200 μL) was added to it and after 10 min, TFMSA (16 μL) was added slowly with vigoros shaking to disperse the heat generated. The reaction mixture was stirred for 2 h at room temperature. The resin was removed by filtration and washed twice with TFA. TFA was removed under reduce pressure up to 1/4th volume. The filtrate was combined and the product was precipitated with cold dry ether. The peptide was isolated by centrifugation and the precipitate was dissolved in de-ionized water (200 μL) and loaded over sephadex G25 column. Fractions of 0.5 mL were collected and the presence of oligomers was detected by measuring the absorbance at 260 nm. The fractions

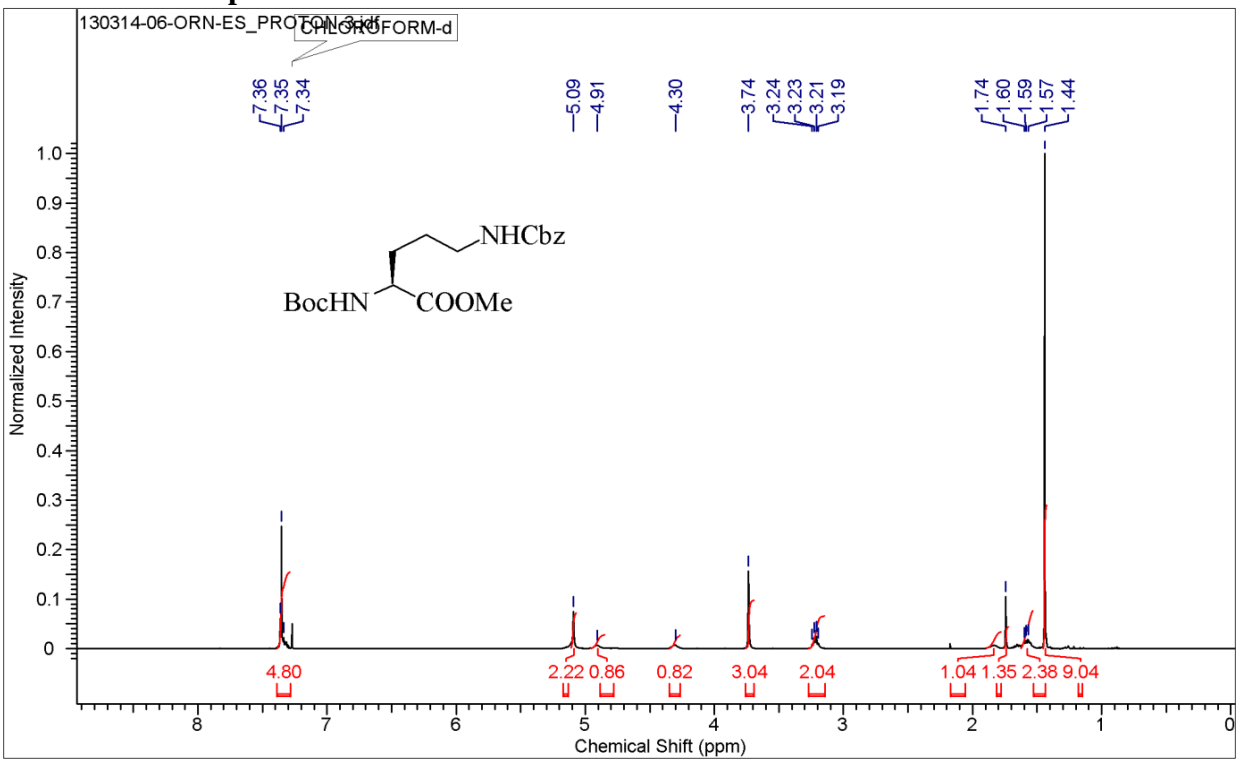
containing oligomers were freeze-dried and the purity of the fractions was assessed by analytical RP-HPLC. If the purity is less than 90%, oligomers were purified by preparative HPLC.

4.4. MALDI-TOF Mass spectrometry:

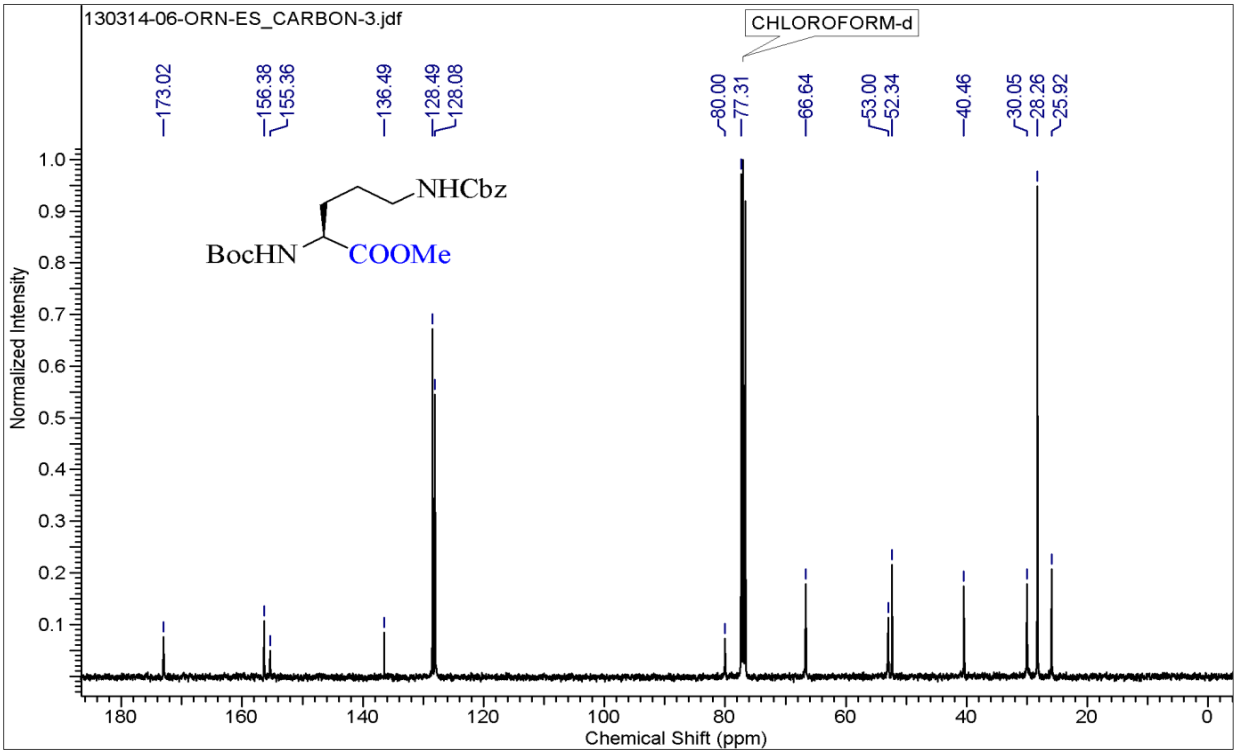
Literature reports the analysis of PNA purity by MALDI-TOF mass spectrometry in which several matrices have been explored, *viz*. Sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid), CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid). Of these, CHCA was found to give the best signal to noise ratio with all other matrices typically producing higher molecular ion signals. For all the MALDI-TOF spectra recorded for the PNAs reported in this chapter, CHCA was used as the matrix on Voyager-De-STR (Applied Biosystems) instrument and was found to give satisfactory results.

4.5. Spectral Data:

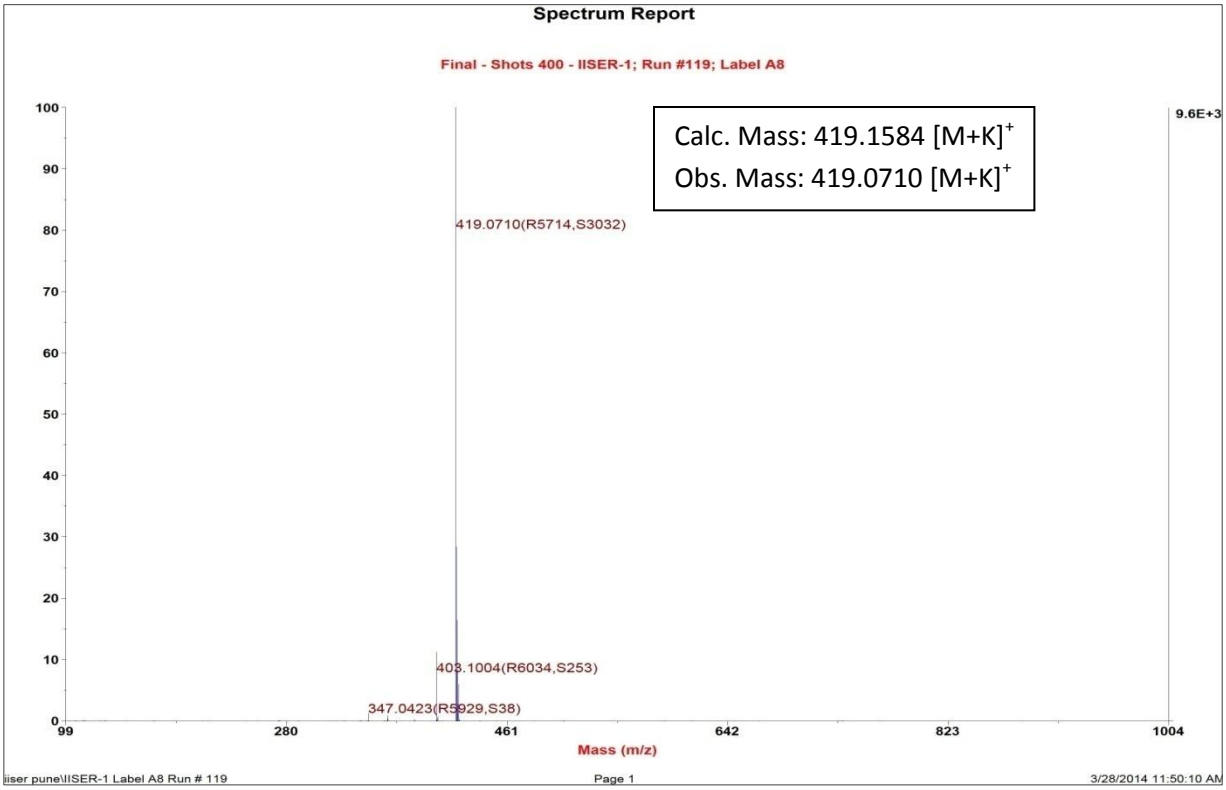
^{1}H NMR of Compound 4 :



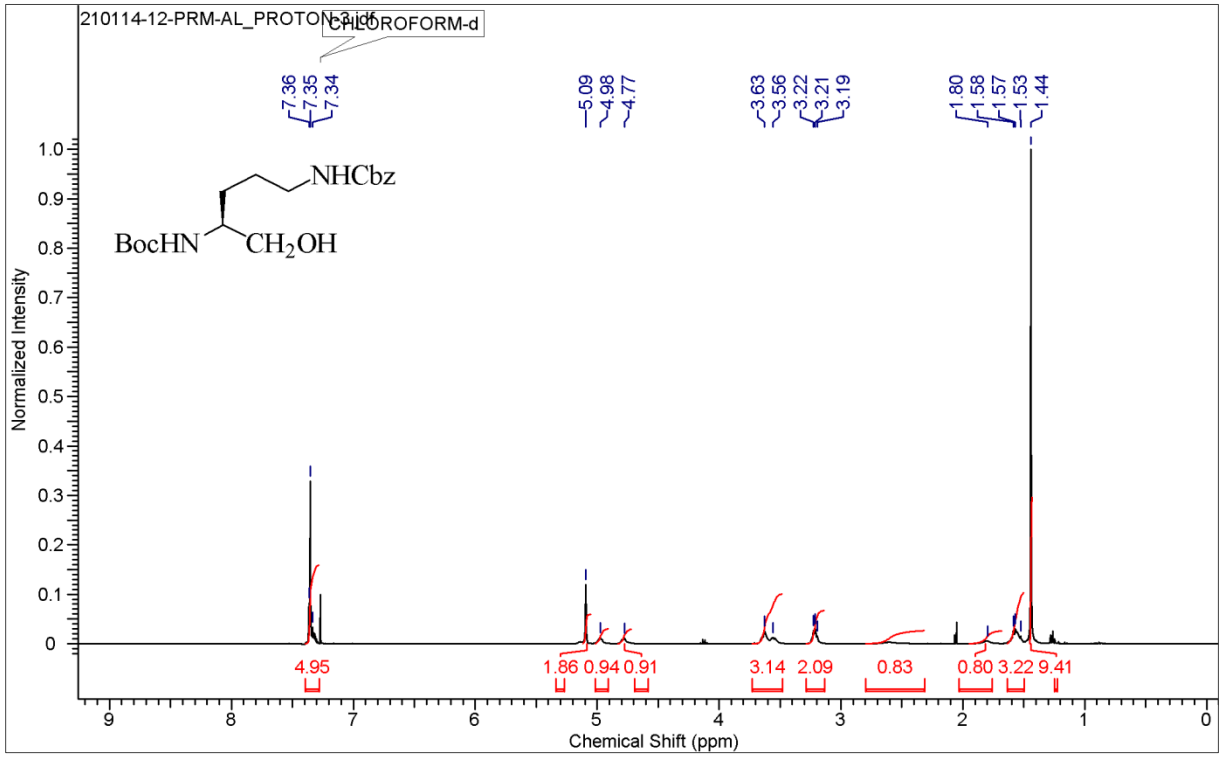
^{13}C NMR of Compound 4 :



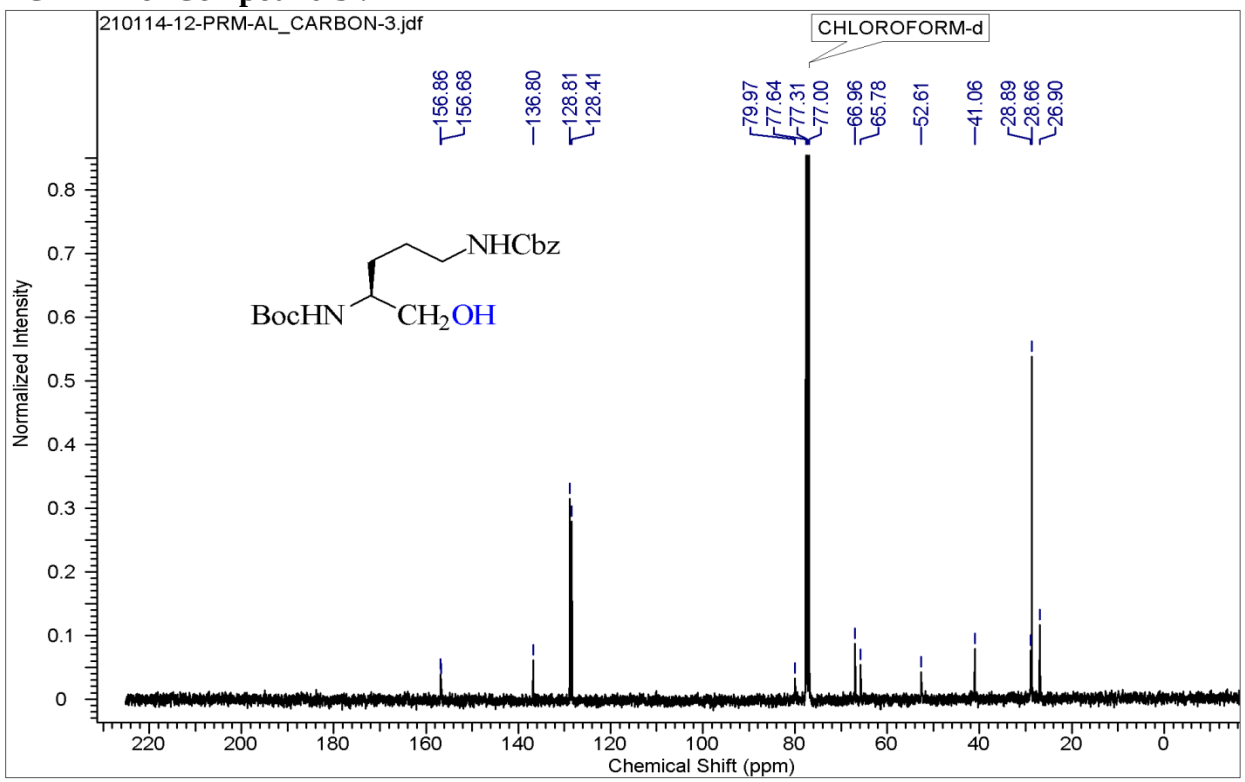
MALDI-TOF spectrum of Compound 4 :



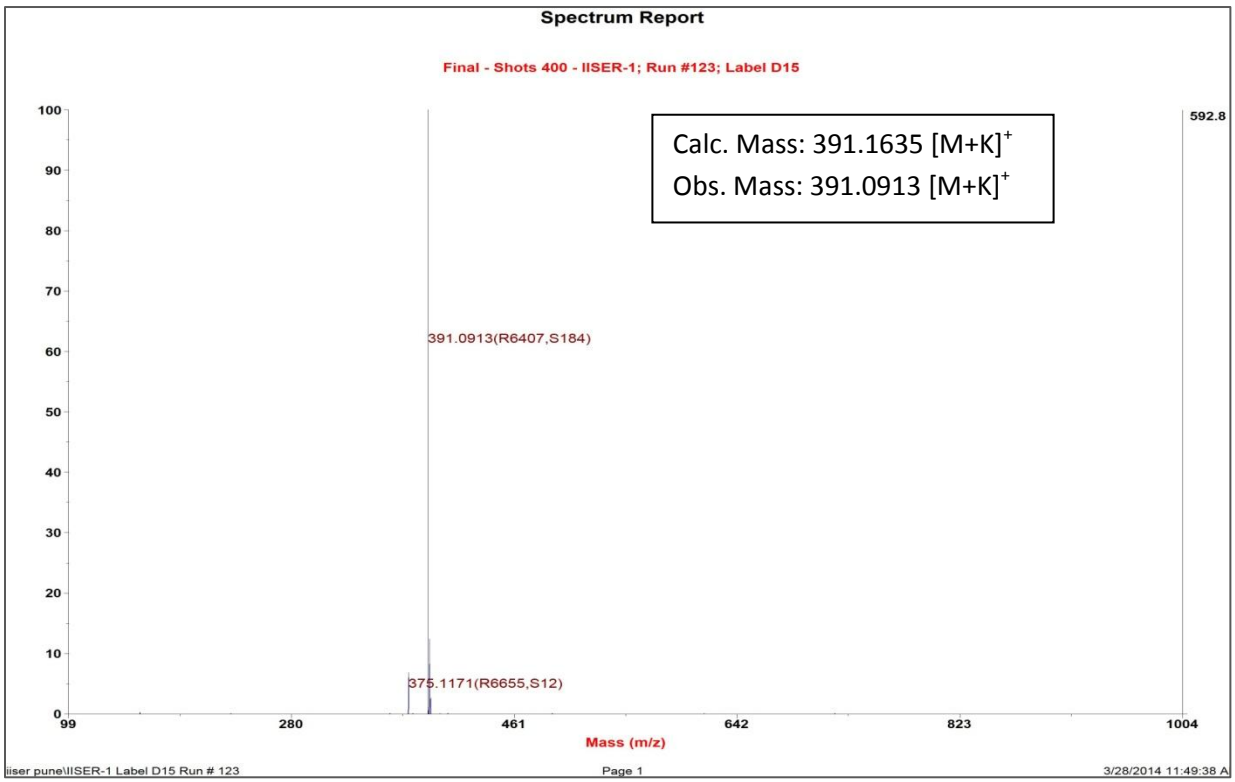
^{1}H NMR of Compound 5 :



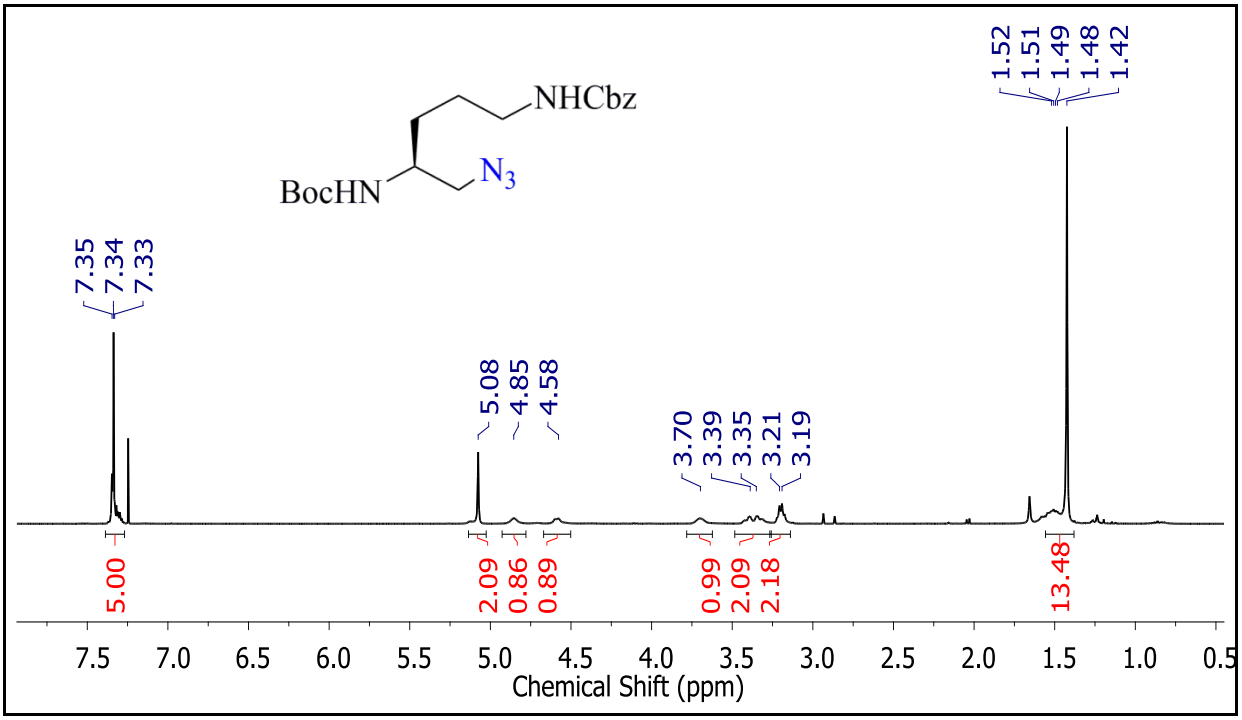
^{13}C NMR of Compound 5 :



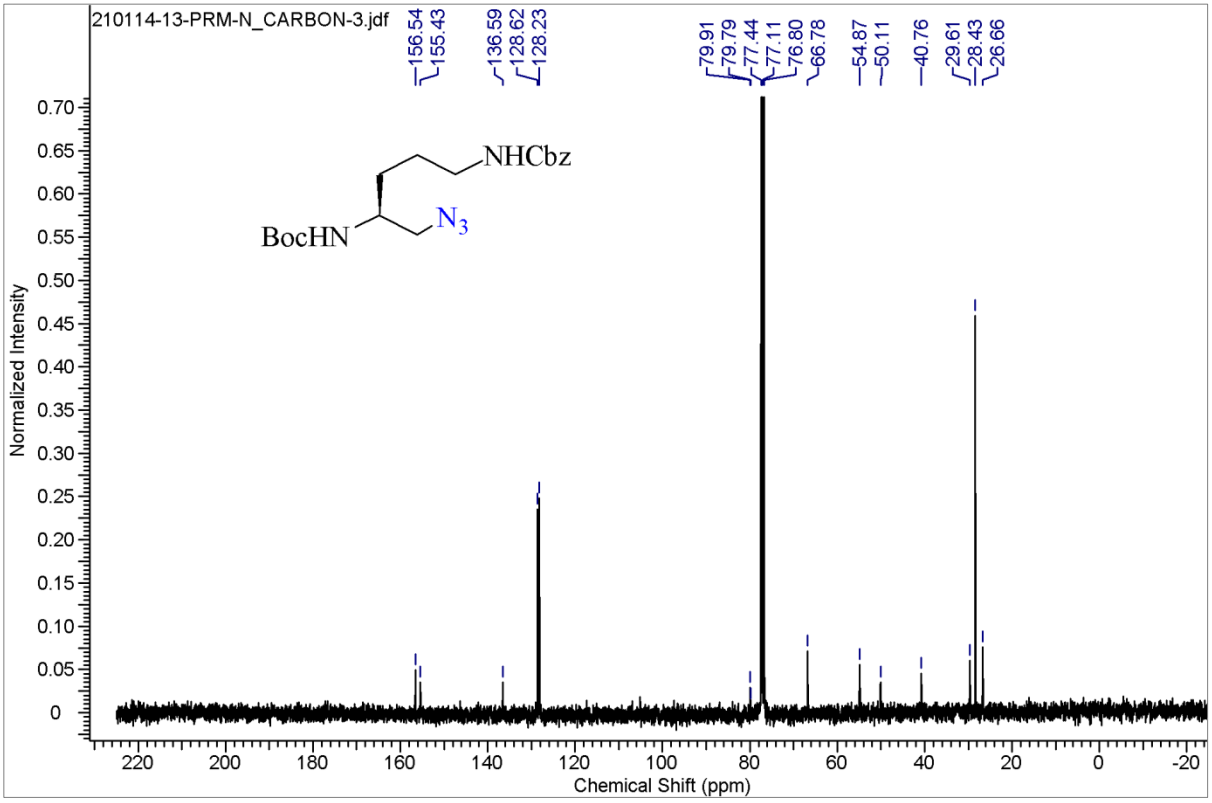
MALDI-TOF spectrum of Compound 5 :



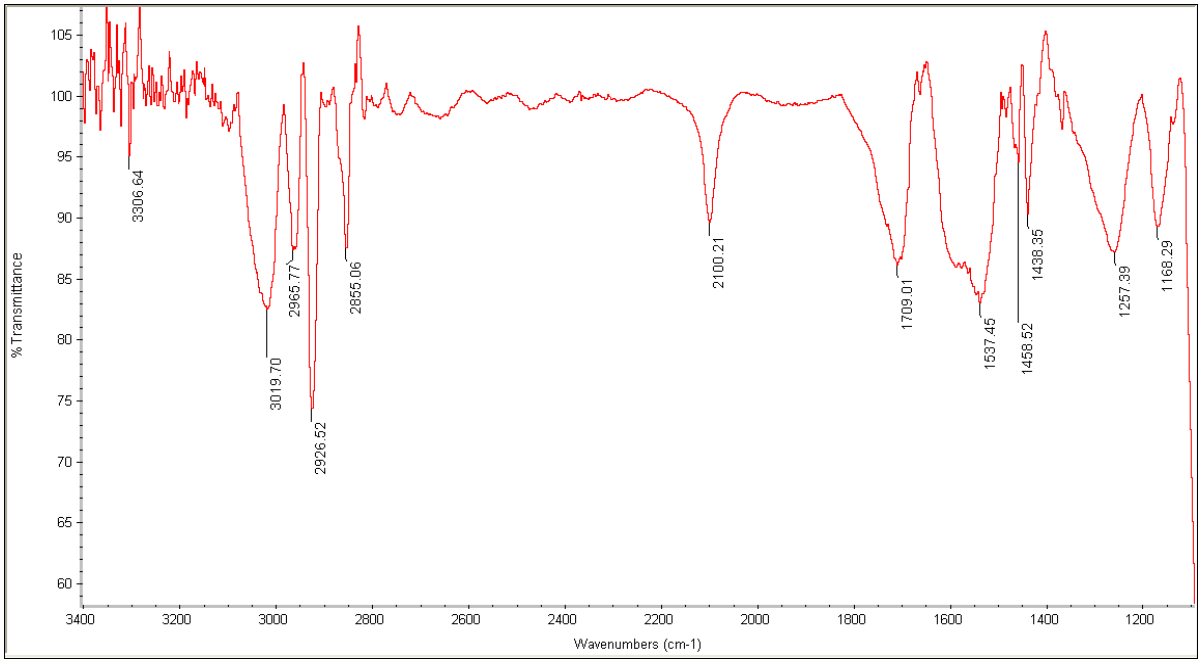
^{1}H NMR of Compound 7:



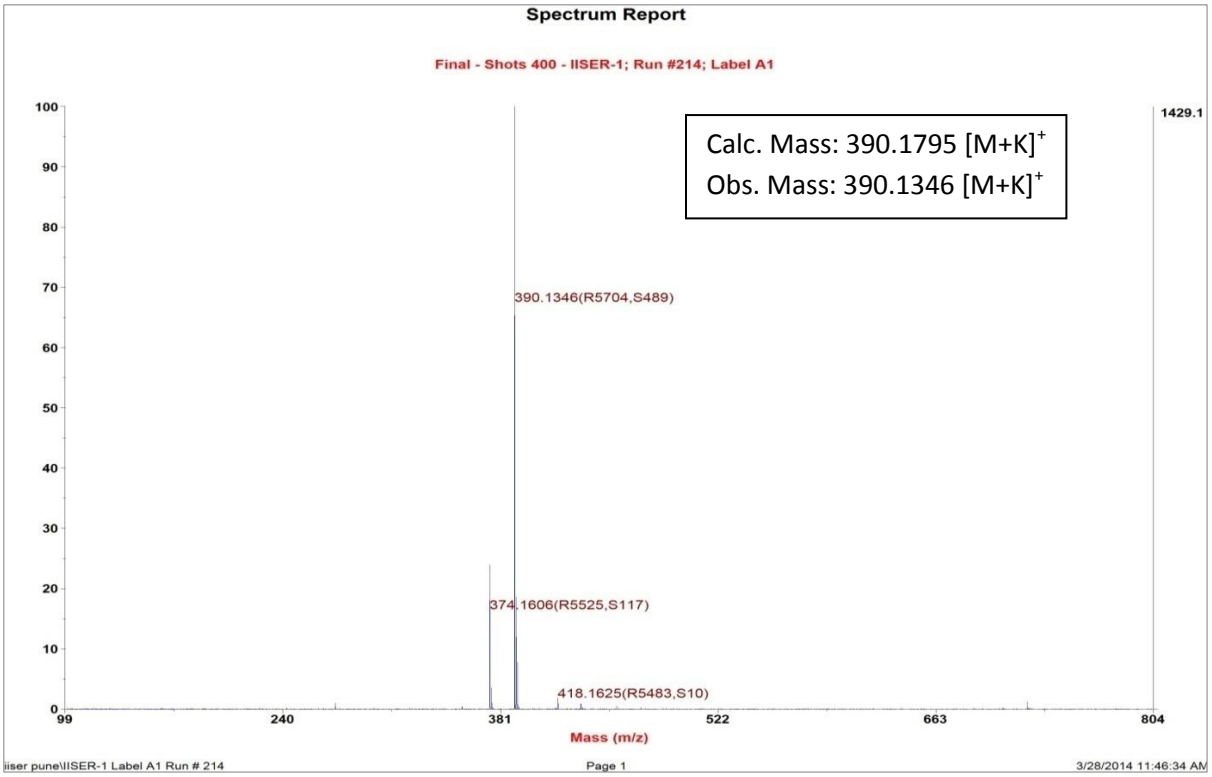
^{13}C NMR of Compound 7 :



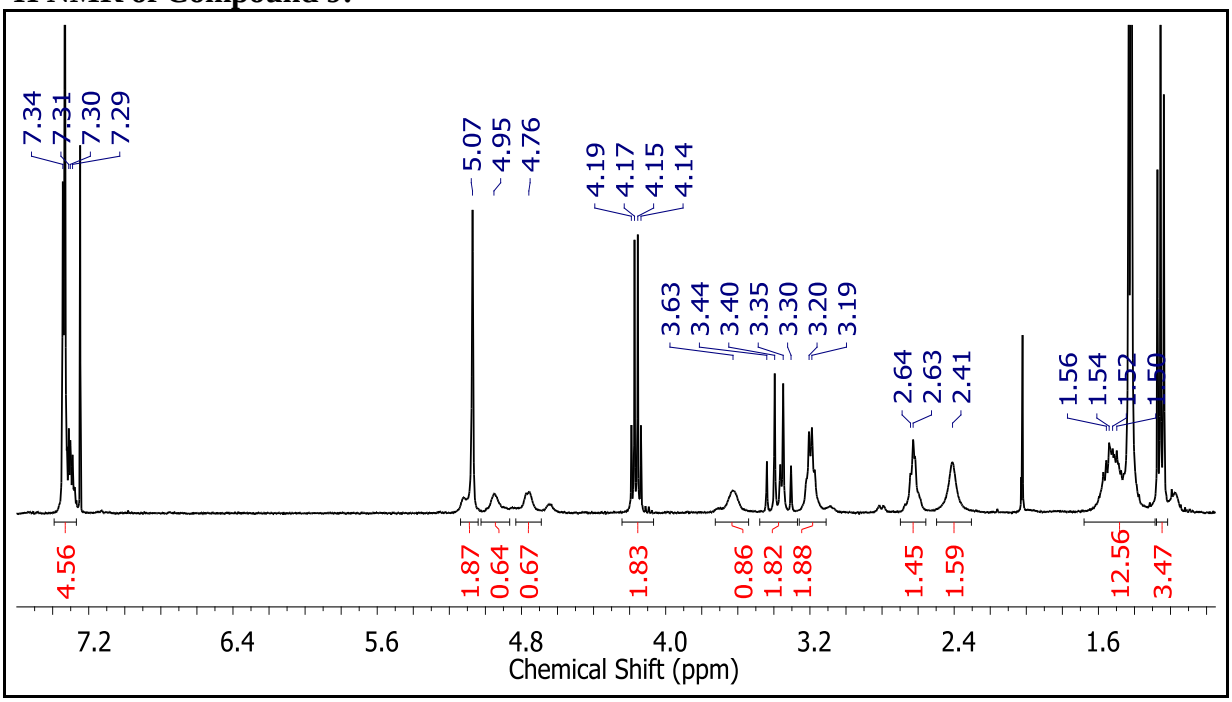
IR spectra of azide derivative compound **7** :



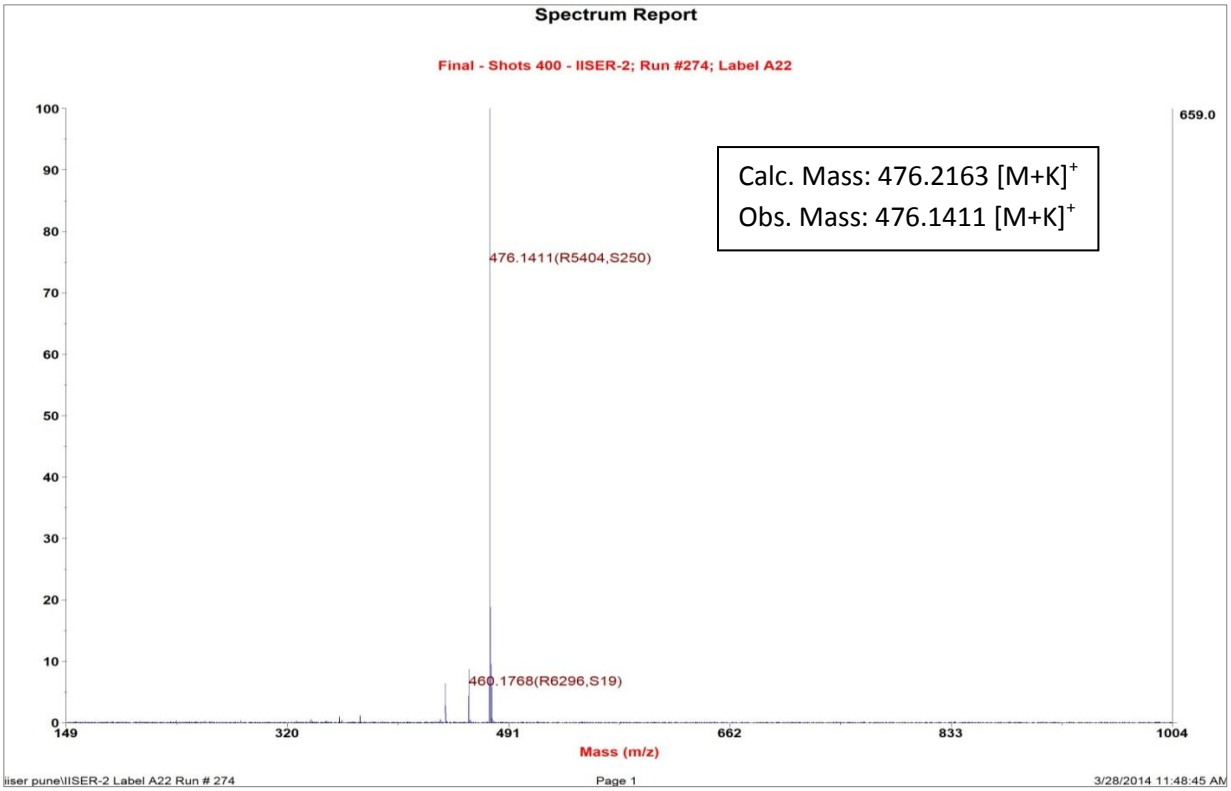
MALDI-TOF spectrum of Compound 8 :



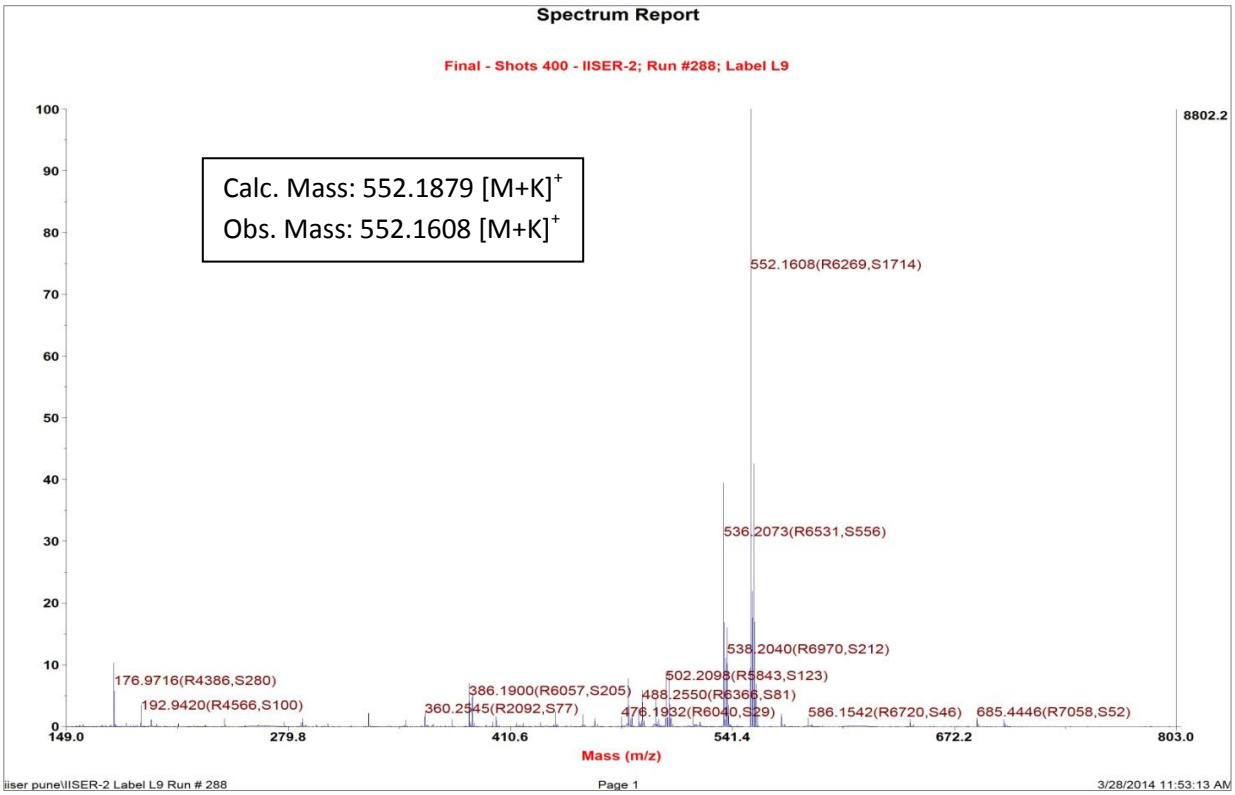
^{1}H NMR of Compound 9:



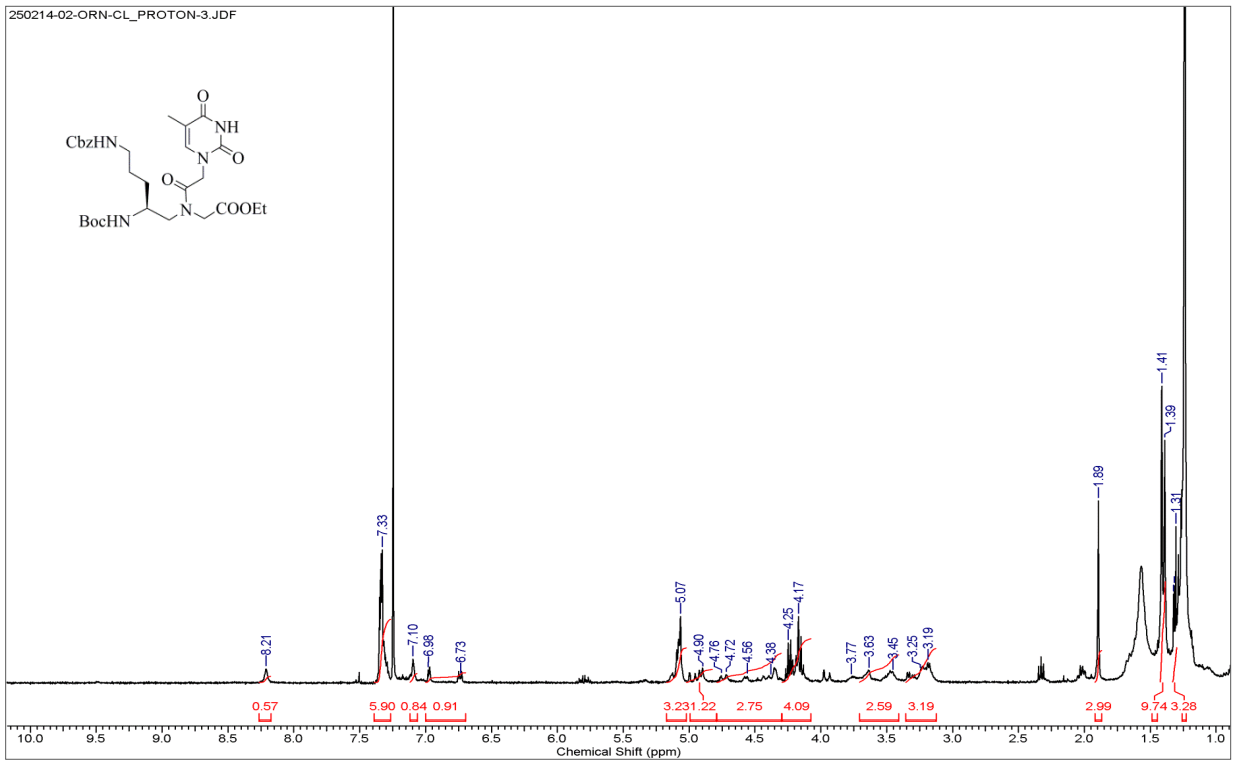
MALDI-TOF spectrum of Compound 9 :



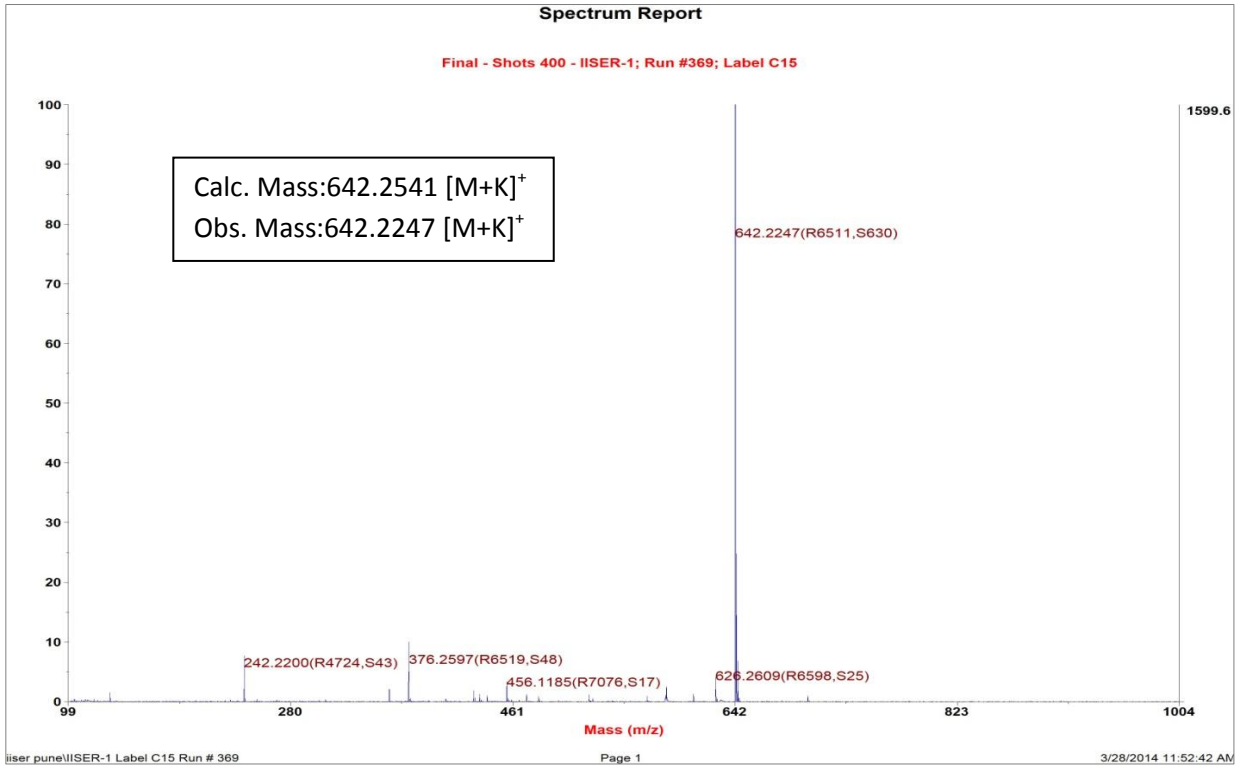
MALDI-TOF spectrum of Compound 10 :



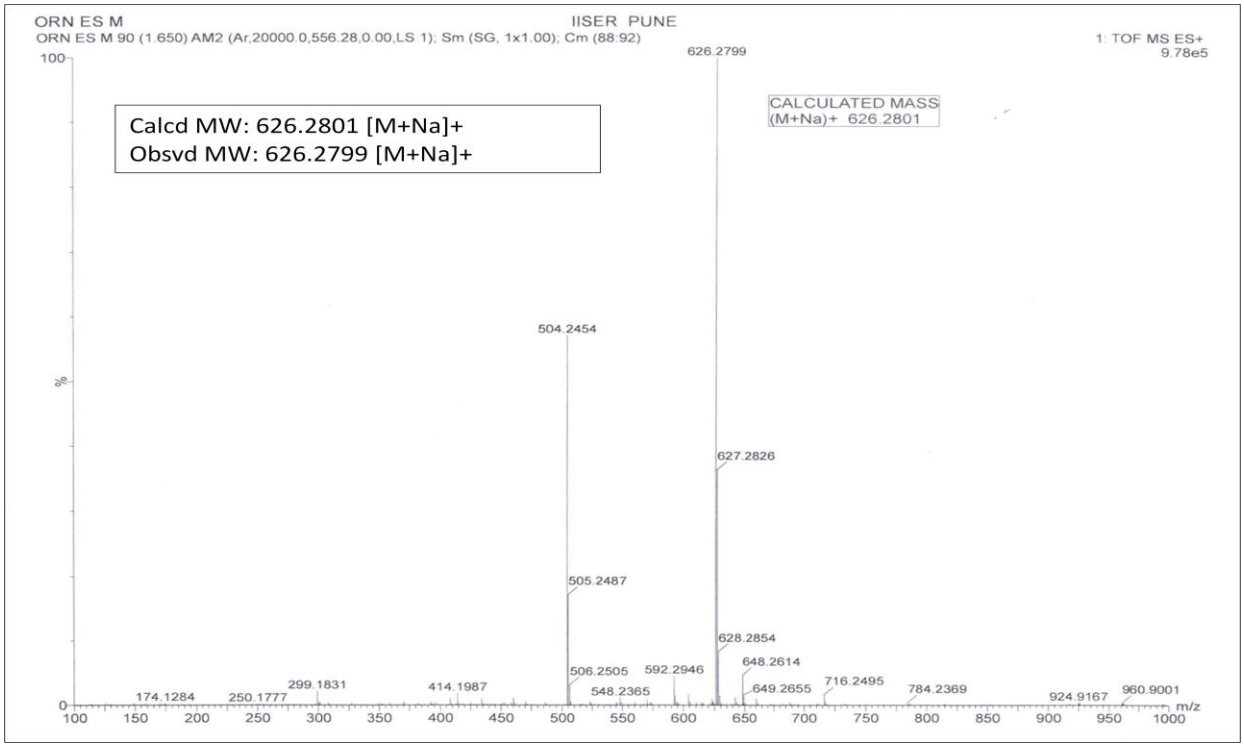
^{1}H NMR of Compound 11:



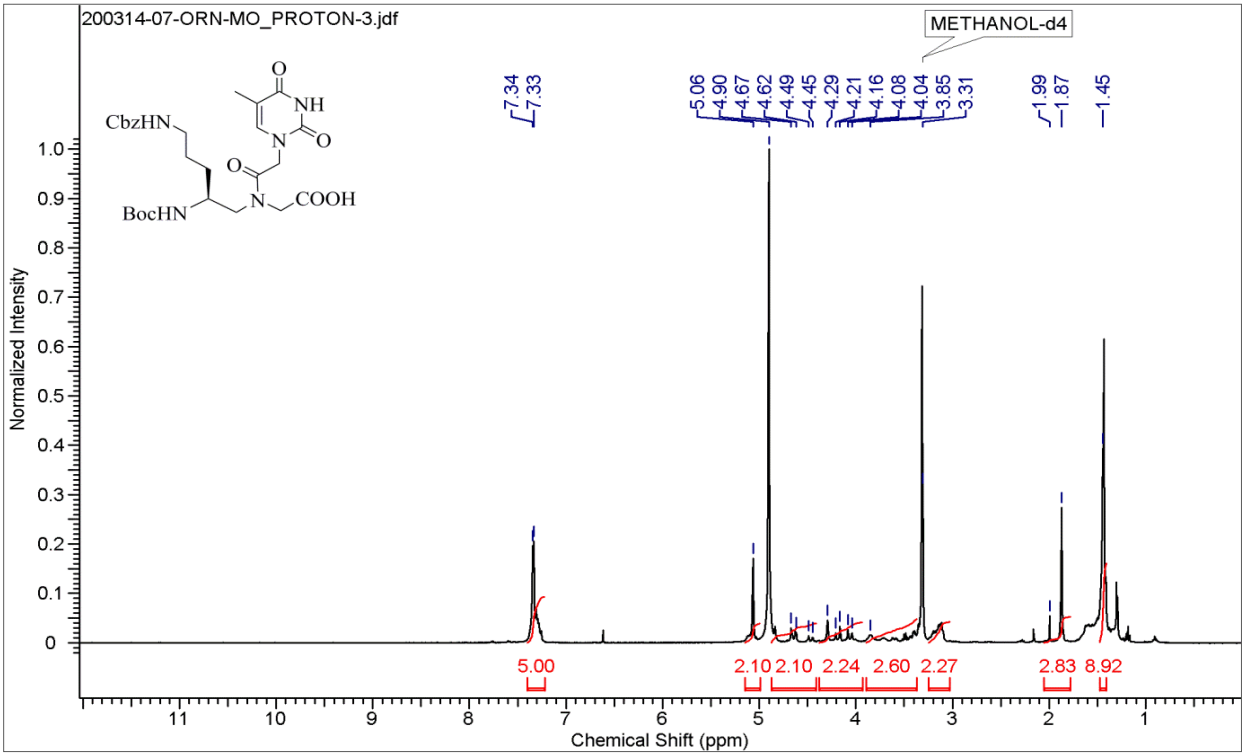
MALDI-TOF spectrum of Compound 11 :



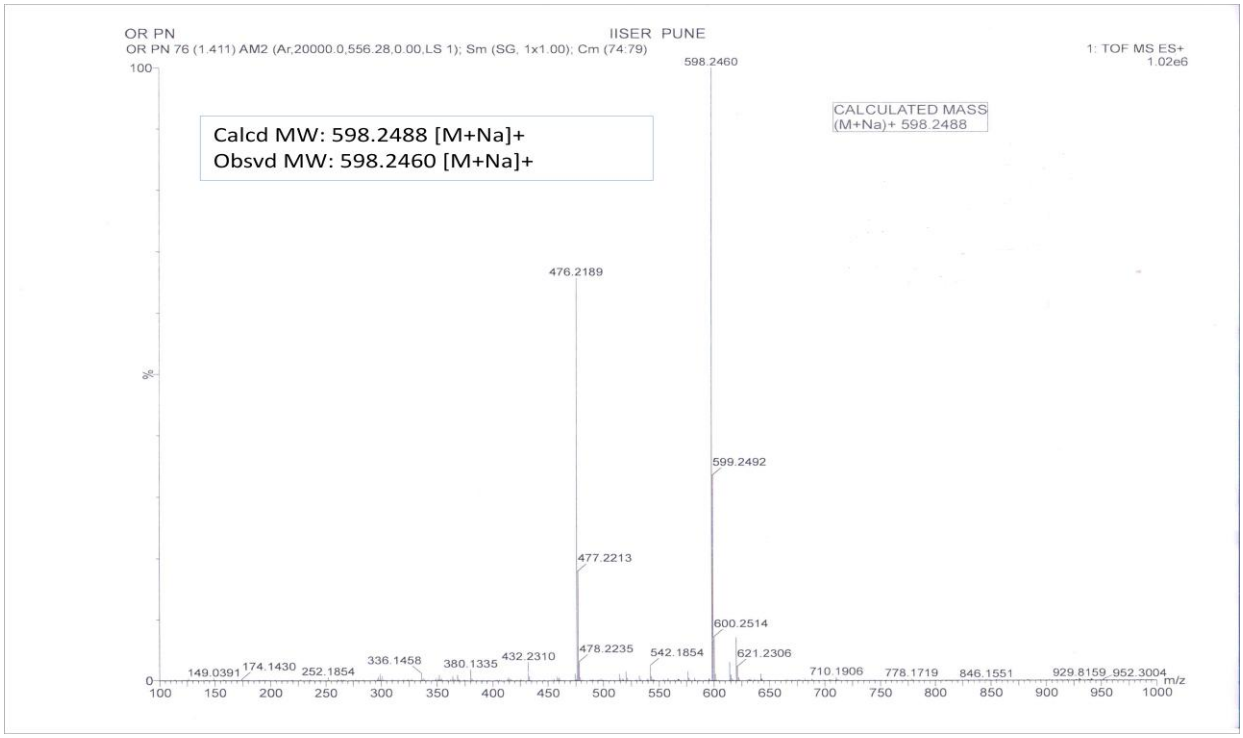
HR-MS SPECTRA OF COMPOUND 11:



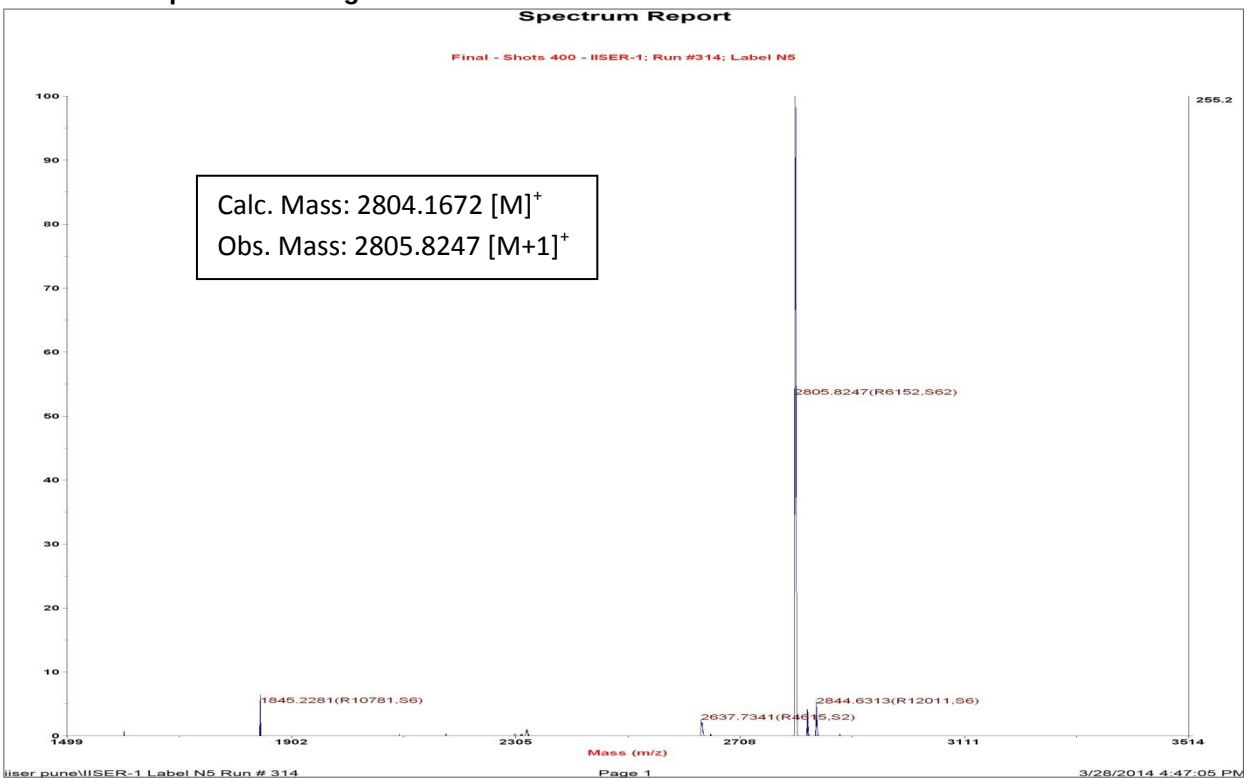
^{1}H NMR of Compound 12:



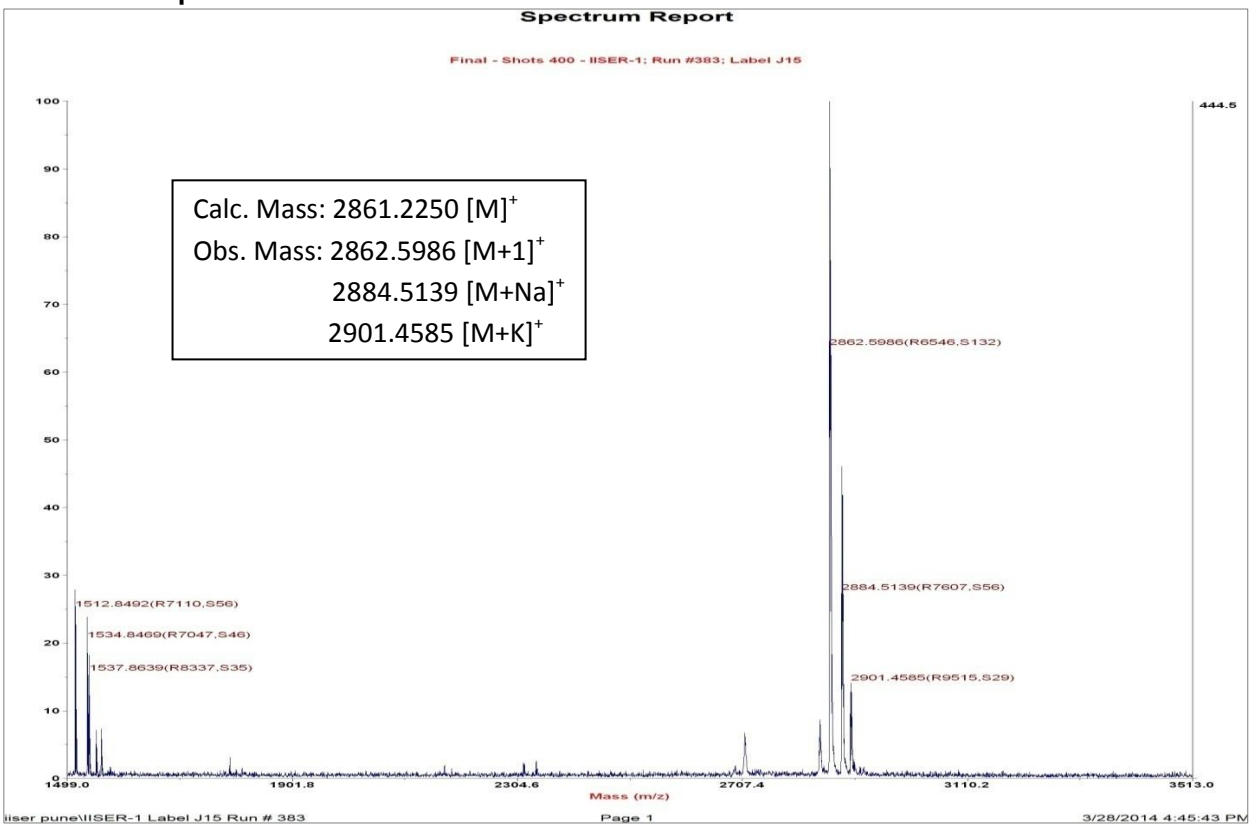
HR-MS SPECTRA OF COMPOUND 12:



MALDI-TOF spectrum of aeg-PNA :



MALDI-TOF spectrum of N-Mod-PNA1 :



4.6. Circular Dichroism :

CD spectra were recorded on JASCO J-715 spectropolarimeter. The CD spectra of the PNA: DNA complexes and the relevant single strands were recorded in 10 mM sodium phosphate buffer, 10 mM NaCl, pH 7.2. The temperature of the circulating water was kept below the melting temperature of the PNA:DNA complexes, i.e., at 10 °C. The CD spectra of the homopurine T single strands, mixed base *am*-PNA single strands and the derived PNA2:DNA triplexes and PNA:DNA duplexes were recorded as an accumulation of 5 scans from 300 to 190 nm using 1 cm cell, a resolution of 0.1 nm, band-width of 1.0 nm, sensitivity of 2 mdeg, response of 2 sec and a scan speed of 50 nm/min.

4.7. UV- T_m measurements :

UV-melting experiments were carried out on Varian Cary 300 UV-spectrophotometer equipped with a Peltier temperature programmer and Julabo water circulator. The sample for T_m measurement was prepared by mixing calculated amount of stock oligonucleotide and PNA solutions together in 2 mL of 10 mM sodium phosphate buffer, 10 mM NaCl (pH 7.1). The samples 2 mL were transferred to quartz cell, sealed with Teflon stopper after degassing with nitrogen gas for 15 min, and equilibrated at the starting temperature for at least 15 min. Nitrogen gas was purged through the cuvette chamber below 15 °C to prevent the condensation of moisture on the cuvette walls. The OD at 260 nm was recorded in steps from 10-85 °C with temperature increment of 0.5 °C/min.

The PNA oligomers and the appropriate DNA oligomers were mixed together in stoichiometric amounts in 0.01M sodium phosphate buffer, pH 7.3 to achieve a final strand concentration of either 0.5 or 1μM each strand. For the AT rich PNAs, *antiparallel* and *parallel* complexes were constituted by employing DNA. The samples were heated at 85 °C for 5 min. followed by slowcooling to room temperature for 2 h. They were allowed to remain at room temperature for at least half an hour and refrigerated overnight prior to running the melting experiments. Each melting experiment was repeated at least twice. The normalized absorbance at 260 nm was plotted as a function of the temperature. The T_m was determined from the first derivative plots of normalized absorbance with respect to temperature and is accurate to ±0.5 °C. The data were processed using Microcal Origin 8.0 and *T*m values derived from the first derivative curve. The concentration of DNA, RNA and PNA were calculated with the help of extinction coefficients, A = 15.4, T = 8.8, C = 7.3 and G = 11.7.

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