Synthesis and Evaluation of Modified Peptide Nucleic Acid (PNA) for Improved DNA/RNA Binding Selectivity

A thesis submitted towards the partial fulfilment 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 modified peptide nucleic acid for improved DNA/RNA binding selectivity**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by "Ms. Isha Dhami at Indian Institute of Science Education and Research, Pune under the supervision of Prof. Krishna N. Ganesh, Professor, Department of Chemistry, IISER Pune during the academic year 2017-2018.

Isha Dhami
(Reg. No. 20131084)

Place: Pune
Date: 20.03.2018

MS Thesis Supervisor

Prof. K. N. Ganesh
IISER Pune

Declaration

I hereby declare that the matter embodied in the report entitled "**Synthesis and evaluation of modified peptide nucleic acid for improved DNA/RNA binding selectivity**" are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education of Research (IISER) Pune, India, under the supervision of Prof. Krishna N. Ganesh and the same has not been submitted elsewhere for any other degree.

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Contents

Abbreviations

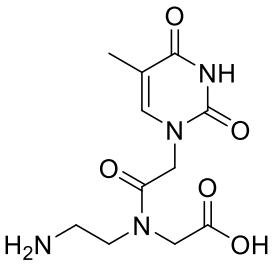
aeg	Aminoethylglycine
aq	Aqueous
Boc_2O	Boc anhydride
Calc.	Calculated
Cbz	Benzyloxycarbonyl
CD	Circular Dichroism
DAIB	Di(acetoxy)iodobenzene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
DMSO	N,N-Dimethyl sulfoxide
DNA	2'-deoxyribonucleic acid
ds	Double stranded
EDC.HCl	N'-ethylcarbodiimide hydrochloride
EtOAc	Ethyl acetate
Fmoc	9-Fluorenylmethoxycarbonyl
g	gram
hrs	Hours
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3 tetramethyluronum-hexafluoro-phosphate
HOBt	N-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
mg	milligram
min	minute
µL	microliter

µM`	micromolar
mL	millilitre
mM	mill molar
mmol	mill moles
MW	Molecular weight
nm	nanometre
NHS	N-Hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
PNA	Peptide Nucleic Acid
RNA	Ribonucleic Acid
RT	Retention time
rt	Room temperature
RP	Reverse Phase (HPLC)
SPPS	Solid Phase Peptide Synthesis
ss	Single stranded
T	Thymine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
THF	Tetrahydrofuran
TLC	Thin layer chromatography
T_m	Melting temperature

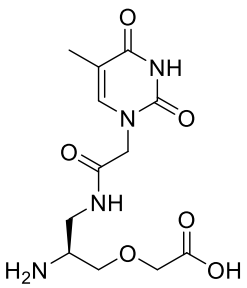
Abstract

Peptide Nucleic Acid (PNA) is an achiral synthetic analogue of DNA / RNA oligonucleotide. Its backbone is made of repeating units of N-(2-aminoethyl) glycine (*aeg*) to which purine and pyrimidine nucleobases are attached. This pseudo-peptide backbone of PNA is uncharged which facilitates binding to cDNA / cRNA duplex or triplex better than the corresponding DNA/RNA due to the decreased electrostatic repulsion. A noticeable property of PNA is that they are resistant to hydrolytic cleavage by nucleases or proteases. Although PNAs have the above mentioned advantages, they still suffer from some limitations like less aqueous solubility, ambiguity in binding orientation and poor cell permeability.

The rationale of my thesis is to synthesize an ether containing PNA backbone which includes synthesis, characterization and biophysical studies. The PNA monomer contains three modifications: the backbone N-3 is replaced with oxygen, the nucleobase is attached at C-γ position and this renders the PNA monomer chiral. A homo-octamer was synthesized with the modified PNA monomer using solid phase synthesis method. UV-T_m studies were performed to check the thermal stability of duplexes or triplexes formed by modified PNA with cDNA / cRNA and compared with *aeg* PNA. Circular dichroism was used to study the conformation of the duplexes or triplexes formed. The conclusion drawn from the present work is that in spite of these drastic changes to the PNA backbone, the modified PNA forms more stable duplexes and triplexes than control *aeg* PNA. The binding is stronger in case of triplex structure as compared to that in duplex structure. Moreover, the modified PNA was found to differentiate between RNA and DNA, by binding specifically only to RNA.



***aeg* PNA backbone**



Modified PNA Backbone

1. Introduction

Nucleic acids are the basic elements of chemistry of life. The flow of genetic information is the basic necessity for all living organisms to sustain. Nucleic acids consist of three major components namely a ribose sugar, a phosphate group and a nitrogenous base. The two fundamental units of information storage, communication and transfer comprise of Ribonucleic Acid (RNA) and Deoxyribonucleic acid (DNA)[1]. According to the central dogma of life, information stored in DNA is transcribed to m-RNA which gets translated into proteins. Naturally, inhibition of protein production is observed if any one of the processes is hampered.

1.1. Nucleic Acids: Structure and Hydrogen Bonding

Both DNA and RNA comprise of four nucleobases: DNA has Adenine (A), Cytosine (C), Guanine (G) and Thymine (T) while RNA has Adenine (A), Cytosine (C), Guanine (G) and Uracil (U) instead of Thymine. Between these nucleobases, there can be Watson-Crick or Hoogsteen hydrogen bonding present depending on the environment; the dominant being Watson Crick hydrogen bonding in most of the cases. Due to the presence of this complementary base pairing between the nucleobases on ribose-phosphate backbone, DNA favours a double helical structure. In 1953, this duplex model of DNA was first proposed by Watson and Crick[2]. Adenine (A) base pairs to Thymine (T) via two hydrogen bonds while Guanine (G) base pairs to Cytosine (C) by three hydrogen bonds. The specific hydrogen bonding with each other is possible due to the donor-acceptor complementarity of the nucleobases. Both types pf hydrogen bonding are shown below:

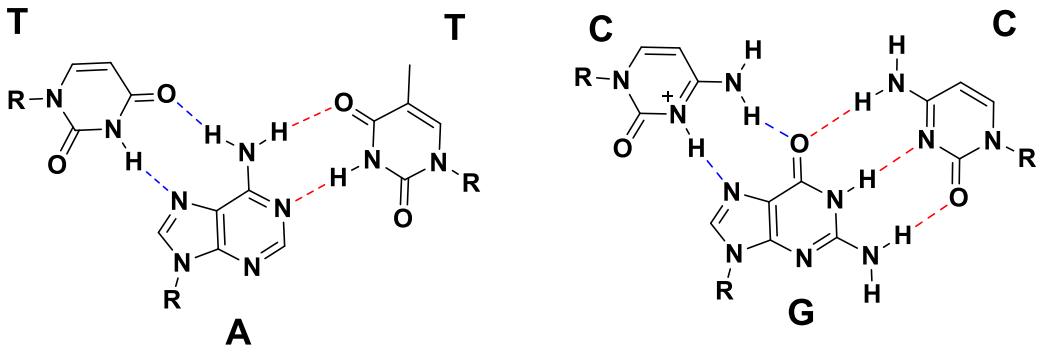


Figure 1: Watson Crick Hydrogen Bonding (RED) and Hoogsteen Hydrogen Bonding (BLUE) between purines and pyrimidines nucleobases

1.2. Peptide Nucleic Acid (PNA)

Peptide nucleic acid is a synthetic polyamide which mimics DNA/RNA. It was invented by Peter Nielsen in 1991.[3] Structurally, it has an uncharged and an achiral flexible pseudo-peptide backbone, instead of chiral sugar phosphate unit as in DNA/RNA, consisting of repeating units of N-(2-aminoethyl) glycine (*aeg*) to which the purines and pyrimidine nucleobases are connected by acetyl spacer via tertiary amide linkage.[4] Just as in DNA, the Watson-Crick hydrogen bonding enables PNA oligomers form complementary hydrogen bonds with each other. The thermal stability of PNA : DNA duplexes is better as compared to normal DNA duplexes due to the presence of uncharged backbone in PNA, which minimizes the electrostatic repulsion that destabilizes DNA/RNA binding. Further, it can also discriminate mismatch sequences, thus resulting in strong duplex formation. PNA is also resistant to hydrolytic cleavage by nucleases and proteases due to the presence of multiple amide bonds (non-peptidic) which make them stable. Currently, PNA is being considered as potential antigene[5] and antisense[6] agents. It can stop the protein production at transcription stage (antisense inhibitor) by binding to complementary DNA or at translation stage (antisense inhibitor) by binding to complementary RNA.

These advantages notwithstanding, PNA still suffers some of the disadvantages.[7] The limitations of PNA include lack of cell permeability, poor water solubility and ambiguity in DNA/RNA recognition for parallel or anti-parallel binding. To overcome some of these limitations, various structural modifications have been performed on PNA, which have proven to improve properties.[8]

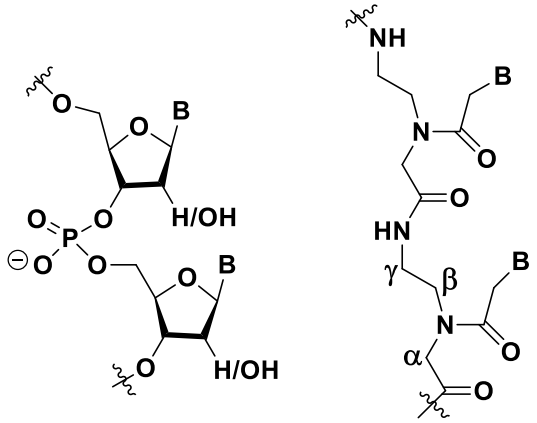


Figure 2: Structure of DNA/RNA (left) and PNA (right)

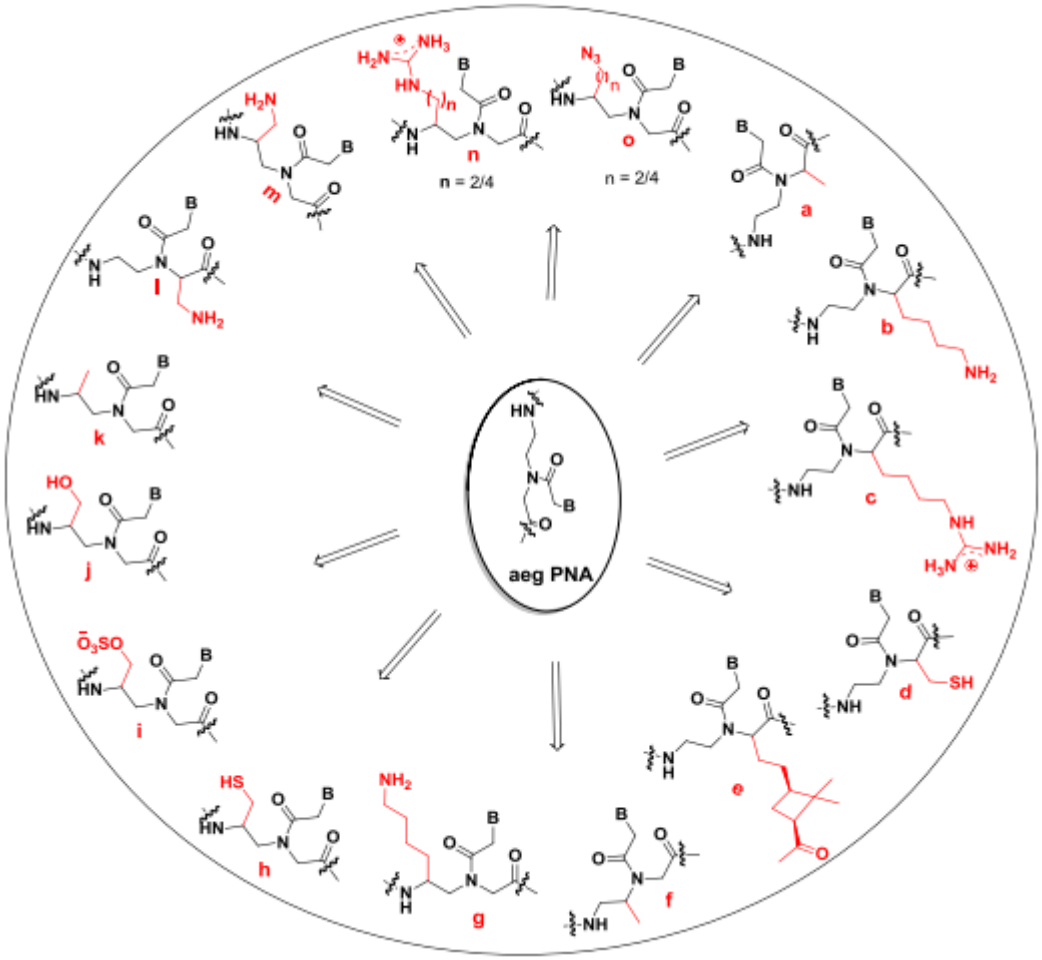


Figure 3: Modified acyclic PNAs[22]

1.3. Solid Phase Peptide Synthesis

Solid Phase Peptide Synthesis (SPPS) is a well-established method to synthesize short peptides which was invented by Robert Bruce Merrifield around 1950-1960.[9] The ease of handling in synthesis to incorporate many groups and couple them efficiently makes SPPS a favorable method to use. Although the direction of synthesis of peptides in cells is from N-terminus to C-terminus, synthesis starts from C-terminus (carboxyl end) to N-terminus (amino end) in SPPS. The protection of building blocks by a stable protecting group is a necessary step. Most frequently used protecting groups are Fmoc (base-labile PG) and Boc (acid-labile PG). The resin used as a solid phase support varies depending on the substrate.

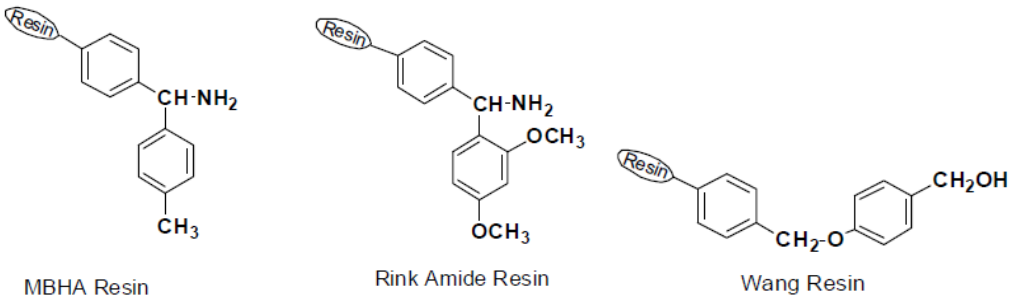


Figure 4: Different types of resins used in SPPS[11]

SPPS offers certain advantages compared to solution phase synthesis which are:

a) Better yield and effectively less loss of yield comparatively since the peptide is not taken out or transferred from the vessel.
b) Faster and efficient method.
c) No purification required after every step; washing removes most of the impurities.

Although SPPS has many advantages compared to solution phase synthesis, much care needs to be taken. The percentage yield may decrease drastically if any mistake is done in between synthesis. Also, this method cannot be used for large scale peptide synthesis. There are two chemical strategies for SPPS namely Boc and Fmoc. Each of the strategies have their specific protocols. Mostly, Fmoc strategy gives a better percentage yield than Boc strategy and the choice of strategy depends on the nature of protecting groups on the sidechain of amino acids. The chosen linker should be flexible and cleave itself along with the side chain protecting groups during the last step of synthesis. Figure 5 shows the protocol for Boc strategy (on the right) and Fmoc strategy (on the left).

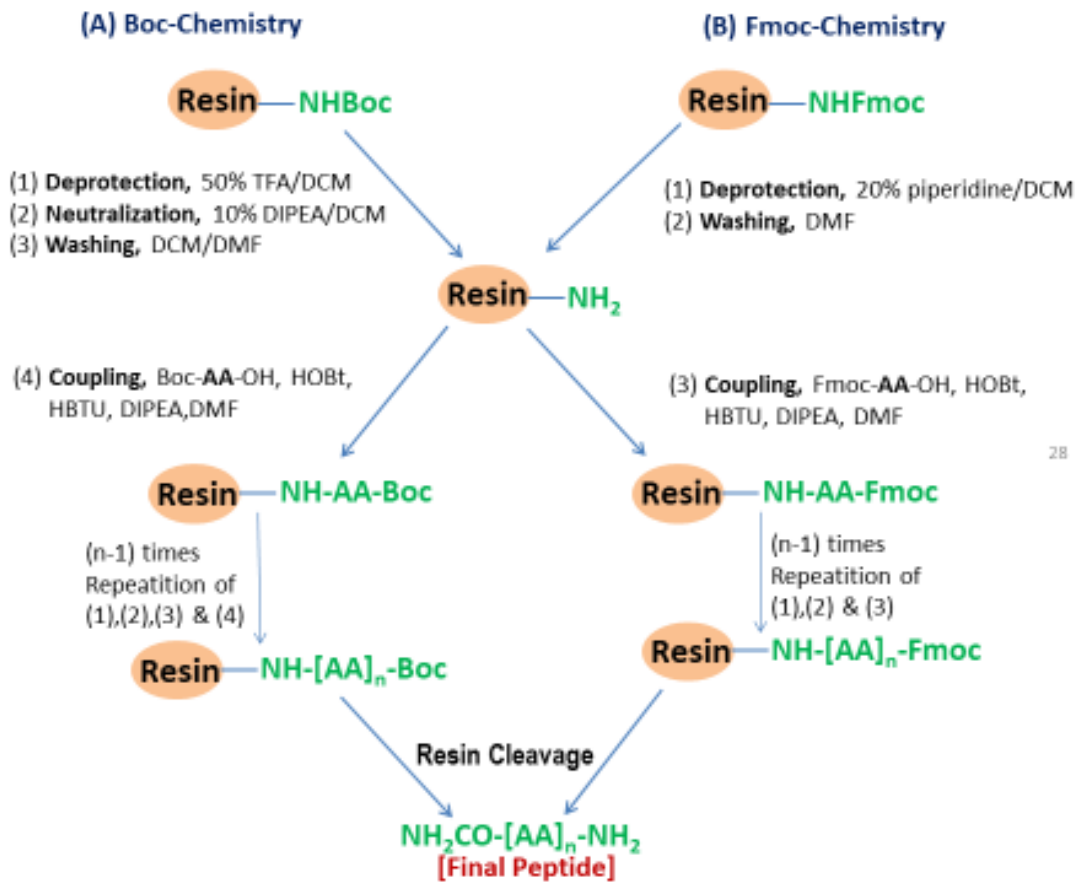


Figure 5: General protocol for SPPS[11] via (A) Boc-chemistry and (B) Fmoc-chemistry

The solid phase synthesis protocol described above for peptide synthesis is successfully used for the synthesis of PNA oligomers.

1.4. High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a purification technique used in analytical chemistry to separate compounds dissolved in a solvent. Reverse Phase (RP) HPLC which consists of using hydrophobic stationary phase (columns) is routinely used to purify large molecules like peptides and PNA based on polarity and molecular size. In HPLC, once the compound is injected, it is eluted under high pressure which makes the process of separation faster and produces better resolution between the peaks compared to conventional column chromatography. The retention time of compounds coming through RP-HPLC varies depending on their

hydrophobicity. Hydrophilic compounds will elute faster as compared to hydrophobic compounds in RP-HPLC. The compounds eluting from HPLC have a high purity.

1.5. Melting temperature (T_m)

In nature, DNA exists in double stranded (ds) helix form. Upon heating, the strands of DNA open up by loss of base pairing to separate out forming two single stranded (ss) DNA. The temperature at which 50% of the population of DNA molecules exists in folded (ds) form while other 50% remains in unfolded (ss) form is known as the melting temperature. Basically, T_m is a measure of thermal stability. It depends upon factors like the length of DNA oligomers, nucleotide sequence (G-C will have higher T_m compared to that of same number of A-T sequence), salt concentration (mostly K^+) and Mg^+) and the concentration of DNA.

UV absorbance increases upon melting as the strands separate out and the hyperchromicity caused by base stacking is removed. Similarly, absorbance decreases while cooling as DNA starts forming duplex again. Graphically, T_m corresponds to the maximum temperature of the derivative plot of absorbance measured vs time. On the other hand, theoretically estimation of approximate T_m is done by Wallace's rule which is:

$$T_m = 2\ ^{o}C\ (A + T) + 4\ ^{o}C\ (G + C)$$

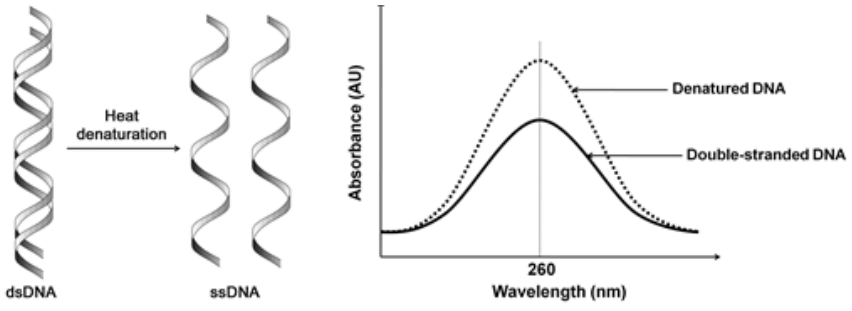


Figure 6: The hyperchromic effect in DNA; denaturation leads to higher absorption [23]

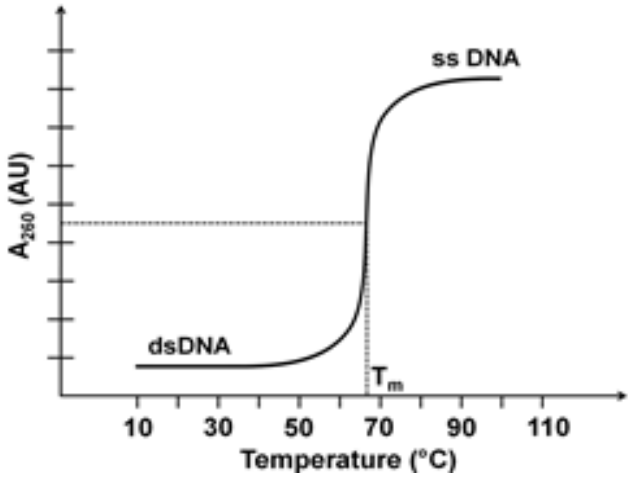


Figure 7: A typical DNA Melting curve; the temperature at which half of the DNA is denatured is called as melting temperature (T_m) [23]

1.6. Circular Dichroism

Circular Dichroism (CD) has been used since many years for studying the properties of DNA and other molecules having chiral chromophores. CD is used to study chiral molecules by measuring the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL). Norden and co-workers showed that variation in CD signals is a powerful tool for extracting information about the duplex and triplex formation of PNA with DNA or RNA.

Normally, achiral single stranded PNA and PNA : PNA duplexes do not give a CD signal. Also, circular dichroism of ss PNA does not show signals in the nucleobase region even on introducing amino acids at the end of an achiral PNA strand, due to the flexibility of these molecules. However, the CD spectrum of an antiparallel PNA : DNA duplex with a terminal L-amino acid is characterized by alternate maxima in the 270-280 nm, 250-260 nm, 240-245 nm, and 220-230 nm regions[10]. It has been shown that two bands are observed while formation of PNA : DNA[11] duplex or PNA : RNA duplex with a positive band in the region of 265 to 275 nm (higher intensity) and a negative band in the region of 220 to 225 nm (lower intensity). The bands are slightly shifted for the PNA : RNA compared to PNA : DNA duplex, but the overall shape is conserved. PNA_2 : DNA triplexes have two characteristic positive bands at 260 nm and 285 nm.[12]

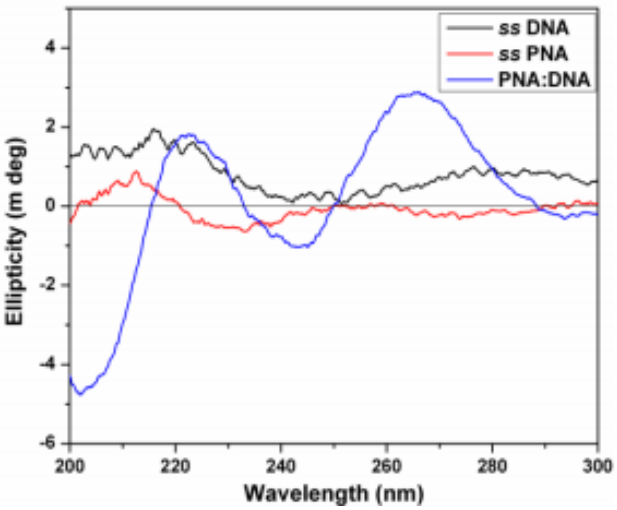


Figure 8: CD signatures of respective ssDNA, ssPNA and PNA : DNA duplex[21]

2. Objectives and rationale of the work

Many modifications have been made on PNA to improve the above mentioned limitations. Nielsen et. al have tried many different modifications such as extending the length of PNA backbone at different substituents[13], making a PNA ester backbone, introduction of ring strain and rigidity in the backbone[14] etc. Many short PNA oligomers are reported with chemical modifications which are structurally close to DNA.[15] Substituents at α, β and γ positions are introduced to check the change in properties of PNA: α, and γ being more common[16]. According to the literature, it has been proven that introduction of a chiral center makes the PNA backbone more rigid.[17,18] It has also been shown that substitution at C- γ with the sidechain of L-lysine improved the solubility of PNA and stabilized the PNA : DNA hybrid duplexes by pre-organizing the PNA backbone to form right handed double helices with the cDNA[19]. Recently, Ganesh *et. al.* have shown how the pendant C-γ cationic side chain positively influences cell permeability and hybridization of PNA backbone with cDNA / RNA.[20] Synthesis and characterization of ethyleneamino / guanidino PNA monomers have been performed and reported from our lab.

Although many reports of modified PNA backbone and side chains are reported till date, structural and conformational requirements that promotes specific hybridization with complementary nucleic acids are yet to be fully deciphered.

In order to explore further the effect of backbone architecture on the ability of the PNA to bind to complementary DNA / RNA, we propose to synthesize a PNA analogue

having an ether backbone with the nucleobase conjugated through an acetyl linker to the sidechain amine substitution at the chiral C-*γ* carbon. By comparing the hybridization properties of PNA oligomers bearing these modifications with the canonical *aeg* PNA, we aim to elucidate the principles of DNA/RNA binding by polyamide analogues.

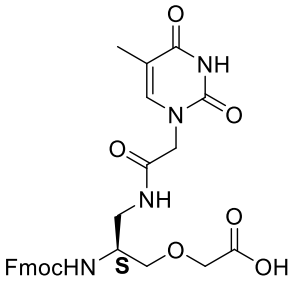


Figure 9: Target PNA monomer

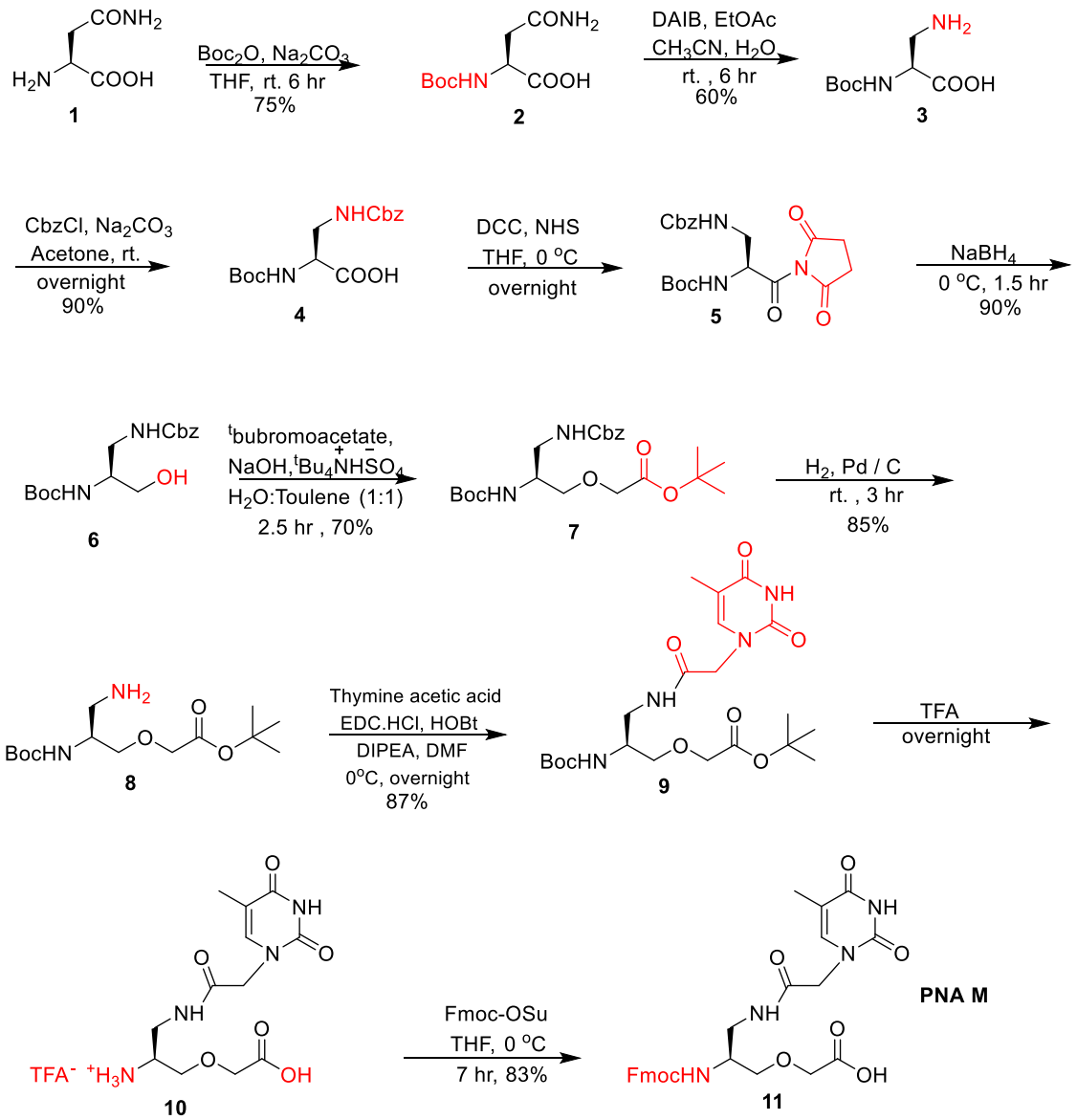
Specific objectives are the following:

- Synthesize a modified PNA monomer with an incorporated heteroatom and *γ* -C substitution
- To synthesize the modified PNA oligomer and control *aeg* PNA oligomer by solid phase peptide synthesis.
- Biophysical evaluation of synthesized PNA oligomer by CD and UV T_m studies.
- To study duplex and triplex formation of modified PNA with cRNA or cDNA.

3. Results and Discussion:

3.1. Synthesis of the PNA monomer

As shown below, we started with L-Asparagine amino acid and made the modified PNA backbone, with thymine attached to its side via ten step procedure.



Scheme 1: Synthesis of PNA Monomer

To synthesize PNA monomer **11**, the commercially available L-Asparagine **1** was reacted with Boc_2O in presence of THF and Na_2CO_3 to get compound **2**. It was subjected to Hofmann rearrangement to get free amine compound **3**, which was then protected using Cbz-Cl to get compound **4**. It was followed by active ester formation of compound 4 using DCC and NHS to get compound **5**, later reduction of ester using sodium borohydride to get primary alcohol **6**. It was then reacted with tert-butyl bromoacetate in presence of aq. NaOH : Toluene to get compound **7**. Compound **7** was reduced with H_2, Pd/C to get amino derivative **8**, which was then immediately coupled with thymine acetic acid using EDC.HCl, HOBT and DIPEA to get compound **9**. Boc and tert-butyl group of compound **9** were de-protected using 95% TFA to get

compound **10**, which was reacted with Fmoc-OSu in presence of Na_2CO_3 and THF to give compound **11**, which was the desired final modified PNA monomer.

3.2. Synthesis of PNA oligomers using SPPS

PNA oligomers were synthesized by the method of SPPS. Initially, Lys-Fmoc monomer was attached to the resin support to deal with the solubility problem. The synthesis of PNA was performed from the *C*-terminus to the *N*-terminus using monomeric units with protected amino and carboxylic acid functions. Since Fmoc strategy proves to be better than Boc strategy in terms of yield and side products, we went ahead with the Fmoc strategy for coupling.

The resin/solid support was taken to be rink amide (RAM) linker 4-((2,4-dimethoxyphenyl)(Fmocamino) methyl) phenoxyacetic acid, on which the monomer coupling was done by *in situ* activation with HBTU/ HOBt to build the oligomers. Commercially available orthogonally protected (Boc/Fmoc) L-lysine was selected as the *C*-terminal spacer-amino acid for the synthesis of all oligomers and it was linked to the resin via amide bond. The amine content on the resin was determined by the picrate assay and was found to be 0.62 mmol/g. Later, partial acetylation of amine content (capping) using calculated amount of acetic anhydride was done to reduce the loading value to 0.02 mmol/g. The uncapped or free $-NH_2$ groups on the resin available for coupling was again calculated before starting solid phase synthesis.

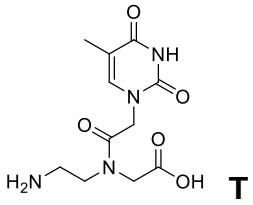
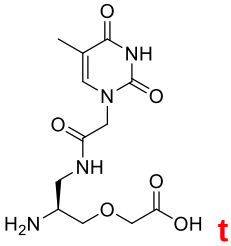
For the purpose of the study of DNA and RNA recognition, the fully modified T_8 PNA homo oligomer was synthesized using monomer (Compound 11) by Fmoc strategy. The unmodified *aeg* PNA homo oligomer T_8 was also made using commercially available Fmoc protected *aeg* T-PNA monomer, which was used as the control sequence for the comparison of the hybridization and discrimination of the properties of modified T_8 PNA homo-oligomer. It was also used to study and investigate the stability upon binding with cDNA / cRNA. (Table **1**, S.No.**2**, PNA M).

3.2.1 Cleavage of the PNA oligomers from the solid support

The PNA oligomers were cleaved from the solid support (L-lysine derivatized Rink Amide resin), using trifluoroacetic acid (TFA - 200µL) in the presence of

triisopropylsilane (TIPS - 20µL), which acted as scavengers to trap the free radicals generated during the cleavage process. The yielded PNA oligomers have L-lysine amide at their C-terminus. Optimum time was found to be 2hrs for cleavage of the oligomers and the side chain protecting groups of lysine. The resin was removed by filtration and washed twice with TFA, which was removed under reduce pressure up to minimum volume. The filtrate was combined and the product was precipitated with cold dry diethyl ether. Finally, isolation of the peptide was performed by centrifugation. The observed precipitate was dissolved in de-ionized water (200 µL) and loaded over sephadex G25 column.

TABLE 1. PNA oligomers with modified/unmodified monomers

S. No.	Sequence Code	PNA Sequence	Monomer Used
1	*aeg* PNA	H-TTTTTTTT-Lys NH_2	 **T**
2	PNA M	H-tttttttt-Lys NH_2	 **t**

3.3. Purification of the PNA oligomers using HPLC

The synthesised crude PNA oligomers had a purity of more than 85% when checked on analytical HPLC (C-18 column, acetonitrile: water system). Further, these PNA oligomers were subsequently purified by RP-HPLC on a semi-preparative C-18 column to give oligomers of 95% purity as determined by analytical RP-HPLC. The method was set as isocratic elution with 10% CH_3CN in 0.1% TFA / H_2O with a flow rate of 1.5 mL/min (linear gradient from A to B in 33 min) and the eluent was monitored at 260 nm.

Solvent A = 0.1% TFA in CH_3CN : H_2O (5:95)

Solvent B = 0.1% TFA in CH_3CN : H_2O (1:1).

HPLC profiles of *aeg* PNA and PNA M sequences are shown below

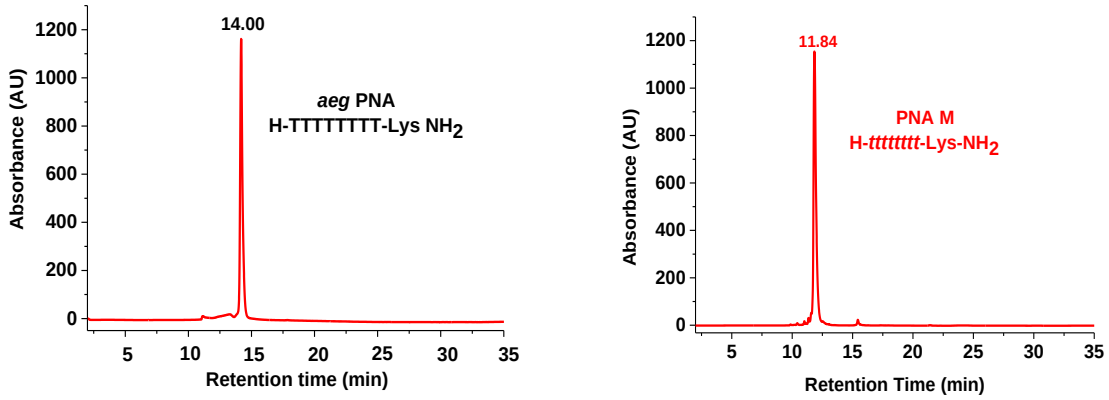


Figure 10: HPLC trace of *aeg* PNA (left) and PNA M (right)

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

S. No.	Sequence Code	HPLC R.T (min)	Mol. Formula	M(Calcd)	M(Obsd)
1	*aeg* PNA	14.00	$C_{113}H_{149}N_{55}O_{33}$	2275.24	2298.15 $[M+Na]^+$
2	PNA M	11.84	$C_{116}H_{156}N_{56}O_{33}$	2515.01	2516.13 $[M+1]^+$

3.4. Comparative study of UV- T_m of Mod-t_8 PNA and *aeg*-T_8 PNA:

The thermal stability of duplexes and triplexes were determined by temperature dependent UV absorption spectroscopy. The complementary DNA / RNA strands used were poly A_8. The PNA strands were taken with complementary DNA / RNA at different stoichiometric concentrations and annealed together in 10 mM phosphate buffered saline (pH 7.0) to form duplexes or triplexes. The absorbance at 260 nm was plotted

against temperature to check for a sigmoidal transition indicating the formation of hybrids. The first derivative of this curve gave the value of melting temperature T_m.

3.4.1. RNA : PNA Duplexes

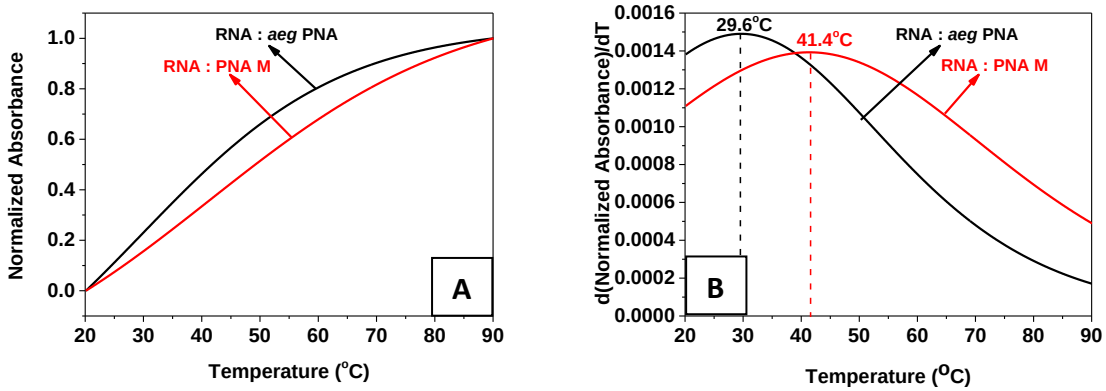


Figure 11: Melting curves for RNA : PNA duplexes with (A) PNA M (B) First derivative curve
(RNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

The RNA : *aeg* PNA duplex has a melting temperature of 29.6 °C, while duplex RNA : PNA M has a melting temperature of 41.4 °C. The 11.8 °C higher T_m of RNA : PNA M duplex compared to RNA : *aeg* PNA duplex suggests that the former has higher thermal stability and binds to RNA better than the latter.

3.4.2. DNA : PNA Duplexes

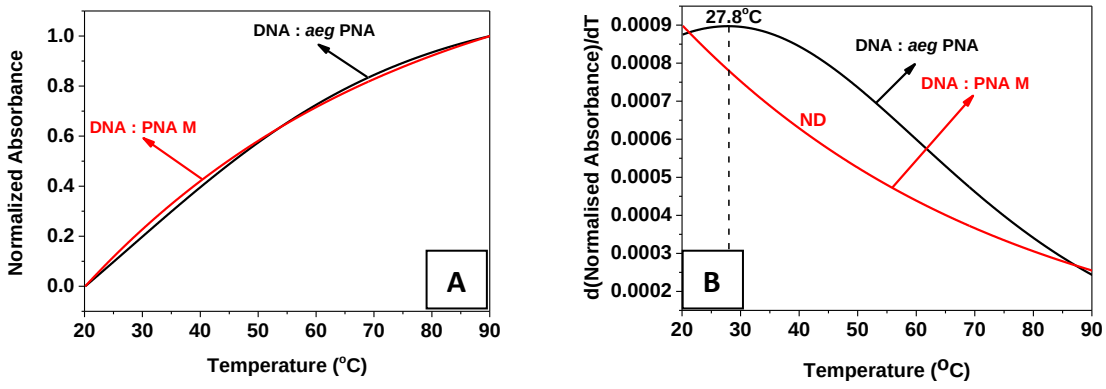


Figure 12: Melting curves for DNA : PNA duplexes with (A) PNA M (B) First derivative curve
(DNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

The DNA : *aeg* PNA duplex shows a melting temperature of 27.8 ^{o}C, while the melting temperature of DNA : PNA M duplex is too low to be determined. The higher T_m of DNA : *aeg* PNA duplex compared to DNA : PNA M duplex suggests that the former has a higher thermal stability and binds to DNA better than the latter. The DNA : PNA M duplexes are thermally very unstable.

3.4.3 RNA : PNA_2 Triplexes

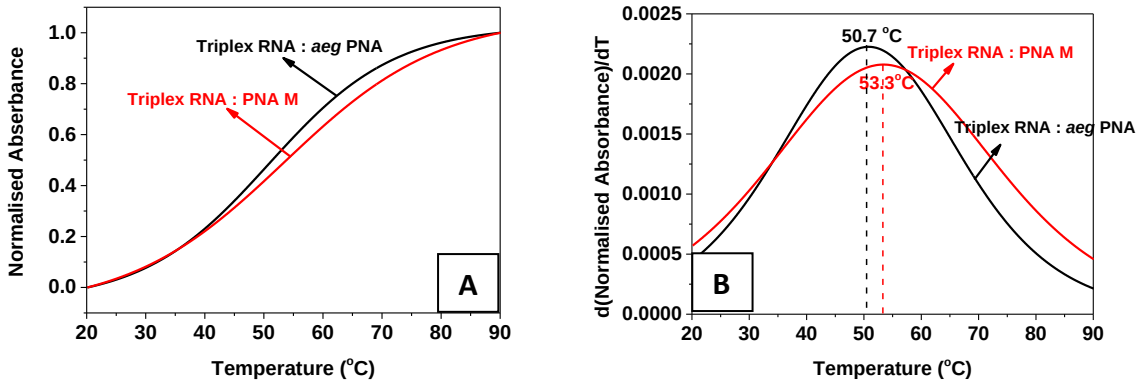


Figure 13: Melting curves for RNA : PNA_2 triplexes with (A) PNA M (B) First derivative curve
(RNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

The RNA : *aeg* PNA_2 triplex shows a melting temperature of 50.7 ^{o}C, while triplex RNA : PNA_2 M shows a melting temperature value at 53.3 ^{o}C. The 2.6 ^{o}C higher T_m of RNA : PNA_2 M triplex compared to RNA : *aeg* PNA_2 triplex suggests that the former has a slightly higher thermal stability.

3.4.4 DNA : PNA_2 Triplexes

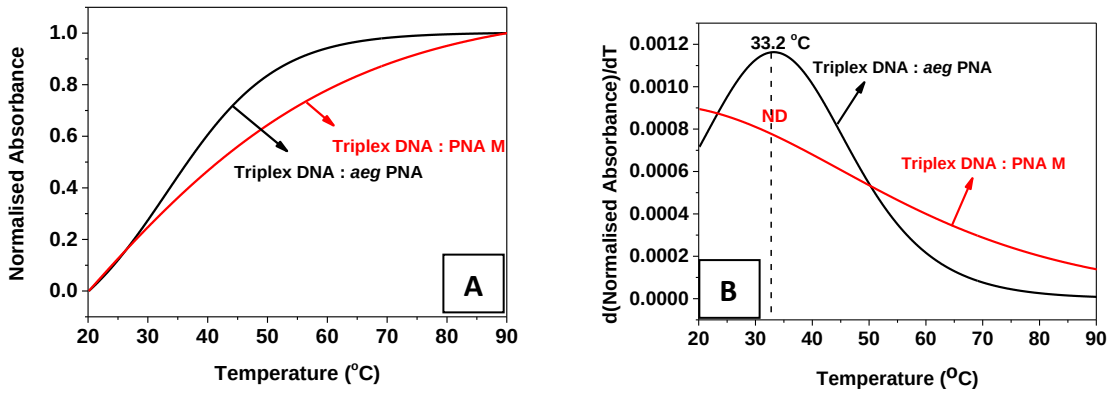


Figure 14: Melting curves for DNA : PNA_2 triplexes with (A) PNA M (B) First derivative curve
(DNA : 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

The DNA : *aeg* PNA_2 triplex shows a melting temperature of 33.2 °C, while the melting temperature of DNA : PNA_2 M duplex is too low to be determined. The higher T_m of DNA : *aeg* PNA_2 triplex compared to DNA : PNA_2 M triplex suggests that the former has a higher thermal stability and binds to DNA better than the latter. The DNA : PNA_2 M triplexes are unstable

Table 3: Melting temperature (T_m) for PNA : RNA and PNA : DNA duplexes and triplexes

-	*aeg* (T_m)	M (T_m)	Δ T_m (M - *aeg*)
Duplex RNA : PNA	29.6 °C	41.4 °C	11.8 °C
Duplex DNA : PNA	27.8 °C	ND	-
Triplex RNA : PNA_2	50.7 °C	53.3 °C	2.6 °C
Triplex DNA : PNA_2	33.2 °C	ND	-

From the above table, we see that triplexes have a higher melting temperature than duplexes indicating that the triplexes are more stable. The duplex and triplex of DNA with modified PNA (PNA M) have a very low melting temperature which is of little relevance. The T_m of PNA M is higher in case of RNA binding, suggesting that RNA

binds stronger as compared to DNA. The modified PNA (PNA M) binds to RNA better, forming duplexes and triplexes compared to control PNA (*aeg* PNA). Moreover, *aeg* PNA binds better to RNA duplex and triplex but not that greatly with DNA. The low T_m of DNA duplexes and triplexes observed maybe due to sequence being AT rich and its short length. However the data from the present work still suggests that the PNA M has an affinity for binding with RNA selectively.

3.5. Comparative study of Circular Dichroism of Mod t_8 PNA and *aeg* T_8 PNA

The conformations of the duplexes and triplexes formed by the PNA oligomers with complementary DNA/RNA were studied using circular dichroism spectroscopy. The annealed PNA and DNA/RNA strands were incubated at 4 ^{o}C for a period of 12 hrs and the spectra were measured at the same temperature to observe the characteristic bands for duplexes and triplexes of the hybrids formed by the *aeg* PNA and modified PNA oligomers.

3.5.1. RNA : PNA Duplexes

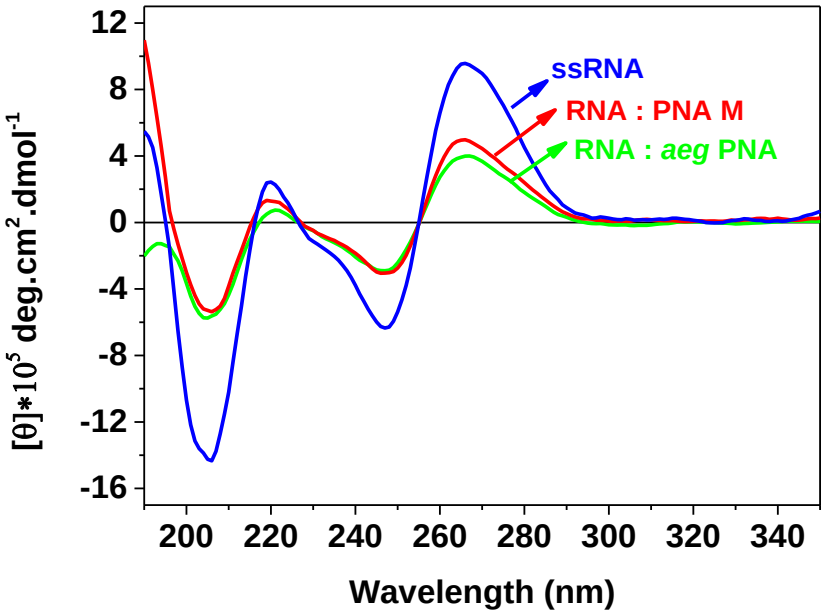


Figure 15: CD spectra of Duplexes of *aeg* PNA (green) and PNA M (red) with ssRNA (blue)

(ss RNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

We see four bands in the CD spectra (Figure 15). A highly intense negative band is present at 205 nm and a low intensity positive band at 225 nm, while a moderate intensity negative band is seen at 255 nm followed by a positive maximum band again at 265 nm. We find a decrease in intensity of PNA : RNA duplexes compared to intensity of ss RNA, confirming duplex formation. The CD spectra of *aeg* PNA has almost similar pattern (only a very slight increase in the intensity of positive bands at 225 nm and 265 nm) as that of PNA M with cRNA. We can conclude that cRNA forms slightly better duplexes with PNA M compared with *aeg* PNA.

3.5.2 DNA : PNA Duplexes

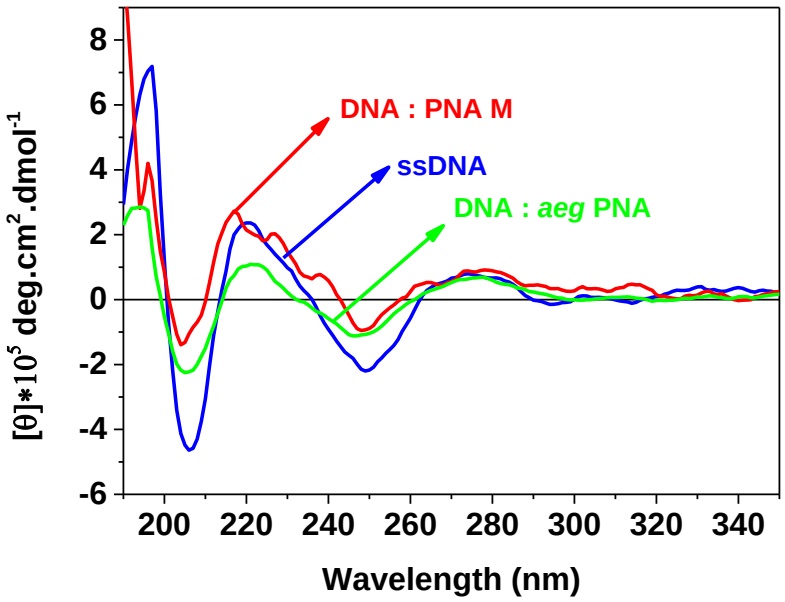


Figure 16: CD spectra of Duplexes of *aeg* PNA (green) and PNA M (red) with ssDNA (blue)

(ss DNA: 5' AAAAAAAA 3'; Buffer:10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

We see four bands in the CD spectra (Figure 16). A highly intense negative band is present at 205 nm and a moderate intensity positive band at 225 nm, while again moderate intensity negative band is seen at 255 nm followed by a positive maximum

band again at 275 nm. The CD spectra of *aeg* PNA and PNA M with cDNA is almost the same with the formation of duplexes since we see the signature bands of PNA : DNA duplex at 280 nm (positive intensity band) and 245 nm (negative intensity band). We can thus conclude that PNA M and *aeg* PNA binds in the same manner to cDNA and do not render any change in conformation.

3.5.3 RNA : PNA_2 Triplexes

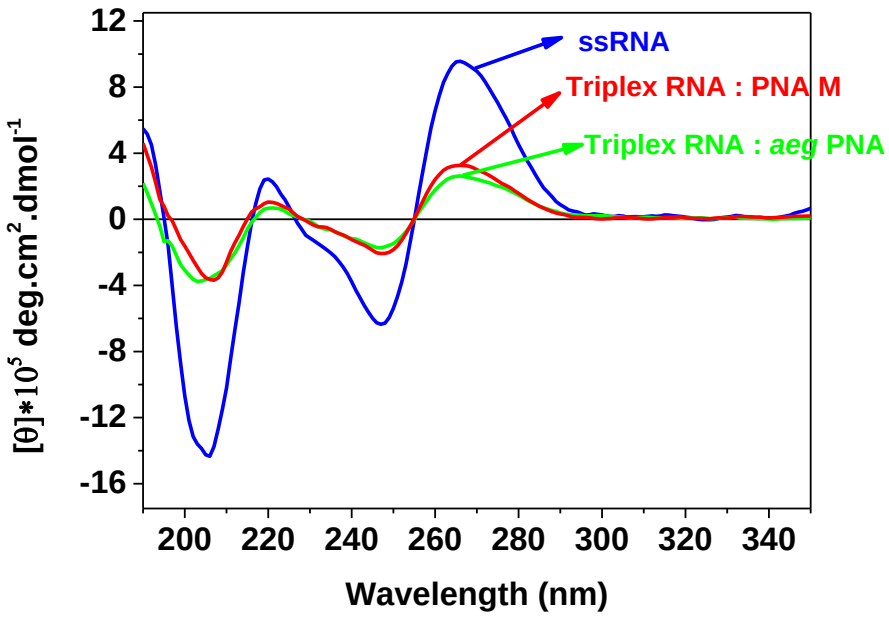


Figure 17: CD spectra of Triplexes of *aeg*-PNA (green) and PNA M (red) with ssRNA (blue)

(ss RNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

We see four bands in the CD spectra (Figure 17). A highly intense negative band is present at 205 nm and a low intensity positive band at 225 nm, while a moderate intensity negative band is seen at 255 nm followed by a positive maximum band again at 265 nm. We see a change in intensity of PNA : RNA triplexes compared to intensity of ss RNA, confirming triplex formation. The CD spectra of *aeg* PNA has a similar pattern as that of PNA M with cRNA. We can conclude that cRNA binds to PNA in the same fashion as that of *aeg* PNA, forming triplexes.

3.5.4 DNA : PNA_2 Triplexes

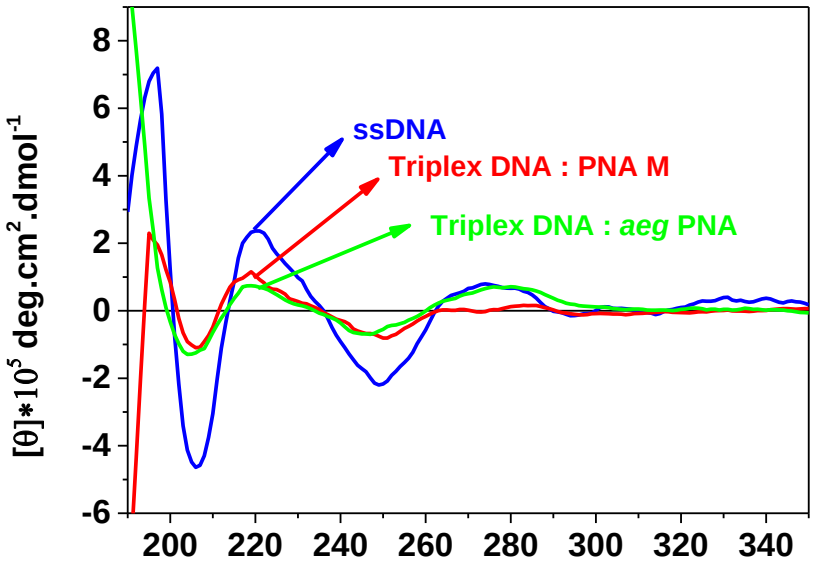


Figure 18: CD spectra of Triplexes of *aeg*-PNA (green) and PNA M (red) with ssDNA (blue)

(ss DNA: 5' AAAAAAAA 3'; Buffer: 10 mM sodium phosphate, pH 7.0, NaCl 10 mM)

We see four bands in the CD spectra (Figure 18). A highly intense negative band is present at 205 nm and a moderate intensity positive band at 225 nm, while again moderate intensity negative band is seen at 255 nm followed by a positive maximum band again at 275 nm. The CD spectra of *aeg* PNA and PNA M with cDNA has a difference at 275 nm. The signature band for PNA : DNA_2 triplexes occur at 255 nm and 285 nm. We can see that there is no 285 nm band for PNA M triplex which indicates that DNA : PNA_2 M triplexes are not formed in this case although control *aeg* PNA is forming triplex with DNA.

From the above CD spectra, we can conclude

a. PNA M and *aeg* PNA duplexes and triplexes are formed with cRNA (PNA M being slightly better than *aeg* PNA.
b. The duplexes formed by PNA M with cRNA / cDNA are similar in conformation to the one formed by *aeg* PNA.

c. PNA M duplex forms but its triplex does not form with cDNA.
d. *aeg* PNA duplexes and triplexes are formed with cDNA.

4. Conclusion

Here we wanted to address the limitations of *aeg* PNA. An ether backbone PNA was rationally designed and synthesized by ten steps protocol. This monomer was incorporated into PNA homo-oligomer by solid phase synthesis method. The DNA/RNA binding properties of the PNA oligomer were studied using UV-T_m and CD spectroscopic techniques. The following conclusions were drawn from these studies:

- Even though we made such drastic changes to the PNA, it still behaves as a DNA mimic and it also shows superior RNA binding ability than *aeg* PNA.
- PNA M could form more thermally stable duplexes and triplexes with cRNA compared to *aeg* PNA, which was evident from UV-T_m and CD studies.
- The modified PNA is able to distinguish between DNA and RNA by showing specific binding with RNA.
- Although *aeg* PNA could form duplex and triplex with cDNA but it has so less melting temperature and thus is of very less significance.
- Further studies with longer sequences and higher CG content could be useful to explore more about this modification to understand how it confers the observed ability to discriminate between the complementary DNA and RNA strands.

5. Experimental Section

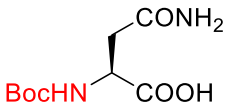
5.1. General

The chemicals used were of laboratory or analytical grade. The purified solvents were used. Ethyl bromoacetate, HBTU, HOBt, EtOAc, DMF, pet-ether were obtained from Sigma or 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 size (Merck). The ^{1}H spectra were recorded on Bruker 500 MHz (or 125 MHz for ^{13}C) and Jeol 400 MHz (or

100 MHz for ^{13}C) using residual solvents signals as an internal reference ($CDCl_3$ δH, 7.26 ppm, δC 77.0 ppm) and DMSO-d_6 (δH 2.50 ppm, δC 39.52ppm). 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.

5.2. Procedures

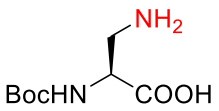
2: (tert-butoxycarbonyl) asparagine



2

A solution of Boc_2O (12.6 mL, 55 mmol) in THF was added to the mixture of L-Asparagine (6.6 gm, 50 mmol) and 10% Na_2CO_3 (10.6 gm, 100 mmol) in a round bottom flask supported by ice-bath. The whole reaction mixture was stirred for 8 - 10 hours at rt. THF was fully removed under reduced vacuum. The crude residue was acidified with 10% HCl to pH 3 - 4 and extracted with EtOAc (3 x 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated to give compound 2 as a white solid (4.4 gm, 75% yield).

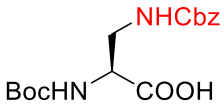
3: 3-amino-2-((tert-butoxycarbonyl)amino)propanoic acid



3

A slurry of Boc-L-aspargine (2) (5 gm, 20.3 mmol), EtOAc (24 mL), CH_3CN (24 mL), H_2O (12 mL), and iodobenzene diacetate (7.87 gm, 24 mmol) was cooled and stirred at 16 °C for 30 min. The temperature was maintained at 20 °C for 4 h, cooled to 0 °C, and filtered. The solid residue was washed with EtOAc and dried in vacuum to obtain compound 3 (2.65 gm, 60% yield).

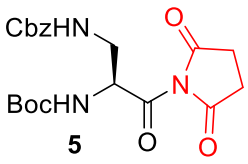
4: 3-(((benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)propanoic acid



4

The solution of 10% aqueous $NaHCO_3$ (2.3 gm, 27 mmol) was added to an ice-cold solution of compound 3 (2 gm, 9.2 mmol) in acetone (25 mL) and stirred for 10 min at 0 °C. To this, benzyl chloroformate (3.7 mL, 11 mmol) in 50% toluene solution was added, and the reaction mixture was stirred overnight at room temperature. Acetone was removed; the aqueous layer was washed with Et_2O (2 × 20 mL), acidified to pH 3 - 4 with saturated aqueous $KHSO_4$ solution and extracted with EtOAc (3 × 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated to give compound 4 as a sticky oil (2.9 gm, 90% yield).

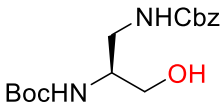
5: benzyl tert-butyl (3-(2,5-dioxopyrrolidin-1-yl)-3-oxopropane-1,2-diyl)dicarbamate



5

To the solution of compound 4 (2.3 gm, 6.8 mmol) in THF, NHS (1.56 gm, 13.6 mmol) was added followed by DCC (1.68 gm, 8.16 mmol) at 0 ^oC. The reaction was stirred for 10 hours, washed and filtered to get compound 5.

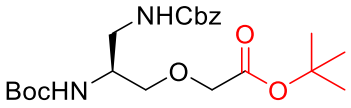
6: benzyl tert-butyl (3-hydroxypropane-1,2-diyl)dicarbamate



6

To the filtered solution of compound 5, $NaBH_4$ (1.3 gm, 34 mmol) dissolved in H_2O was added and stirred for 90 min at 0 ^oC to get compound 6 (2.5 gm, 90% yield).

7: tert-butyl 2-(3-(((benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl) amino) propoxy) acetate

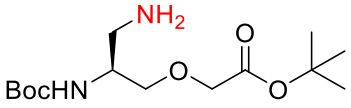


7

1N NaOH (0.9 gm, 1 mmol) in H_2O (5 ml) and toluene (8 ml) was added at 0^oC, followed by addition of phase transfer catalyst ($^tBu_4NH^+SO_4^-$, 34 mg, 0.1 mmol). To this mixture, t-butylbromoacetate (0.16 ml, 1.1 mmol) was added and stirred for 10 min. A solution of compound 6 (0.32 gm, 1 mmol) in toluene was added and stirred for 2.5 hours. Later, 10% HCl solution was added and extracted thrice with EtOAc. The combined organic layer was washed with brine solution, dried under Na_2SO_4 and concentrated to give compound 7 (0.25 gm, 75% yield).

^{1}H NMR *(400MHz, $CDCl_3$)* δ 7.37-7.29 (m, 6H, J=32Hz), 5.83 (s, 1H), 5.37-5.35 (d, 1H, J=8Hz), 5.12-5.09 (s, 2H, J=12Hz), 3.95-3.93 (s, 2H, J=8Hz), 3.66-3.63 (m, 1H, J=12Hz), 3.55-3.39 (m, 3H, J=64Hz), 1.48 (s, 9H), 1.44 (s, 9H). **^{13}C NMR** *(100 MHz, $CDCl_3$)* δ 170.1, 157.4, 155.5, 136.7, 128.3, 127.2, 82.2, 79.1, 76.8, 71.5, 68.2, 66.4, 50.5, 42.6, 28.1, 14.4. **HRMS** *(ESI-TOF)* m/z calcd for $C_{22}H_{34}N_2O_7$ $[M + H]^+$ 439.2444, found 439.2451.

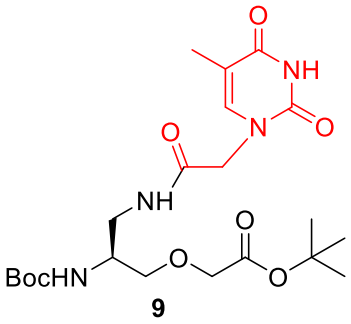
8: tert-butyl 2-(3-amino-2-((tert-butoxycarbonyl)amino)propoxy)acetate



8

To a solution of compound 7 (1 gm, 2.2 mmol) in MeOH, catalytic amount of Pd / C (0.2 gm) was added under ice bath conditions under H_2 atmosphere and stirred for 3 hours. The solution was later filtered off by methanol, and filtrate was concentrated to get compound 8 (0.6 gm, 90% yield).

9: tert-butyl2-(2-((tert-butoxycarbonyl)amino)-3-(2-(5-methyl-2,4-dioxo-3,4 dihydro pyrimidin-1(2H)-yl)acetamido)propoxy)acetate

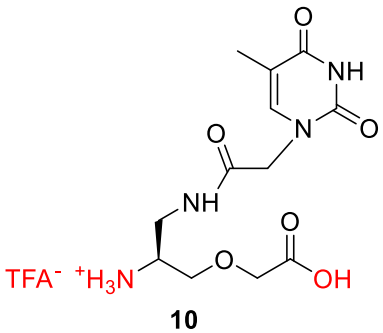


9

Thymine acetic acid (83 mg, 0.45 mmol) was dissolved in DMF (3 ml) at 0°C under N_2 atmosphere and stirred for 10 min. A mixture of HOBt (61 mg, 0.45 mmol) and EDC.HCl (86 mg, 0.45 mmol) was added to the reaction mixture followed by DIPEA (0.31 ml, 1.8 mmol) dropwise. After 5 min, compound 8 (0.15 g, 0.5 mmol) in DMF was added to the round bottom flask and stirred for 8 - 10 hr. Later, 10% HCl solution was added and extracted thrice with EtOAc. The combined organic layer was washed with 10% Na_2CO_3 and brine solution, dried under Na_2SO_4 and concentrated to give compound 9 as white solid (0.17 gm, 87% yield).

^{1}H NMR *(400MHz, $CDCl_3$)* δ 7.07 (br, 1H), 5.42 (s, 1H), 4.36-4.34 (d, 2H, J=8Hz), 3.96 (s, 2H), 3.64-3.61 (m, 1H, J=12Hz), 3.52 (m, 1H), 3.43 (m, 1H), 1.90 (s, 3H), 1.47 (s, 9H), 1.41 (s, 9H). **^{13}C NMR** *(100 MHz, $CDCl_3$)* δ 170.3, 167.1, 163.9, 155.8, 151.0, 140.7, 111.1, 82.8, 79.7, 76.6, 71.5, 67.9, 50.1, 29.8, 27.7, 12.0. **HRMS** *(ESI-TOF)* m/z calcd for $C_{21}H_{34}N_4O_8$ $[M + H]^+$ 471.2455, found 471.2463.

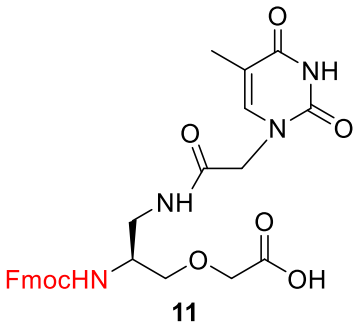
10: 2-(2-amino-3-(2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl) acetamido) propoxy)acetic acid



10

TFA (5 ml) was added to compound 9 (350 mg) at 0^{o} C and stirred for 6 hr. Then, TFA was evaporated to obtain compound 10.

11: 2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(2-(5-methyl-2,4-dioxo-3,4 dihydropyrim idin-1(2H)-yl)acetamido)propoxy)acetic acid



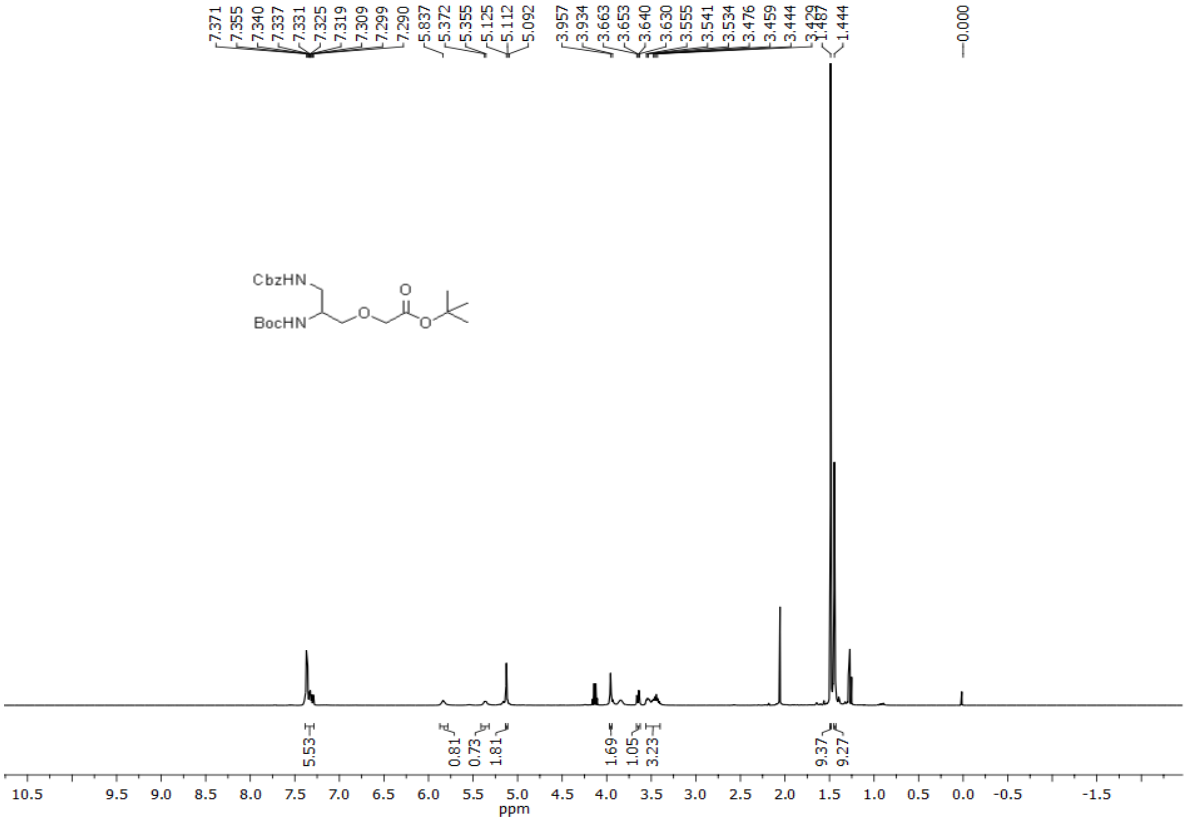
11

Compound 10 (0.6 gm, 1.27 mmol) was dissolved in THF at 0^{o}C. The pH was maintained around 8 by addition of 10% Na_2CO_3 solution. After 5 min, a solution Fmoc-OSu (0.39 gm, 1.14 mmol) in THF was added and stirred for 7 hr. Later, 10% HCl solution was added and extracted thrice with EtOAc. The combined organic layer was washed with brine solution, dried under Na_2SO_4 and concentrated. The compound, after addition of EtOAc and Pet ether was decanted and dried using high vacuum to give a white solid as compound 11 (0.7 gm, 83% yield).

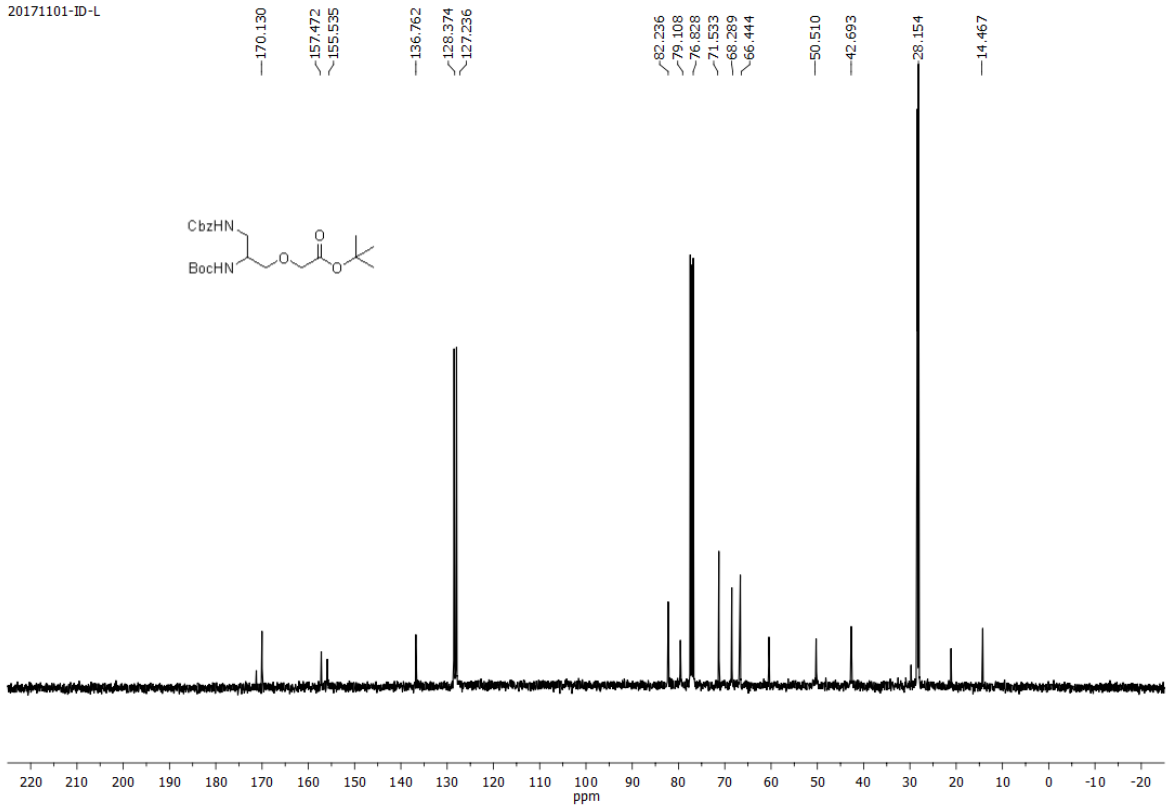
^{1}H NMR (400MHz, $CDCl_3$) δ 12.66 (br, 1H), 11.25 (s, 1H), 7.89-7.87 (d, 2H, J=8Hz), 7.71-7.69 (d, 2H, J=8Hz), 7.42-7.38 (m, 3H, J=16Hz), 4.28-4.21 (m, 5H, J=28Hz), 4.01 (s, 2H), 3.69 (m, 1H), 3.45-3.43 (d, 2H, J=8Hz), 3.33 (s, 1H), 3.13 (m, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 171.6, 167.5, 164.9, 156.2, 151.7, 144.0, 142.5, 140.7, 127.9, 127.1, 125.3, 120.6, 108.0, 70.9, 67.9, 65.8, 50.9, 49.6, 46.5, 12.3**. HRMS** *(ESI-TOF)* m/z calcd for $C_{27}H_{28}N_4O_8$ $[M + H]^+$ 537.1985, found 537.1995.

5.3. Spectral Data

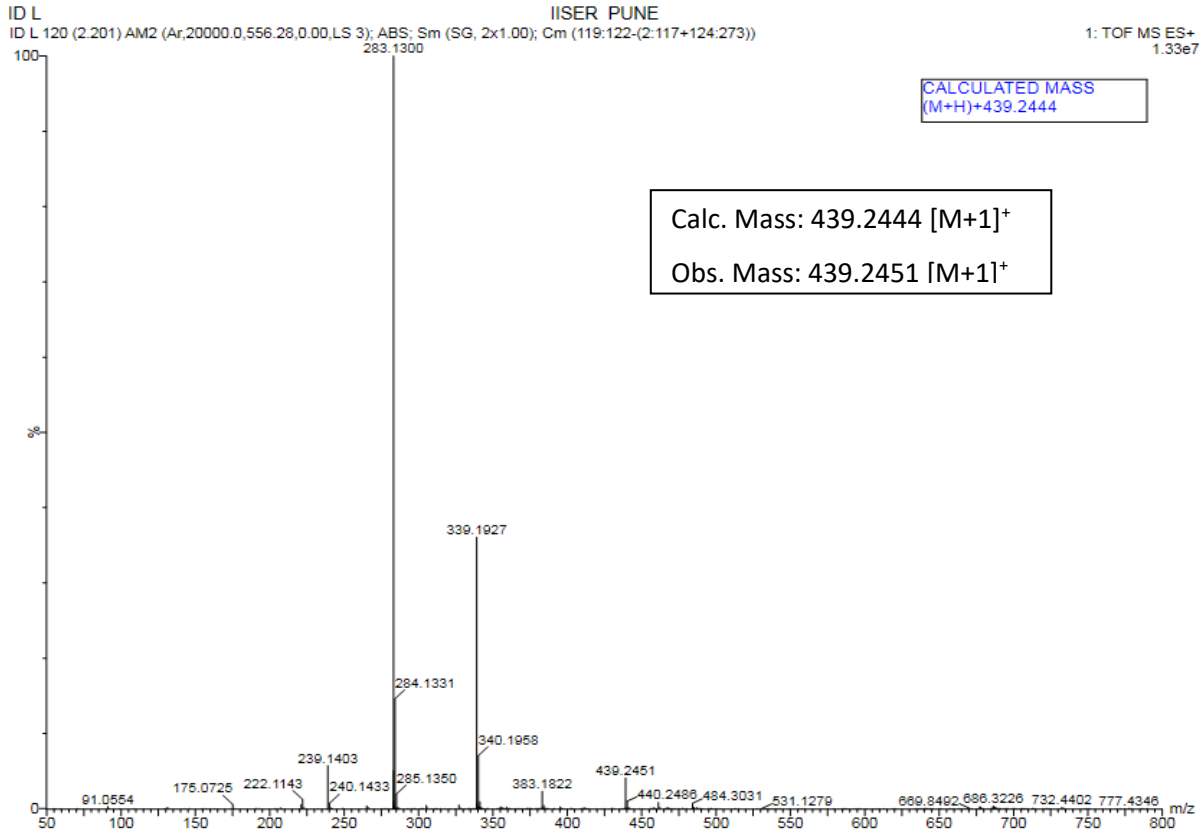
^{1}H NMR of Compound 7



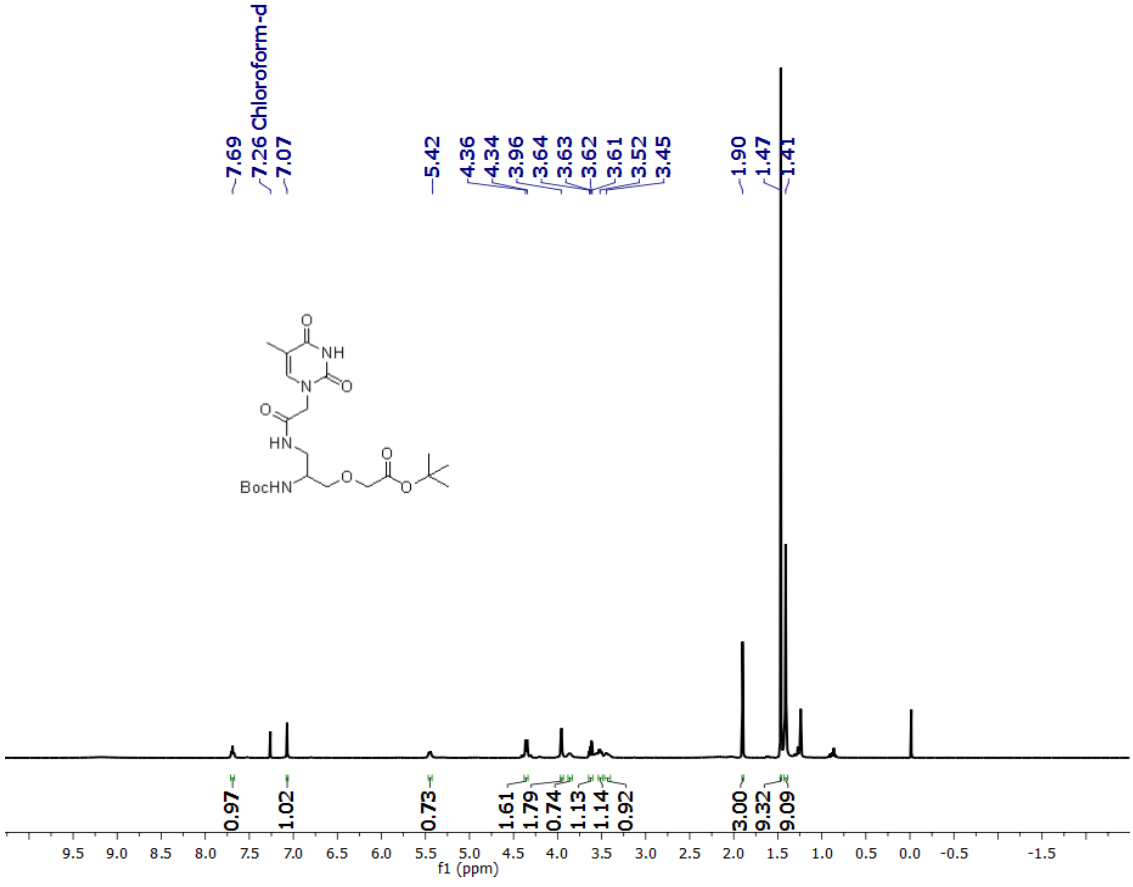
^{13}C NMR of Compound 7:



HRMS of Compound 7:



^{1}H NMR of Compound 9:



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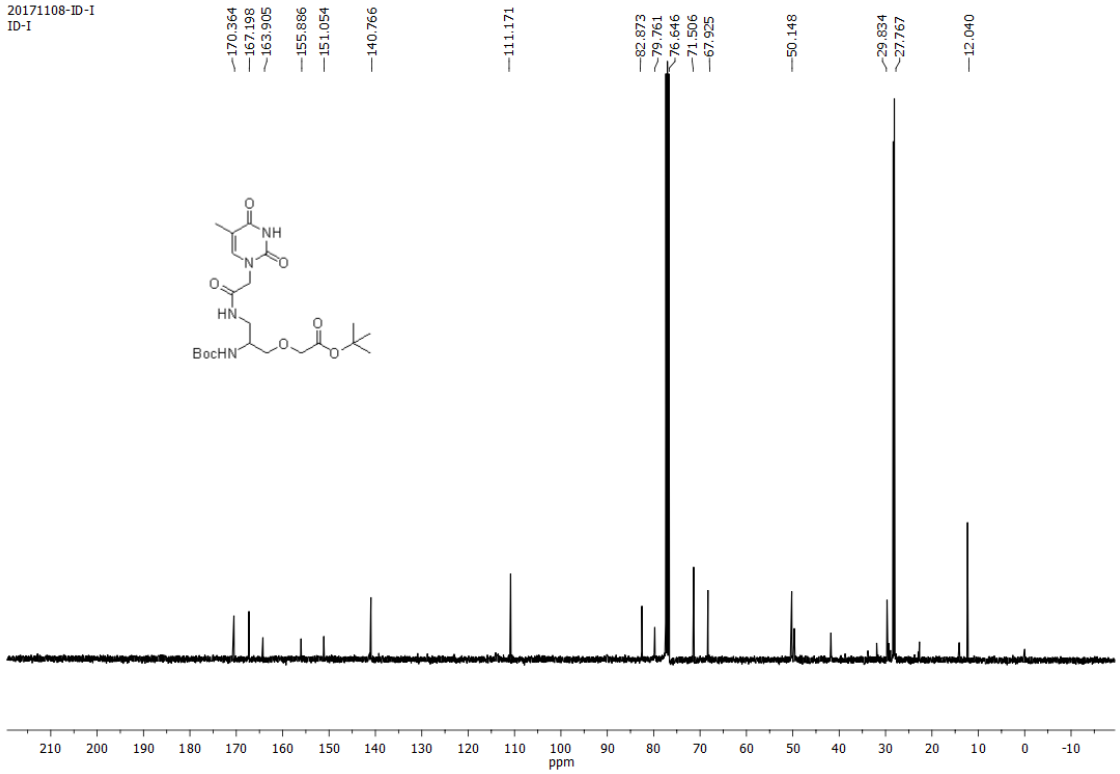
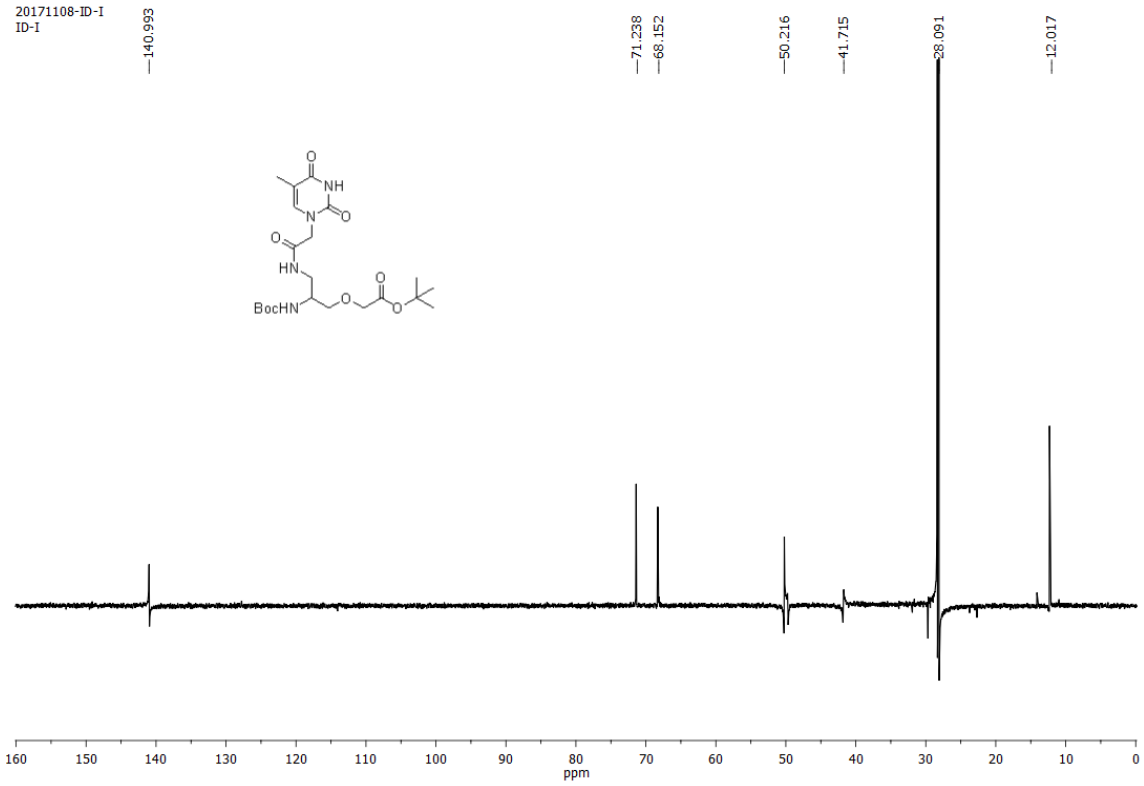


DEPT-135 NMR of Compound 9:



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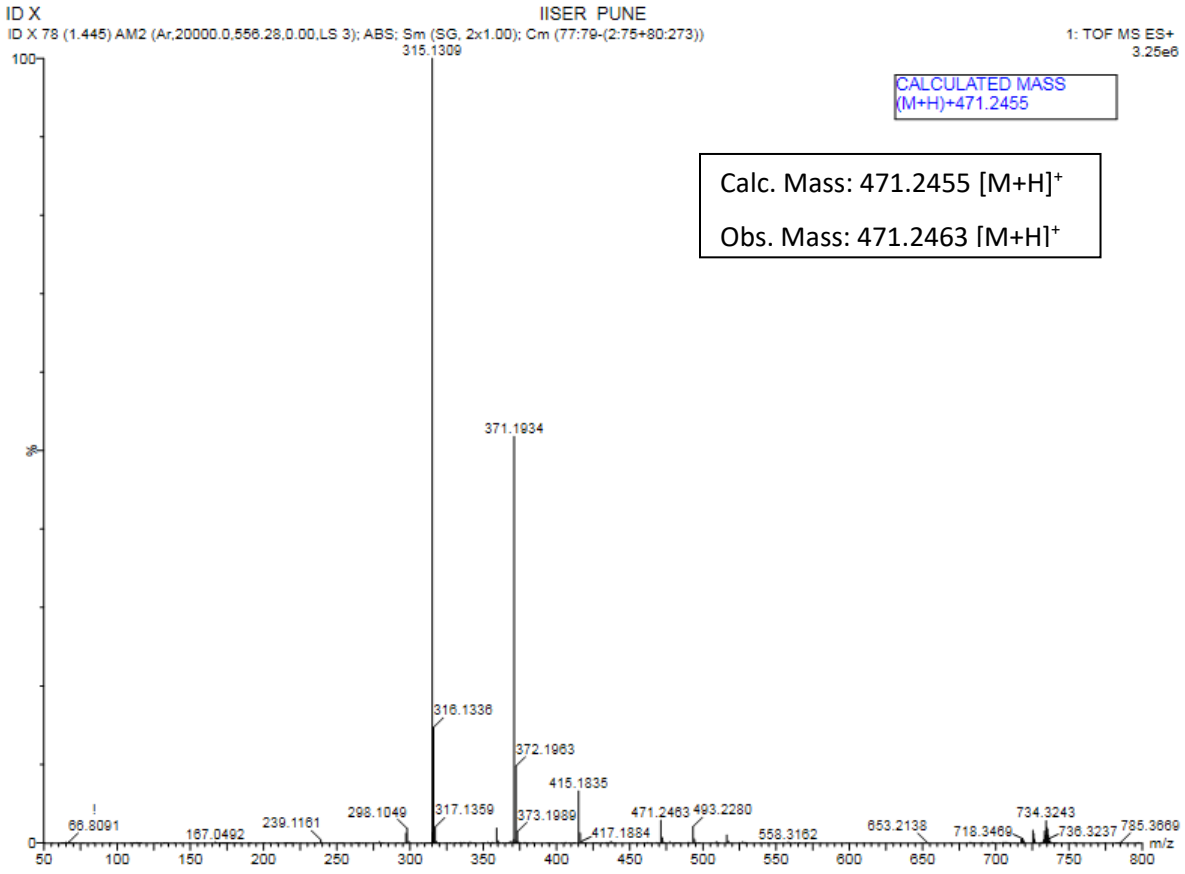

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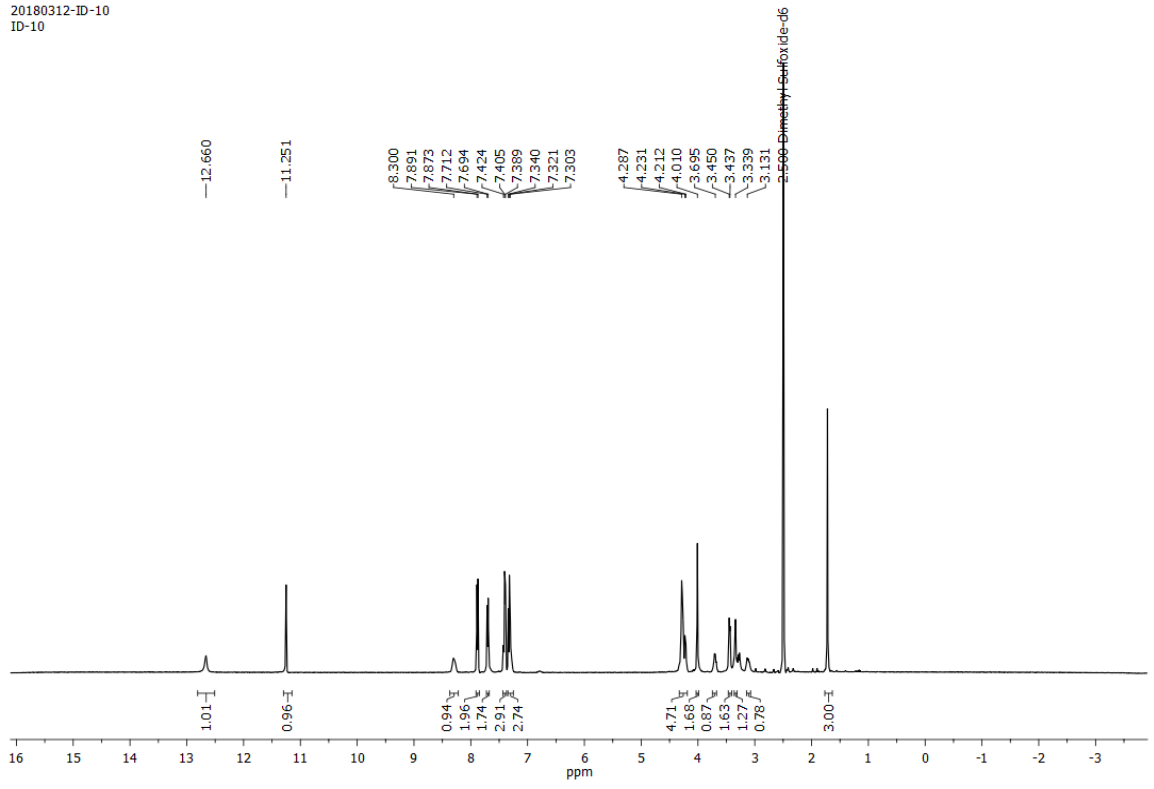

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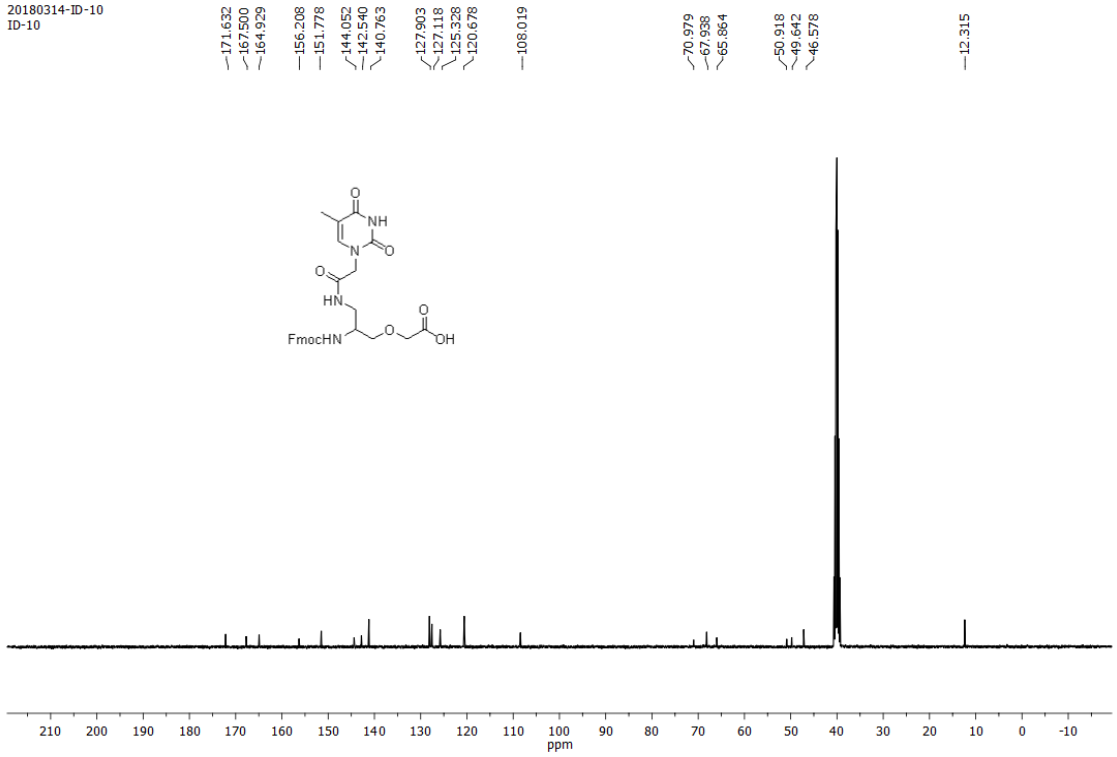

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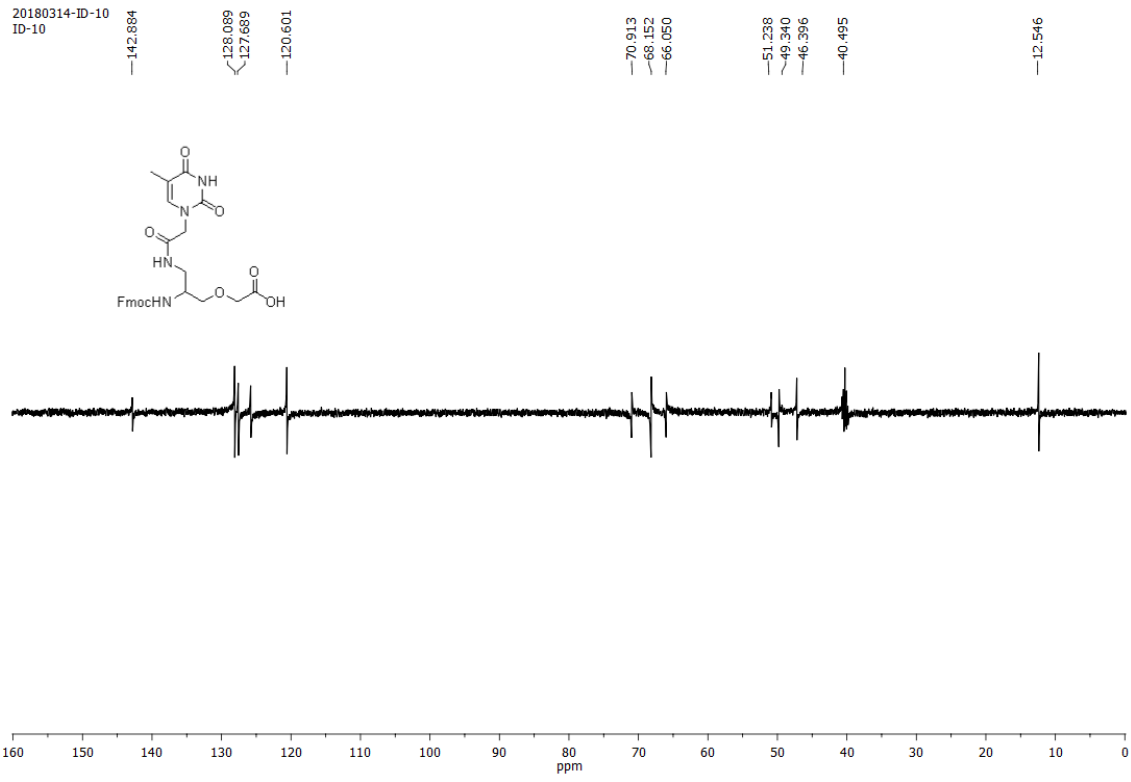

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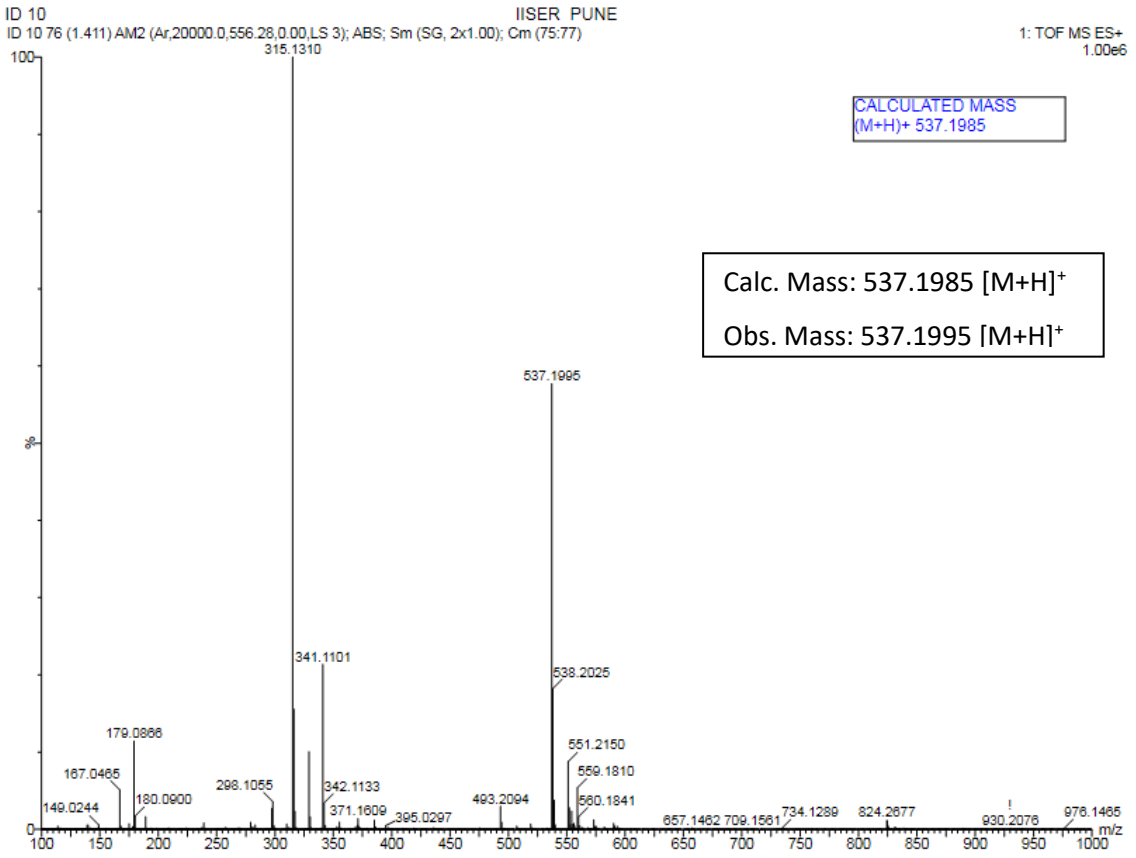
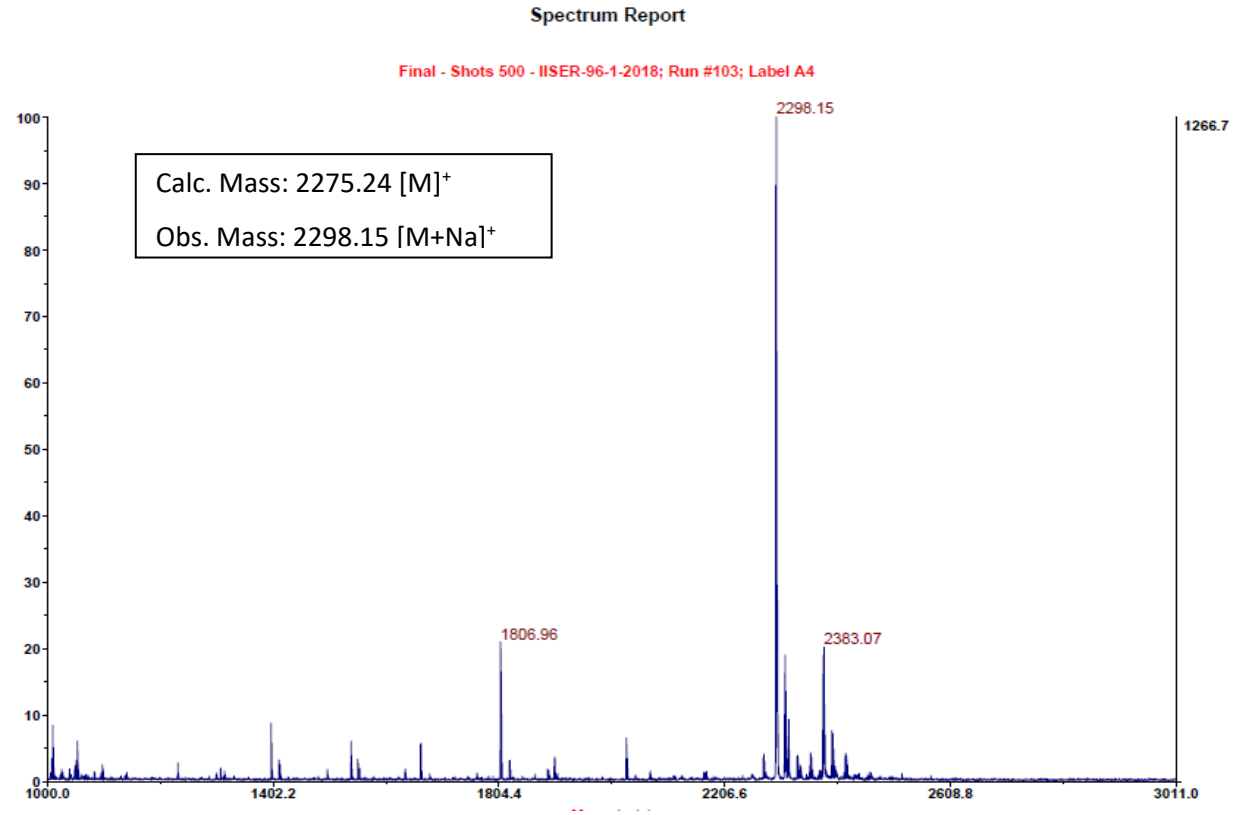
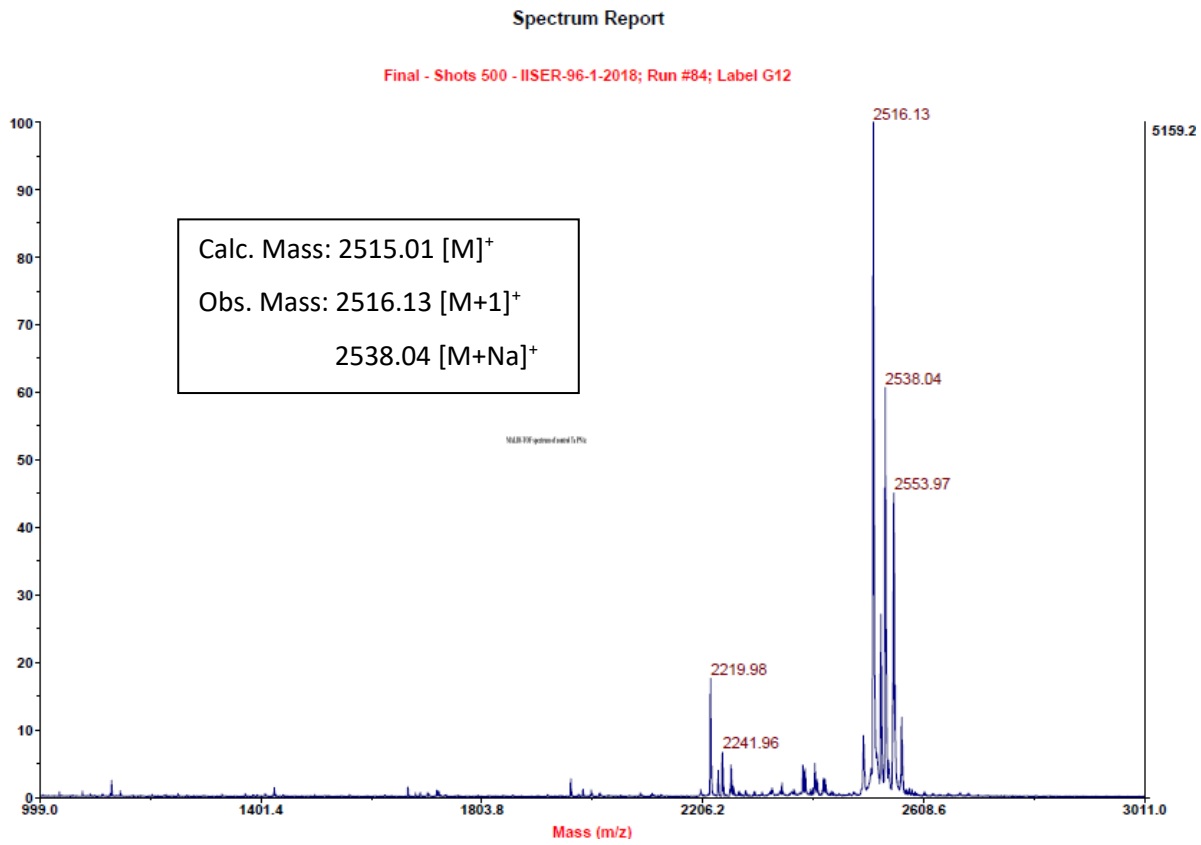


MALDI-TOF spectrum of control T_8 PNA:



MALDI-TOF spectrum of Mod-T_8 PNA:



5.4. Instruments

5.4.1. UV-T_m

For UV-Tm experiment, Varian Cary 300 UV-spectrophotometer having a Peltier temperature programmer was used for performing the measurements. The samples were prepared by calculating and later mixing the required amount of PNA solutions (*aeg* PNA or PNA M) and stock oligonucleotide (ssDNA or ssRNA) together in a 1.5ml microfuge tube with 10 mM NaCl at pH 7.0 and 10mM of sodium phosphate buffer. The final volume was made to 500µL by addition of milliQ water. These samples were heated at 90^{o}C for 5min and left for slow cooling (8hr) till they reach room temperature followed by refrigerating them for 12hr at 4^{o}C. The samples were later pipetted to the quartz cell and sealed/capped with Teflon stopper. The OD of the samples were recorded at 260nm in steps starting from 20^{o}C to 90 °C and later cooling with an increment of 1.0 °C/min in temperature. Each experiment was repeated twice for getting a reproducible data. Determination of T_m was done with the help of first

derivative plots of normalized absorbance at 260nm vs temperature using Origin 9.0. Beer-lamberts law and values of extinction coefficients was used to determine the concentrations DNA, RNA and PNA.

5.4.2. Circular Dichroism

JASCO J-715 spectropolarimeter was used for recording the CD spectra of the prepared samples. The relevant control single strands and PNA:DNA or PNA:RNA complexes were recorded in a concentration of 10 mM NaCl, 10 mM sodium phosphate buffer at pH 7.0. The circulating waster was maintained at a temperature of 4 °C, which was below the melting temperature of strands. The homo-oligomer of Mod PNA and *aeg* PNA was mixed with ssDNA or ssRNA at the required ratio to make duplex or triplex structures for recording CD spectra, which had an accumulation of 3 scans from 350 to 190 nm using 1 cm cell, a resolution of 0.1 nm, sensitivity of 2 mdeg, band-width of 1.0 nm, a scan speed of 50 nm/min, and response of 2 sec and. Each CD experiment was repeated twice for having a reproducible data.

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