

Supramolecular assemblies of C α -bimodal PNAs to tetraplexes and triple duplexes and biophysical studies of C5 substituted uracil PNA oligomers

A Thesis

**Submitted in Partial Fulfillment of the Requirements
of the Degree of**

Doctor of Philosophy

By

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January 2022



Indian Institute of Science Education and Research Pune

dedicated to *my Late* grandfather

CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Supramolecular assemblies of C α -bimodal PNAs to tetraplexes and triple duplexes and biophysical studies of C5 substituted Uracil PNA Oligomers**” Submitted by **Iranna Annappa Todkari** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

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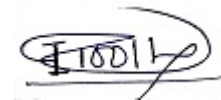


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Table of Contents

Chapter 1: Introduction to *bimodal* Peptide Nucleic Acids

1.1	Introduction	1
1.2	Base pairing in nucleic acids	2
1.3	Metal mediated base pairing	5
1.4	Secondary structures of nucleic acids	6
1.5	Applications of nucleic acids	9
1.6	Chemical modifications of DNA	12
1.7	Peptide nucleic acids (PNA)	17
1.8	Applications of PNA	20
1.9	Chemical modifications of PNA	24
1.10	Synthetic heterocycles as Janus nucleobases	31
1.11	Bimodal peptide nucleic acids	37
1.12	Present work: Supramolecular assemblies of bm-PNAs	39
1.13	References	43

Chapter 2: Synthesis and Characterization of Ca(S)-ethyl azido PNA monomers: precursor for bimodal PNA oligomers

2.1	Introduction	57
2.2	Rationale of the present study	59
2.3	Objectives of the present work	62
2.4	Synthesis of C α bimodal PNA monomers	62
2.4.1	Synthesis of C α (S-eaz)-aeg- ^{Cbz} NH monomer	62
2.4.2	Synthesis of N ^{Boc} ,N'(N1-acetamido-T) aminoethyl (Ca-eaz) glycine methyl ester (11) and N ^{Boc} ,N'(N ⁴ Cbz-N1- acetamido C) aminoethyl (Ca-eaz) glycine methylester 16)	63

2.4.3	Synthesis of (N ^{Boc} , NH ^{Cbz}) aminoethyl glycine (23)	64
2.4.4	Synthesis of nucleobase acetic acids	65
2.4.5	Synthesis of N1-propargyl C/T and N9-propargyl G	65
2.5	Experimental section	66
2.6	Summary	80
2.7	Appendix-I: Index	80
2.8	References	103

Chapter 3: Solid phase synthesis, Purification and Characterization of *bimodal* PNA Oligomers.

3.1	Introduction to Solid phase synthesis	106
3.2	Objectives of the present work	108
3.3	Results and discussion	109
3.3.1	Rationale for choice of sequences	109
3.3.2	Solid phase synthesis of PNA oligomers	111
3.3.3	Synthesis of bimodal PNA having polycytosine on triazole face by click chemistry on solid phase	112
3.3.4	Synthesis of bimodal PNA having Polycytosine on Amide face by click chemistry on solid phase	115
3.3.5	Synthesis of PNA analogue with nucleobase on only on triazole face (<i>iso</i> -PNA)	116
3.3.6	Cleavage of PNA oligomers from the solid support	117
3.3.7	Purification and characterization of the PNA oligomers	118
3.4	Summary	120
3.5	Experimental methods	120
3.5.1	Solid phase synthesis protocol	120

3.5.2	Solid phase synthesis method for <i>bm</i> -PNA 2	121
3.5.3	Synthesis of <i>bimodal</i> PNA oligomers on solid support by ‘click’ reaction	122
3.5.4	Cleavage of the <i>bimodal</i> PNA oligomers from solid support	122
3.5.5	Purification of the <i>bimodal</i> PNA oligomers by RP-HPLC	122
3.6	References	123
3.7	RP-HPLC and MALDI-TOF of PNA Oligomers	125
 Chapter 4: <i>i</i>-Motif formation from <i>bimodal</i> PNAs with all Cytosine on one face		
4.1	Introduction	134
4.2	Rationale and Objectives of the present work	137
4.3	Results and discussion	139
4A.3	Section A: <i>bm</i> -PNA <i>i</i> -motifs	139
4A.3.1	UV Spectra of dTC ₅ , <i>aeg</i> -PNA-C ₅ , <i>iso</i> -PNA 1, <i>bm</i> -PNA 1 and <i>bm</i> -PNA 2	
4A.3.2	pKa determination of dC ₆ , <i>aeg</i> -PNA-C ₅ and <i>iso</i> -PNA 1	140
4A.3.3	pH dependent UV-T plots of <i>i</i> -motifs of dTC ₅ / <i>aeg</i> / <i>iso</i> / <i>bm</i> -PNAs	140
4A.3.4	Circular dichroic spectra of bimodal PNA <i>i</i> -motifs	147
4A.3.5	Summary of thermal stability of bimodal PNA <i>i</i> -motifs	148
4B.3	Section B: DNA: <i>bm</i> -PNA hybrid <i>i</i> -motifs	150
4B.3.1	UV Spectra of dTC ₅ : <i>aeg</i> -PNA-C ₅ , dTC ₅ : <i>iso</i> -PNA 1 and dTC ₅ : <i>bm</i> -PNA 1 hybrid tetraplexes	
4B.3.2	pH dependent UV-T plots of hybrid <i>i</i> -motifs of dTC ₅ : <i>aeg</i> / <i>iso</i> / <i>bm</i> -PNAs	151
4B.3.3	Circular dichroic spectra of hybrid <i>i</i> -motifs	154
4B.3.4	Summary of thermal stability of hybrid <i>i</i> -motifs	157
4.4	Conclusions	158

4.5	Experimental section	159
4.5.1	pKa determination by pH dependent UV-measurements	159
4.5.2	Temperature dependent UV absorbance and determination of T_m	159
4.5.3	Circular Dichroism (CD) spectroscopy	160
4.6	References	161
 Chapter 5: Silver and Mercury mediated Supramolecular assemblies of bimodal-PNAs		
5.1	Introduction	166
5.2	Rationale and Objectives	168
5.3	Results and discussion	169
5A.4	Section A: Silver mediated <i>bm</i>-PNA complexes	171
5A.4.1	Thermal stability of dTC ₅ , <i>aeg</i> -PNA-C ₅ and <i>iso</i> -PNA 1 Silver duplexes by UV-T.	171
5A.4.2	UV T studies of Ag ⁺ mediated duplex of <i>bm</i> -PNA 1	173
5A.4.3	UV T _m of <i>bm</i> -PNA with poly C on amide face: double duplex of <i>bm</i> -PNA 2	175
5A.4.4	Comparative thermal stability of Ag ⁺ mediated hybrid duplexes and Watson Crick duplex	177
5A.4.5	Circular Dichroism (CD) spectroscopy of Ag ⁺ mediated complexes	178
5A.4.6	Morphological study by Field Emission Electron Microscope (FESEM) and Elemental composition by EDAX analysis	181
5B.4	Section B: Mercury mediated <i>bm</i>-PNA complexes	186
5B.4.1	Thermal stability of dTC ₅ , <i>aeg</i> -PNA-C ₅ , <i>iso</i> -PNA 1 mercury mediated duplexes by UV-T	186
5B.4.2	UV-T studies of Hg ⁺⁺ mediated double duplex of <i>bm</i> -PNA 1	187
5B.4.3	UV-T studies of Hg ⁺⁺ mediated double duplex of <i>bm</i> -PNA 2	189
5B.4.4	Morphological study by Field Emission Electron Microscope (FESEM)	191

5.4	Conclusions	194
5.5	Experimental procedure	195
	5.5.1 Temperature-UV Absorbance Measurement	195
	5.5.2 Circular Dichroism	195
	5.5.3 Field emission scanning electron microscopy (FESEM) studies	196
5.6	References	197
 Chapter 6: Synthesis and Characterization of -C₅-Uracil Substituted PNA Monomers, their Oligomerization and Biophysical study		
6.1	Introduction	202
6.2	Rationale	205
6.3	Aim of the present work	205
6.4.	Results and discussion	206
6A.4	Section A	206
	6A.4.1 Synthesis of ethyl <i>N</i> 1-Boc-aminoethyl-5-NHCbz uracil glycine (5NHCbzU- <i>aeg</i>)	206
	6A.4.2 Synthesis of N1(Boc)-N2(carbonylmethyl-N ¹ (5F/5CF ₃ uracil) aminoethyl glycine (10, <i>aeg</i> -5F/CF ₃ -U)	207
	6A.4.3 Solid phase Synthesis of PNA oligomers	207
	6A.4.4 Purification and characterization of PNA oligomers	208
6B.4	Section B	211
	6B.4.1 Thermal melting of PNA/DNA, PNA/RNA and PNA/PNA duplexes	211
	6B.4.2 Thermal stability (UV- <i>T_m</i>) of PNA:DNA duplexes	211
	6B.4.3 Thermal stability (UV- <i>T_m</i>) of PNA:RNA duplexes	212
	6B.4.4 Thermal stability (UV- <i>T_m</i>) of PNA:DNA duplexes PNA:PNA duplexes	213

6B.5	Circular dichroism spectra of C5-U substituted PNA duplexes	215
6B.5.1	Circular dichroism (CD) of PNA:DNA duplexes	215
6B.5.2	Circular dichroism (CD) of PNA:RNA duplexes	216
6B.5.3	Circular dichroism (CD) of PNA:PNA duplexes	216
6B.6	Discussion	217
6B.6.1	Electronic and local microenvironmental effects of C ₅ substituents	217
6B.6.2	Comparative effects of C ₅ substitution on duplex stability	218
6.7	Conclusions	221
6.8	Experimental section	222
6.8.1	General procedures	222
6.8.2	Solid phase synthesis protocol	222
6.8.3	Biophysical studies	223
6.8.4	Experimental procedures and spectral data	224
6.9	References	229
	HPLC and MALDI	240

Abbreviations

A	Adenine
Abs.	Absolute
AcOH	Acetic acid (glacial)
Ac ₂ O	Acetic anhydride
CAN	Acetonitrile
<i>Aeg</i>	Aminoethylglycine
Eaz	Ethylazide
Tz	Triazolyl
Etz	Ethyltriazolyl
Ap	Antiparallel
<i>Aq</i>	Aqueous
(Boc) ₂ O	Boc anhydride
Bn	Benzyl
BIAB	(Diacetoxyiodo)benzene
Bt	Benzotriazole
C	Cytosine
Calcd	Calculated
Cbz	Benzyloxycarbonyl
CD	Circular Dichroism
CHCA	α -cyano-4-hydroxycinnamic acid
CuAAC	Copper mediated azide-alkyne cycloaddition
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DHB	2,5-dihydroxybenzoic acid
DhbtOH	3-Hydroxy-1,2,3-benzotriazin-4(3H)-one
DIPEA	N,N-Diisopropylethylamine
DMAP	N,N-Dimethyl-4-aminopyridine
DMF	N,N-dimethylformamide

DMSO	N,N-Dimethyl sulfoxide
DNA	2'-deoxyribonucleic acid
Ds	Double stranded
EBA	Ethylbromo acetate
EDTA	Ethylene diamine tetraacetic acid
Et	Ethyl
EtOAc	Ethyl acetate
G	Gram
G	Guanine
Gly	Glycine
H	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
in situ	In the reaction mixture
in vivo	Within the living
ITC	Isothermal Titration Calorimetry
IR	Infra-red
L-	Levo-
Lys	Lysine
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
MBHA	4-Methyl benzhydryl amine
Mg	Milligram
MHz	Megahertz
μL	Microliter
μM	Micromolar
Min	Minutes
mL	Milliliter

mM	Millimolar
Mmol	Millimoles
Mp	melting point
MW	Molecular weight
N	Normal
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
Obsd	Observed
Ons	Oligonucleotides
P	Parallel
PCR	Polymerase chain reaction
Pd	Palladium
PIDA	iodosobenzene diacetate (phenyliodonium diacetate
Ppm	Parts per million
PNA	Peptide Nucleic Acid
RP	Reverse Phase (-HPLC)
Rt	Room temperature
RT	Retention time
S	Sinister
SPPS	Solid Phase Peptide Synthesis
Ss	Single strand/single stranded
T	Thymine
TBAc	tert-Butyl bromoacetate
TEA/Et3N	Et3N/Triethylamine
TEMPO	Tetramethylpiperidine 1-oxyl
TFA	Trifluoroacetic acid
Tf2O	Triflicanhydride
TfN3	Trifluoromethanesulfonyl azide
TFMSA	Trifluoromethane sulfonic acid

THF	Tetrahydrofuran
TLC	Thin layer chromatography
<i>T_m</i>	Melting temperature
UV-Vis	Ultraviolet-Visible

Summary

The thesis entitled “**Supramolecular assemblies of C α -bimodal PNAs to tetraplexes and triple duplexes and biophysical studies of C5 substituted Uracil PNA Oligomers**” comprises of a systematic study of formation of *i*-Motif structures and silver mediated higher order assemblies from bimodal peptide nucleic acid (*bm*-PNA) and the impact of C5-Uracil modification on PNA:DNA/RNA/PNA hybrids.

The bimodal PNAs (*bm*-PNA) are a new class of PNA molecules which can bind two different cDNAs to form double duplexes. The *bimodal* PNA oligomers having all-Cytosines (C) on C α side chains are shown to have potential to form *i*-Motif structure (C:H⁺:C) at acidic pH. Such *i*-Motifs from triazole face of *bm*-PNA possess nucleobases on amide face to which can bind to a complementary DNA strand with potential to form duplexes from outer side.

bm-PNA having all-C can also self-pair through mediation of metal ions at neutral pH. This leads to a *bm*-PNA:Ag⁺:*bm*-PNA duplex, which can in turn form outer duplexes with DNA leading to formation of triple duplexes. Such metal mediated base pairing (C:M⁺:C) of *bm*-PNA presents a promising strategy to generate self-assembling supramolecules with metal-based functionality. The work also describes effects of hydrophilic, hydrophobic, lipophilic C5-U modifications on PNA:DNA/RNA/PNA hybrids.

Abstract

This thesis is presented in six chapters as follows

Chapter 1: Introduction

Chapter 2: Synthesis and characterization of C α (S)-ethyl azido PNA monomers: precursor for bimodal PNA oligomers.

Chapter 3: Solid phase synthesis, purification and characterization of *bimodal* PNA oligomers.

Chapter 4: *i*-Motif formation from *bimodal* PNAs with all-Cytosines on one face

Chapter 5: Silver and mercury mediated supramolecular assemblies of *bimodal*-PNAs

Chapter 6: Synthesis and characterization of C5-Uracil substituted PNA monomers, their oligomerization and biophysical study of their DNA /RNA /PNA hybrids.

Chapter 1: Introduction

In the area of nucleic acids, there have been several backbone modified analogues are well studied. As unmodified oligonucleotides are rapidly cleaved by the action of nucleases that primarily hydrolyse the phosphodiester linkage, backbone modified nucleic acids offer protection against such events and hence valuable for antisense technology. Furthermore, the ability of the negatively charged DNA to cross phospholipid cell membrane is poor. Hence structural modifications are needed to enable oligonucleotides to be used as antisense therapeutic agents in biological systems. The main structural changes to achieve this include modifications in phosphodiester linkage and/or the sugar moiety (Figure 1).¹⁻¹¹ Among these, modifications, peptide nucleic acids (PNA) mimic many of the oligonucleotide properties. They are composed of repeating units of *N*-(2-aminoethyl)glycine units to which natural nucleobases are attached *via* methylenecarbonyl linker (Figure 1, *aeg*-PNA), an acyclic sugar-phosphate backbone modification.^{12, 13}

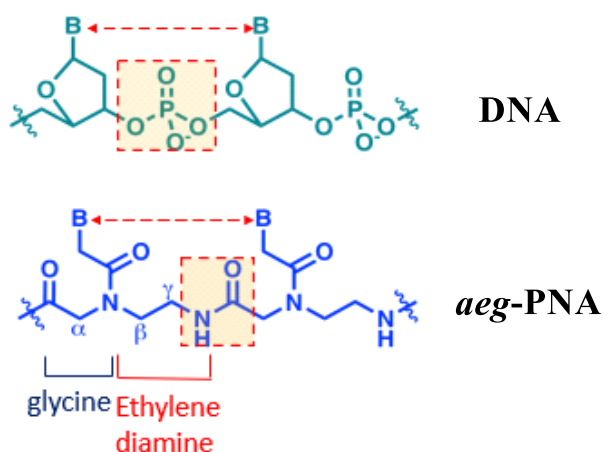


Figure 1. Chemical structures of DNA and PNA.

PNA binds to complementary DNA and RNA to form duplexes *via* Watson-crick base pairs and triplexes through Watson-Crick and Hoogsteen hydrogen bonding and exhibit thermal stability higher than that of DNA:DNA and DNA:RNA complexes.¹³ Recently, a new class of PNAs known as “bimodal PNA” have been introduced in which each aminoethylglycyl unit of *aeg*-PNA carries a second nucleobase linked at either C α through a triazole spacer (Figure 2a) or at C γ via an amide spacer chain (Figure 2b), The bimodal PNAs with mixed base sequences can target two complementary DNAs simultaneously to form double duplexes.^{14, 15}

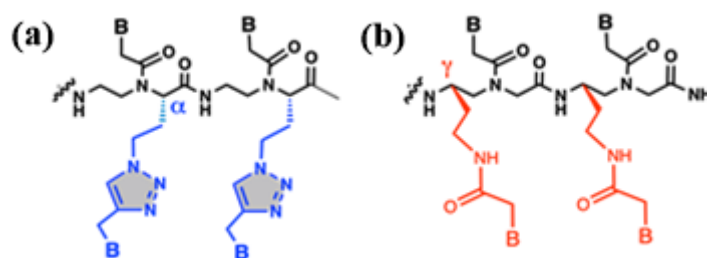


Figure 2. Structures of (a) bimodal-C α -tz-PNA (b) bimodal-C γ -amide-PNA.

This chapter gives an overview of structures of DNA and PNA, their chemical modifications and their effects on their properties and the applications.

Chapter 2: Synthesis and characterization of C α (S)-ethyl azido PNA monomers: precursors for bimodal PNA oligomers.

This chapter deals with synthesis of precursor monomers needed for target *bm*-PNA oligomers. These were made from C α -S-(ethylazido)-aeg-T/C//G protected monomers (**1-3**) which were used for C α -S-(ethylazido)-PNA oligomer synthesis. It was then coupled with N1-propyne-T/ C^{N4(Cbz)} and N9-propyne A/G (**4-6**) to obtain the desired *bm*-PNA oligomers

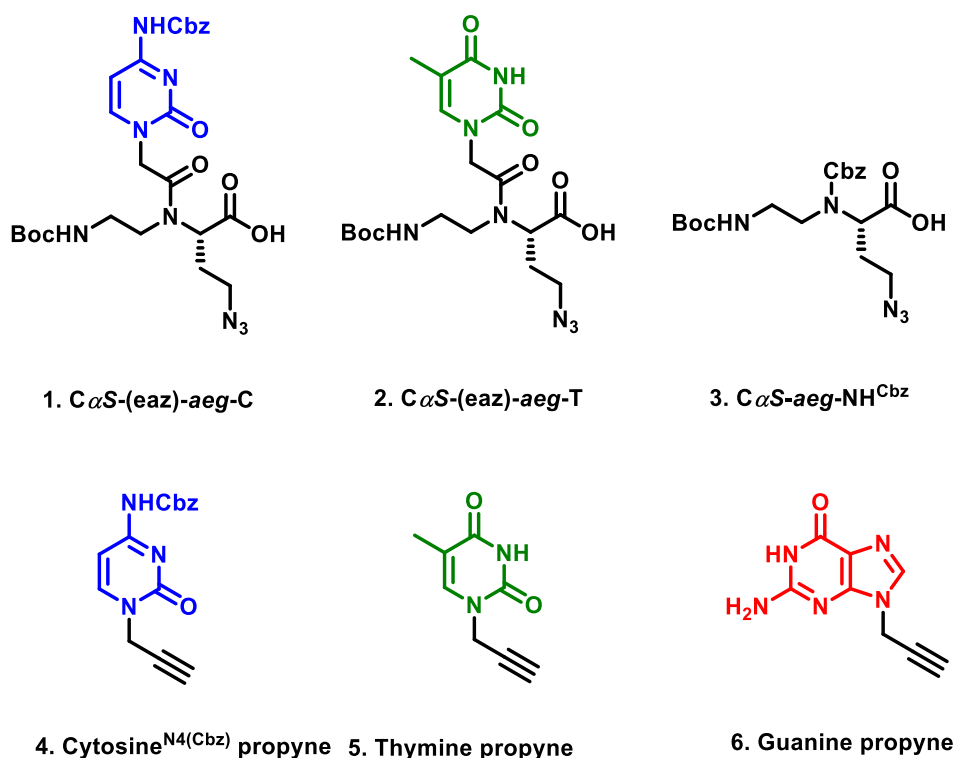
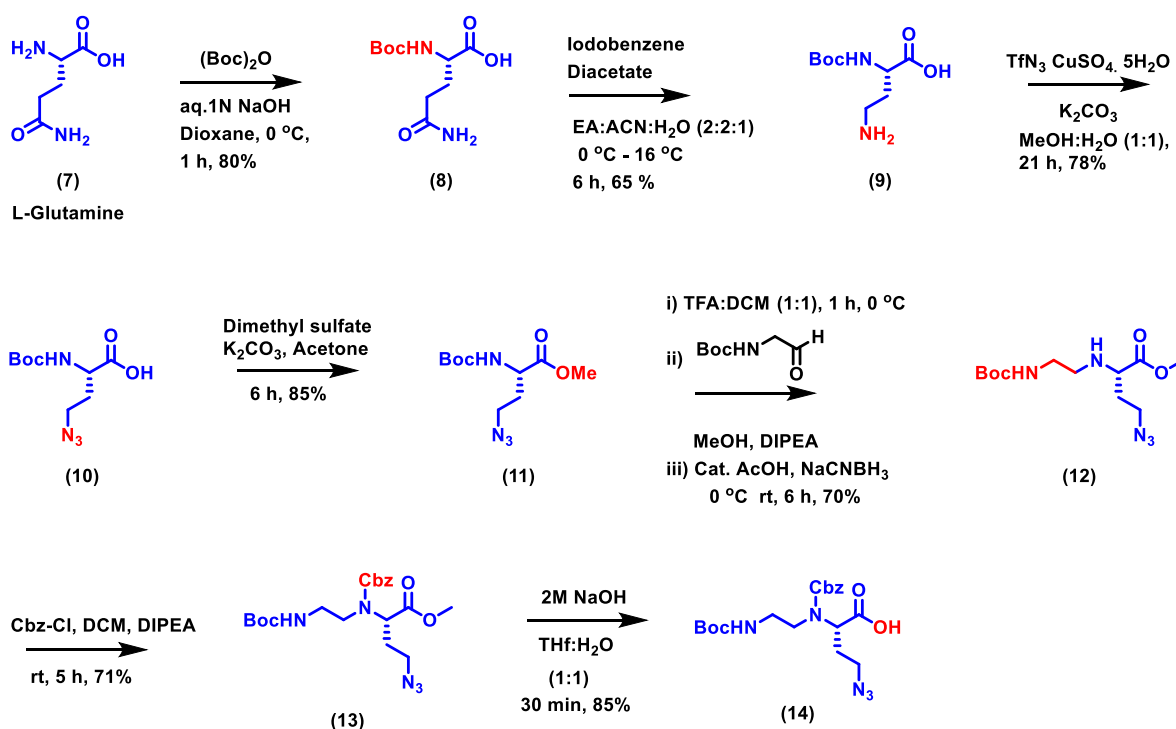


Figure 3 *bm*-PNA monomers used for study

Synthesis of $\alpha(S\text{-}eaz)\text{-}\alpha eg\text{-NH}^{Cbz}$ monomer (14)¹⁴

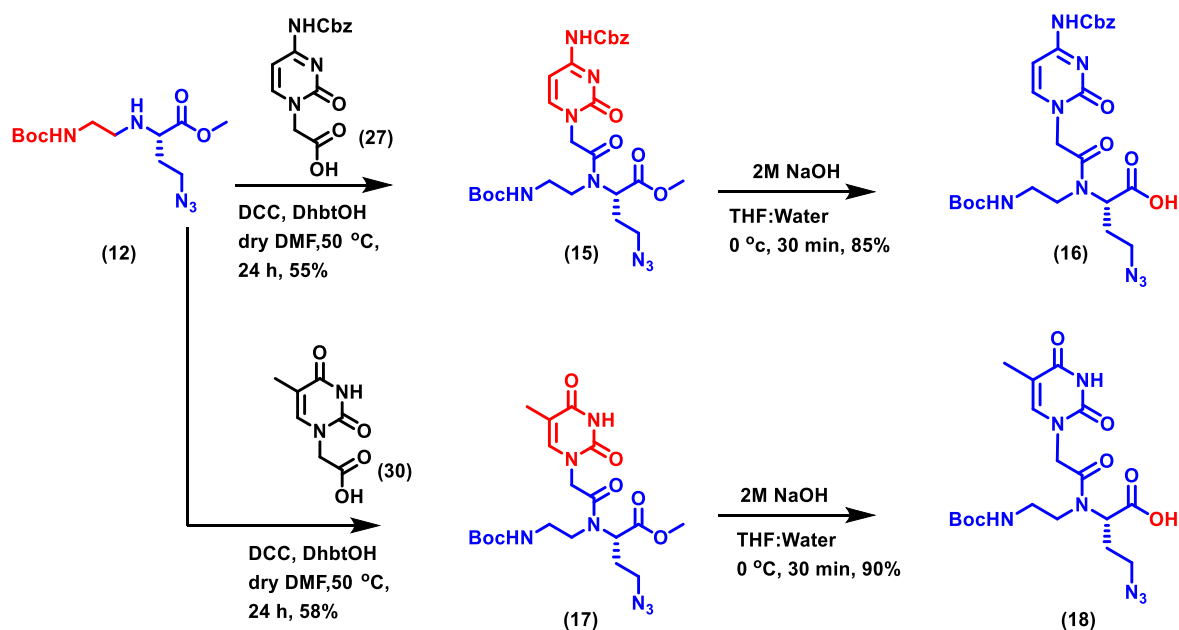
The synthesis of desired compound **8** was started from commercially available L-glutamine **7** (Scheme 2.1). After treatment with di-*t*-butyl decarbonate [(Boc)₂O] it gave protected NHBoc-L-Glutamine **8**. The amido group in **8** was converted to amino function, by reaction with iodobenzenediacetate (IBA) to obtain 4-amino-2-(NHBoc) butanoic acid **9**. This was followed by transformation of the primary amine in **9** to azide **10** through diazo transfer reaction by treatment with triflic anhydride and sodium azide (*in-situ* generation of triflyl azide) in presence of CuSO₄·5H₂O.¹⁶ The resultant acid **10** was treated with dimethyl sulfate to form the methyl ester derivative **11**. This compound reacted with NHBoc aminoacetaldehyde to yield α -substituted aminoethyl glycylic derivative **12** in good yield. The secondary amino group in **12** was protected as Cbz group to get compound **13** that was hydrolysed under basic conditions to afford the desired N1^{Boc},N2^{Cbz} aminoethyl ($\alpha\text{-}eaz$) glycine **14** in very good yield. This monomer was used for solid phase synthesis of *iso*-PNA which has nucleobases only on the triazole face and for further synthesis of other target *bimodal* PNA monomers.



Scheme 2.1 Synthesis of $\alpha(S\text{-}eaz)\text{-}\alpha eg\text{-NH}^{Cbz}$ monomer (14)

Synthesis of N1^{Boc},N2(N1-acetamido-T) aminoethyl (C α -eaz) glycine methyl ester (11) and N1^{Boc},N2(N⁴Cbz-N1- acetamido-C) aminoethyl (C α -eaz) glycine methylester 16)

The compound **12** was coupled with N⁴(Cbz)-Cytosine N-acetic acid (**27**) and Thymine N1-acetic acid (**30**) at high temperature using DhbtOH and DCC as coupling agent (Scheme 2.2)¹⁷ to obtain the esters **15** and **17**. These were hydrolyzed under basic conditions to afford the C α -S-(ethylazido)-C (**16**) and C α -S-(ethylazido)-T (**18**) acid monomers.



Scheme 2.2. Synthesis of C α -eaz-aeg-PNA T monomer

Chapter 3: Solid phase synthesis, purification and characterization of *bimodal* PNA oligomers.

The different *bimodal* PNA oligomers that were synthesized for study are shown in table 1.

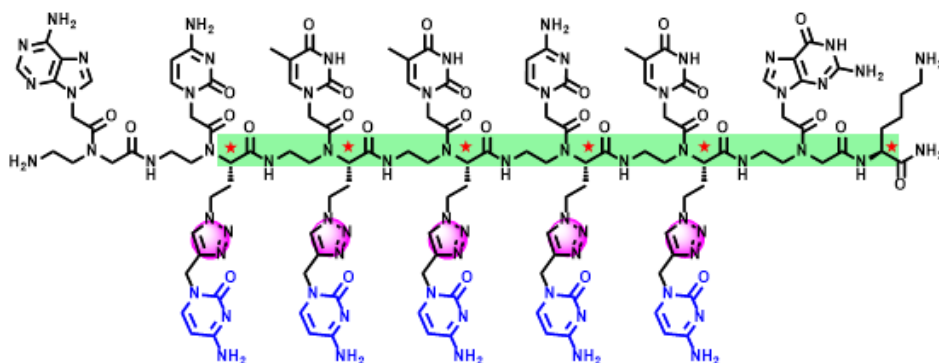


Figure 4 Presentation of *bm*-PNA 1 oligomer

TABLE 1 The sequences of *bimodal*-PNA oligomers

Entry	Sequence Code	PNA Sequences
1	<i>bm</i> -PNA 1	
2	<i>bm</i> -PNA 2	
3	<i>bm</i> -PNA 3	
4	<i>bm</i> -PNA 4	
5	<i>iso</i> -PNA 1	
6	<i>iso</i> -PNA 2	
7	<i>aeg</i> -PNA-C ₅	
8	<i>aeg</i> -PNA-G ₅	

9	<i>aeg</i> -M-PNA	
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Synthesis of bimodal PNA having polycytosine on triazole face by click chemistry on solid phase (*bm*-PNA 1)

The strategies for the synthesis of various kinds of *bimodal* PNA oligomers has been described in this section. PNA monomers were incorporated into 7-mer PNA sequence by solid phase synthesis on L-lysine derivatized MBHA functionalized resin having 0.22 mmol/g loading value. Deprotection, neutralization followed by coupling of nucleobases (Figure 5) and repetition of the same steps gave the oligomer *bm*-PNA having five ethyl azide sidechains. In this PNA, both C and N termini used unmodified *aeg*-PNA monomers to avoid the steric hindrance in synthesis and DNA hybridization reactions. In the subsequent step, a global click reaction of ethyl azido group on solid phase with N1-propynyl cytidine (**4**) gave solid phase bound MBHA *bm*-PNA **1** which was cleaved from the resin by reaction with TFA/TFMSA to yield *bimodal* PNA oligomer *bm*-PNA **1** (Figure 6).

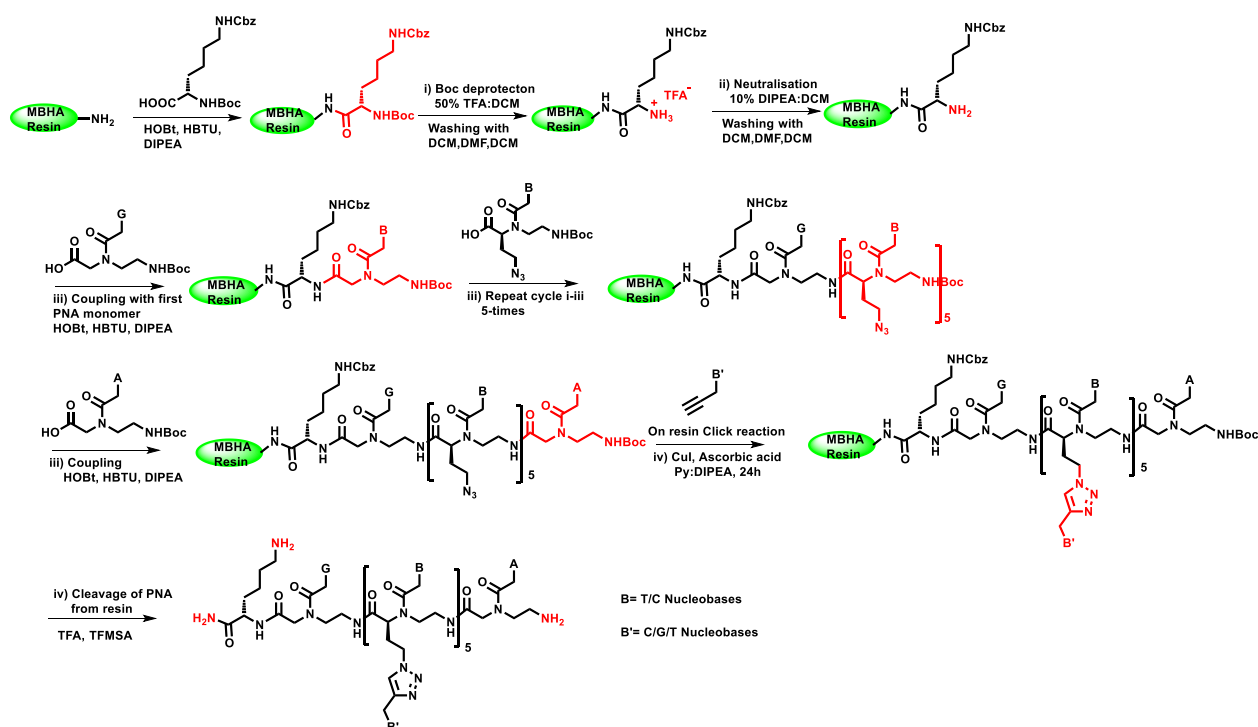


Figure 5 Solid phase synthesis protocol of *bimodal* PNA by *Boc* strategy.

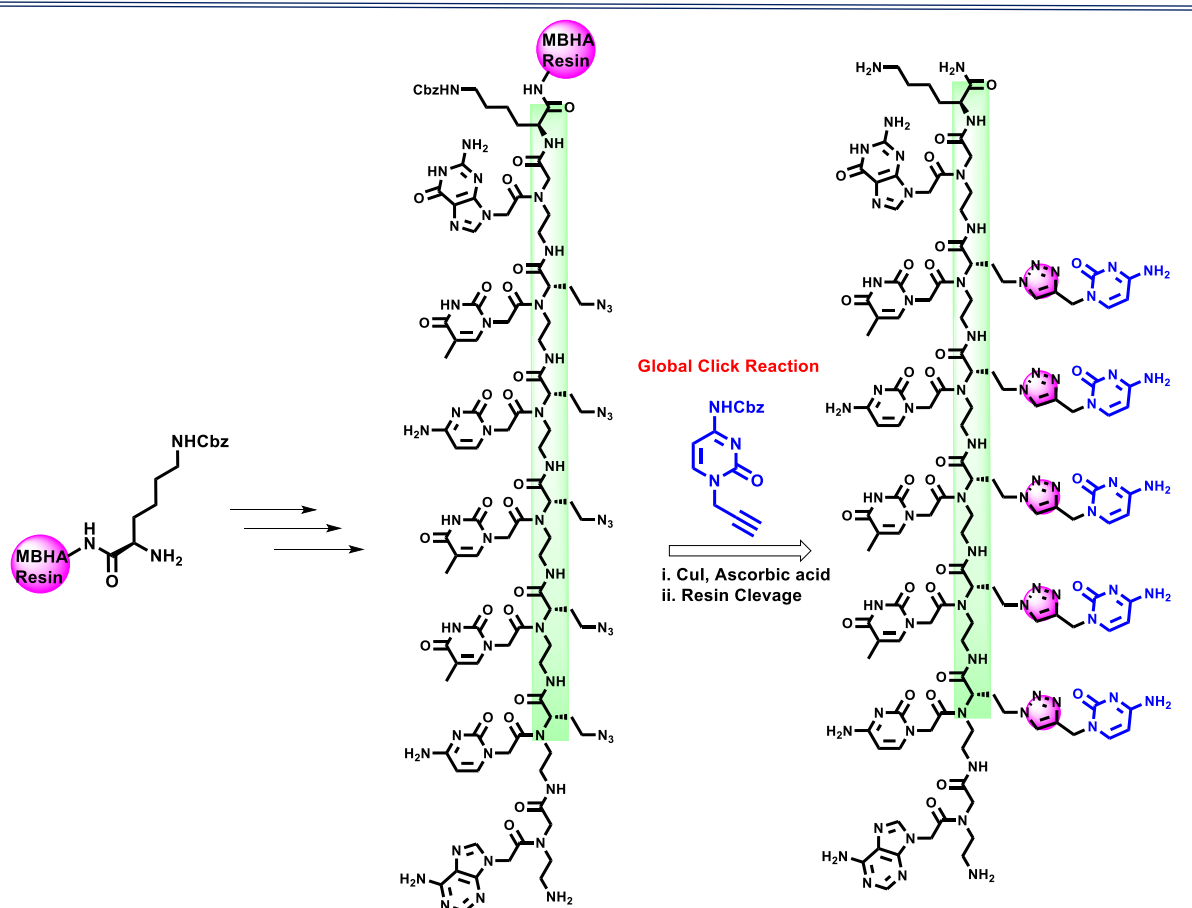


Figure 6 Solid phase synthesis of *bm*-PNA 1

Synthesis of bimodal PNA having Polycytosine on Amide face by click chemistry on solid phase (*bm*-PNA 2)

The *bimodal* PNAs that possess mixed pyrimidine bases on the triazole faces and poly C₅ on the amide face were synthesized by the same solid phase protocol as above, using MBHA resin and (C^{N4Cbz}N1-acetamido) aminoethyl (CαS-ethylazido) *aeg*-PNA monomer (**1**). As compared to strategies shown in (Figures 5 and 6), click reactions were done with appropriate propyne bases (T/C) at desired positions in PNA sequence after each coupling step of monomers (Figure 7) to assemble the triazole face mixed pyrimidine sequence. One step, click reaction was avoided, as it would lead to homo pyrimidine sequence on triazole side rather than mixed sequence. This sequence of reactions leads to mixed sequences on the triazole face and homo polycytosine sequence on amide face to yield resin bound MBHA *bm*-PNA 2 (Figure 8). The PNA oligomer was deprotected, cleaved from the resin, purified by HPLC and characterized by mass spectral data. To ensure successful and efficient coupling at all steps, a small amount of the resin was cleaved at intermediate stages of synthesis at difficult steps of coupling and the intermediate products

characterized by mass spectral data. All PNA oligomers were purified by HPLC and characterised by mass spectral data.

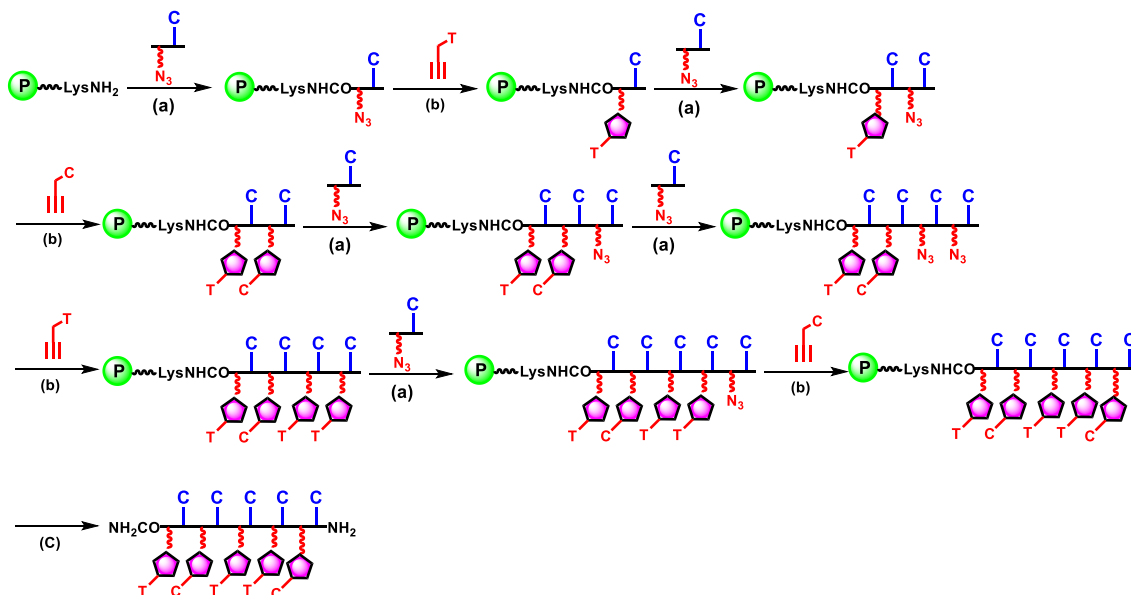


Figure 7 Solid phase synthesis protocol for *amide face ploy C* (bm-PNA 2, P-MBHA resin, (a) Coupling reaction, (b) Click reaction (c) Resin cleavage

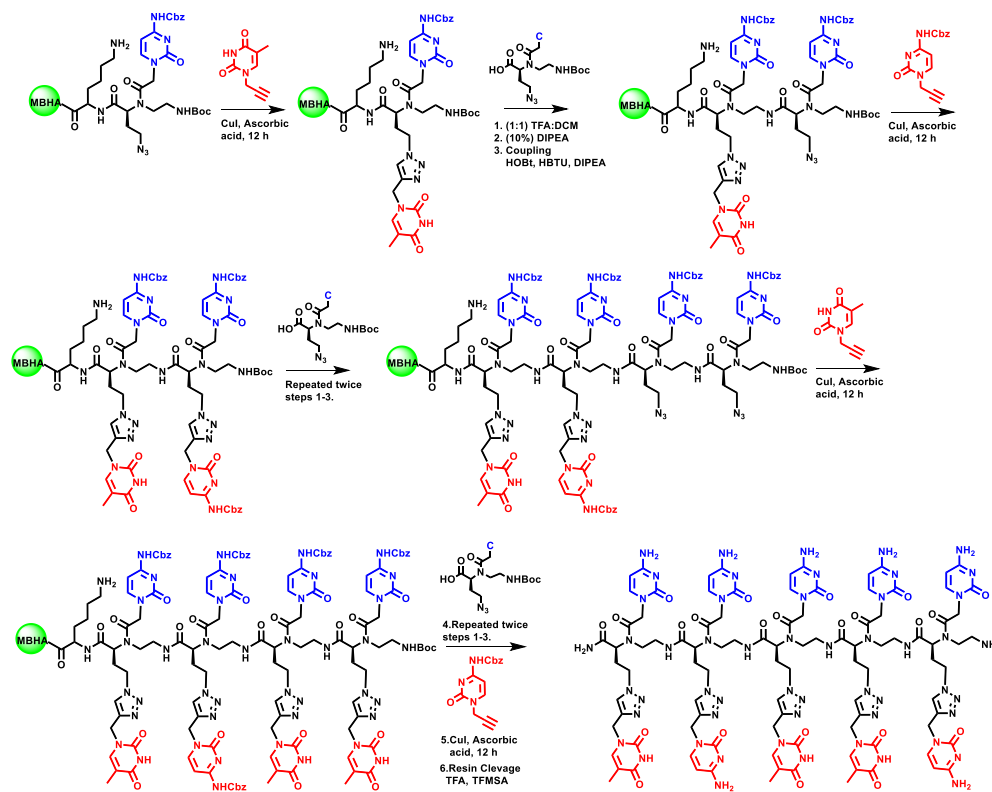


Figure 8 Solid phase synthesis of *bm-PNA 2*

Chapter 4: *i*-Motif formation from *bimodal* PNAs with all-Cytosine on one face and mixed sequence on the other face

In this chapter, it is shown that *bm*-PNAs having all-C nucleobases on both faces are able to form *i*-motif structure. The various PNA constructs used for study as follows: standard *aeg*-PNA- C_5 (Figure 9a) has 5 cytosines (C) linked via t-amide to *aeg* backbone. The *iso*-PNA 1 (Figure 9b) has 5 Cytosines attached at C_α of *aeg* backbone by triazole spacer, without any nucleobases on t-amide side. The bimodal *bm*-PNA 1 has mixed base sequence on t-amide side and 5 cytosines (C) on the triazole face (Figure 9c) similar to *iso*-PNA 1. *bm*-PNA 2 (Figure 9d) has reverse structure with poly C sequence on t-amide side and mixed base sequence at C_α on triazole face (reverse of *bm*-PNA 1).

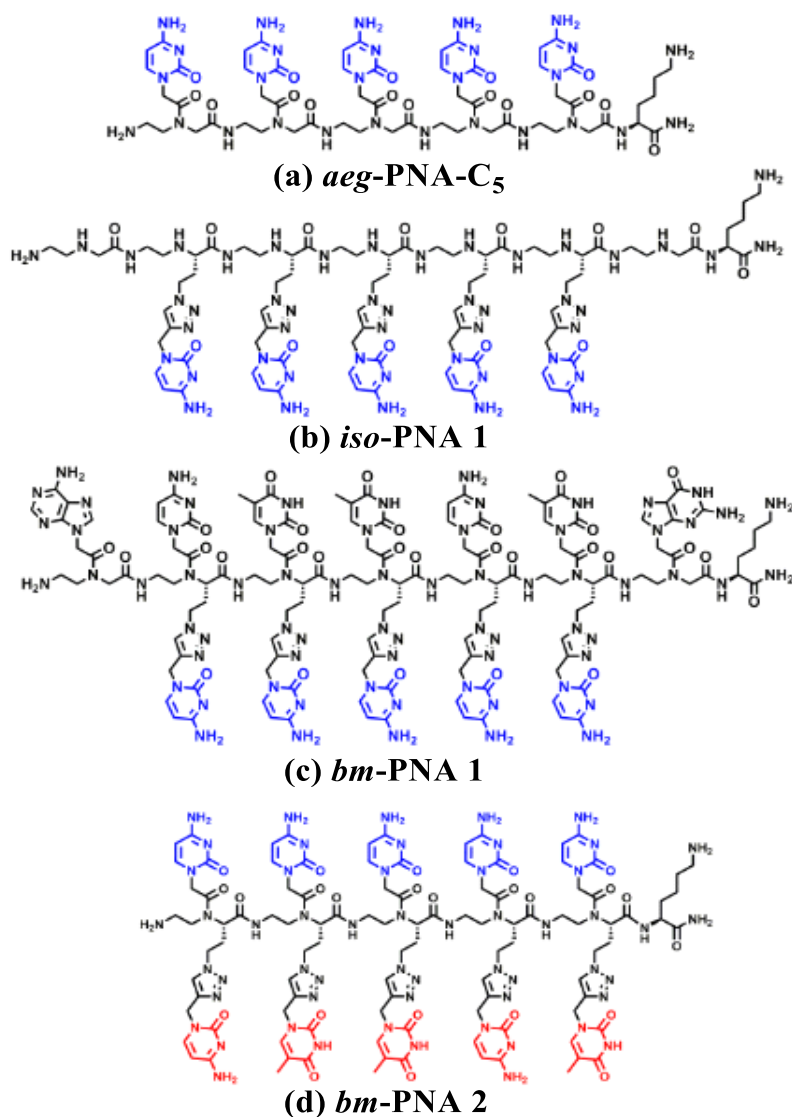


Figure 9. Structures of (a) *aeg*-PNA- C_5 (b) *iso*-PNA 1 (c) *bm*-PNA 1 and (d) *bm*-PNA 2

Thermal stability (T_m) of self-bimodal PNA *i*-motifs

The thermal stabilities of *i*-motifs were examined by temperature dependent UV absorbance (295 nm / 260 nm) at different pHs. A negative sigmoidal transition at 295 nm and positive transition at 260 nm are characteristic of tetraplex. Positive sigmoidal transitions at both 295 nm and 260 nm signify duplex formation. Figure 10 shows the comparative pH dependent T_m data for PNA oligomers *aeg*-PNA, *iso*-PNA 1, *bm*-PNA 1 and *bm*-PNA 2 at different pHs. The T_m s at pH 3.6 and 4.5 corresponding to *i*-tetraplex formation were followed at 295 nm, while at pHs 5.5 and 7.3 duplex formation were followed at 260 nm. For comparison of *i*-motif stability with DNA dTC₅ were measured from UV-T plot.

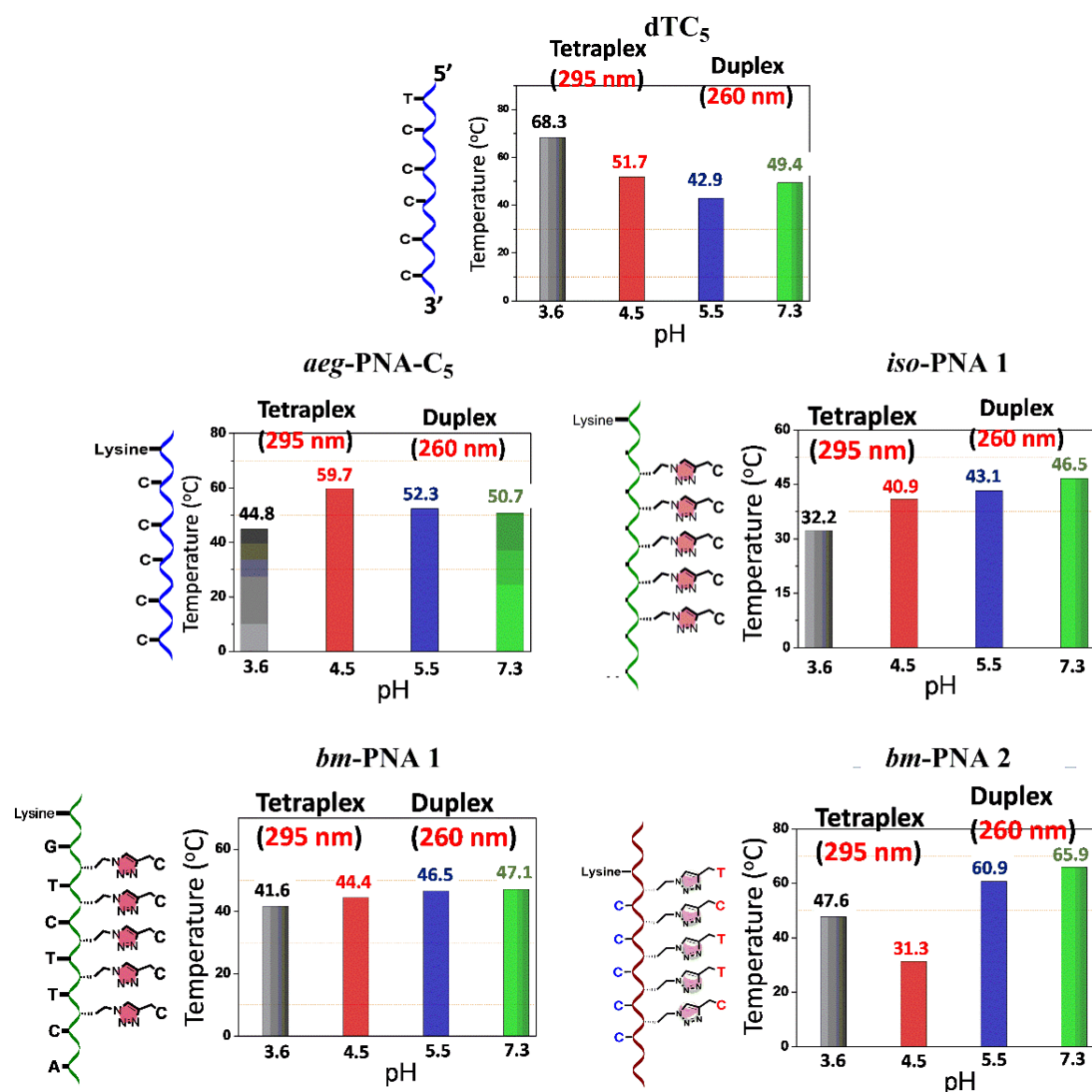


Figure 10. Summary of UV-T plots of dTC₅, *aeg*-PNA-C₅, *iso*-PNA 1, *bm*-PNA 1 and *bm*-PNA 2

Thermal stability of hybrid DNA:bimodal PNA i-motifs

The stability of hybrid motifs comprised from 1:1 mixture of dTC₅ and *aeg*-PNA/*iso*-PNA/*bm*-PNA were also measured. These involve a DNA:DNA duplex intercalated into PNA:PNA duplex. Figure 11 summaries the pH dependent thermal stability data for hybrid complexes. dTC₅:*aeg*-PNA-C₅ hybrid is more stable at acidic pH condition while dTC₅:*iso*-PNA **1** hybrid is stable at neutral pH. The hybrid complex dTC₅:*bm*-PNA **1** has moderate stability from pH 3.6 to pH 7.3. All of them form tetraplex at pH 3.6 and 4.5, but a DNA:PNA duplex at pH 7.3.

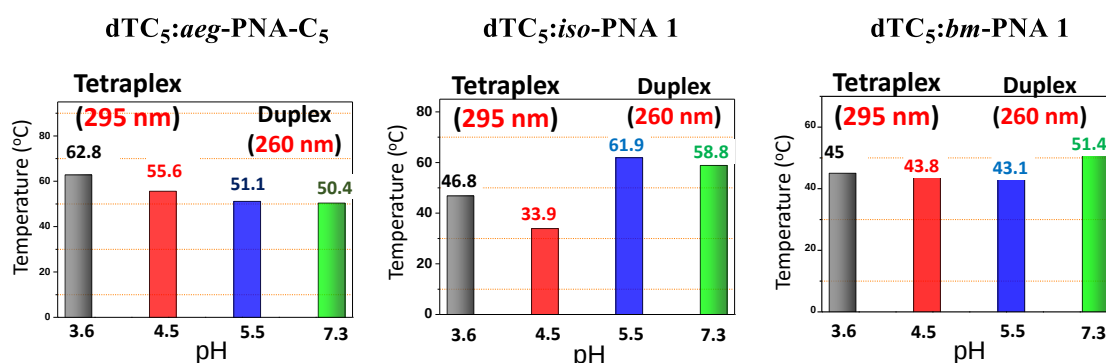


Figure 11. Summary of UV-T plots of dTC₅:*aeg*-PNA, dTC₅:*iso*-PNA **1**, and dTC₅:*bm*-PNA **1**

Chapter 5: Silver and Mercury mediated Supramolecular assemblies of *bimodal*-PNAs

This chapter describes the thermal stability of metal mediated duplexes, triple duplexes and synergistic effect in triple duplex by UV- T_m , sequential formation of complex by CD and morphological behaviour by FESEM and elemental composition characterised by EDX analysis.

Section A: Silver mediated base pairing

Thermal stability of mercury mediated triple duplexes

The bimodal *bm*-PNA **1** formed the expected WC duplex from mixed base sequence on t-amide face with complementary DNA **1** with T_m of 36 °C (Figure 12) and when complexed with Ag⁺ the *iso*-PNA **1** and *bm*-PNA **1** having all-C on triazole side formed silver mediated duplex (*bm*-PNA **1** -C₅:Ag⁺: *bm*-PNA **1** Figure 12b) that exhibited single transition with T_m of 68.3 °C. Compared to its self-melting, T_m of [*bm*-PNA **1**]₂:Ag⁺ complex showed a higher stability with T_m of 68.3 °C (ΔT_m +38.3 °C). The silver complex *bm*-PNA **1**:Ag⁺: *bm*-PNA **1**

was mixed with two equivalents of complementary DNA **1**. The UV-T plot of the resulting complex interestingly showed double sigmoidal transition with T_{ms} at 49.3 °C and 81.5 °C. This indicated the formation of triple duplex compose of two DNA:*bm*-PNA **1** with WC duplex joined by *bm*-PNA **1**:Ag⁺:*bm*-PNA **1** duplex.

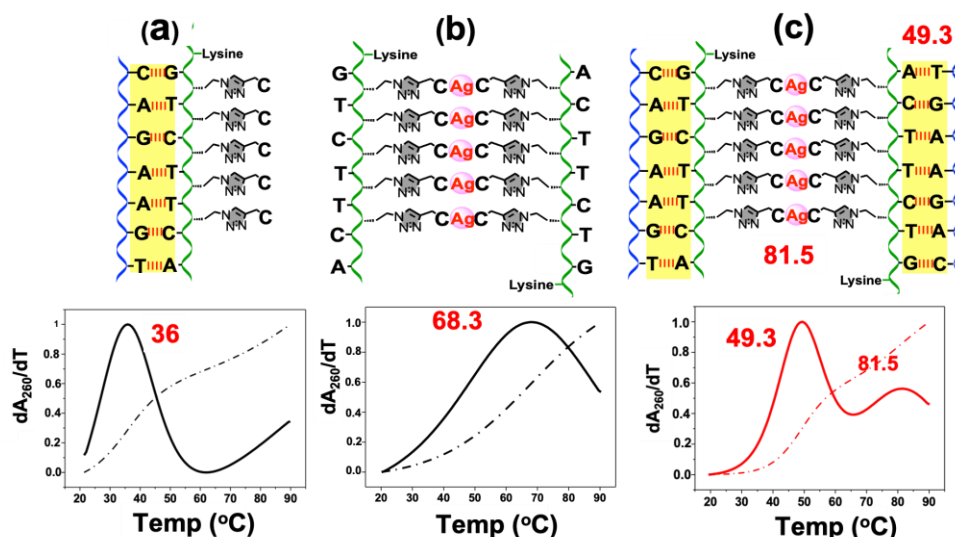


Figure 12 UV-T plots of *bm*-PNA **1** with poly C on triazole face (a) duplex DNA **1**: *bm*-PNA **1** (b) silver complex [*bm*-PNA **1**]₂:Ag⁺ and (c) silver complex of double duplex [DNA **1**: *bm*-PNA **1**]₂:Ag⁺.

CD studies of DNA **1**: *bm*-PNA **1**:Ag⁺: *bm*-PNA **1**:DNA **1**:

Upon complexation to form DNA **1**:*bm*-M-PNA-tz-C₅ duplex, the CD spectrum shows a broad negative band at 283 nm and a positive band at 263 nm (Figure 13, curve **b**) which is different from the CD spectrum of DNA alone (Figure 13, curve **a**). Adding AgNO₃ to the above duplex generated the complex DNA **1**: *bm*-PNA **1**:Ag⁺: *bm*-PNA **1**:DNA **1** and the CD showed appearance of a large negative band with maxima at 290 nm (Figure 12 curve **c**).

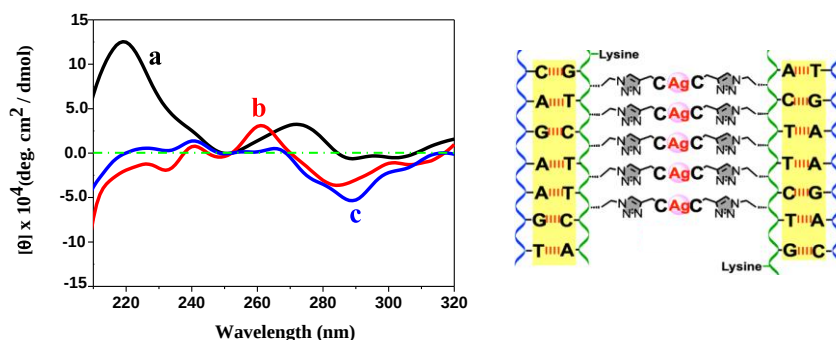


Figure 13. CD spectra of *bm*-PNA: DNA complexes, single stranded DNA **1** (a), duplex DNA **1**: *bm*-PNA **1** (b) and its complex with silver (c).

UV-*tm* studies of Ag⁺ mediated *bm*-PNA with all- C on amide face: triple duplexes of *bm*-PNA 2

It is seen that individual face duplexes showed a single transition in the UV-T plot, with T_m of 45.6 °C for triazole face DNA duplex (Figure 14a) and T_m of 67.1 °C for the t-amide face poly C silver complex (Figure 14b). In presence of Ag^+ , the DNA:bm-PNA duplex DNA 2:bm-PNA 2 dimerized with t-amide face all-C side chains held by Ag^+ to form a single complex comprising of three duplexes (Figure 14c) DNA 2:bm-PNA 2: Ag^+ :bm-PNA 2:DNA 2. This complex also exhibited double transition with T_{ms} at 38.4 °C and 75.4 °C.

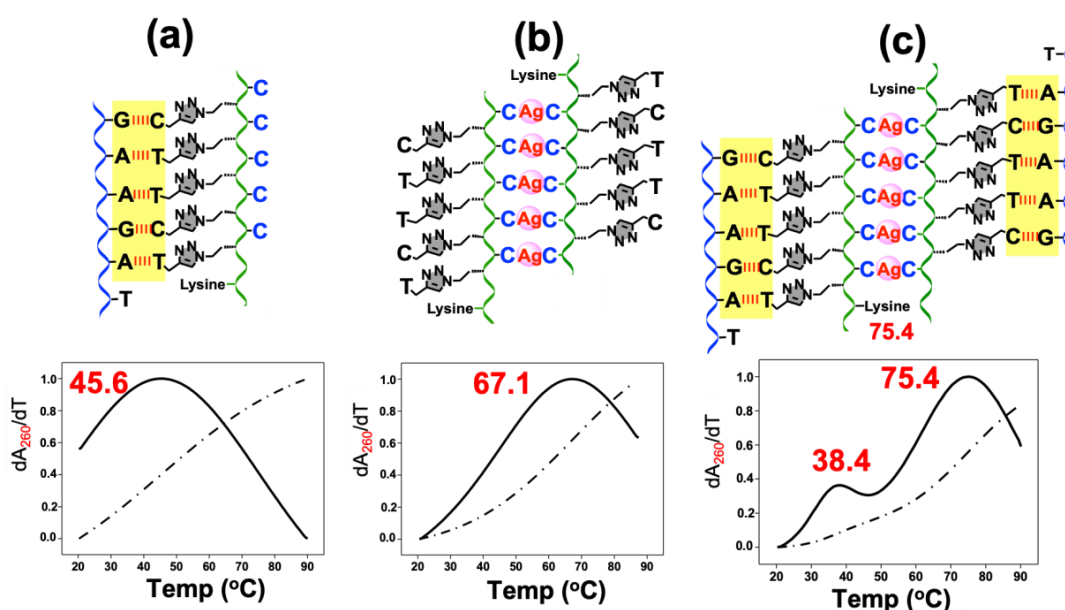


Figure 14 UV-T plots of *bm*-PNA **2** with poly C on t-amide face (a) duplex DNA **2**: *bm*-PNA **2** (b) silver complex [*bm*-PNA **2**]₂:Ag⁺ and (c) silver complex of duplex [DNA **2**: *bm*-PNA **2**]₂:Ag⁺.

CD studies of DNA 2: *bm*-PNA 2:Ag⁺: *bm*-PNA 2:DNA 2

The CD spectra of triple duplexes (Figure 15) are weak due to the fact that the two *bm*-PNA **2**:DNA **2** duplexes are perhaps oriented in opposite directions, mutually cancelling the signals. In conjunction with UV-T results, they support the formation of the double duplex with successive addition of *bm*-PNAs and AgNO₃ to complementary DNA **2**.

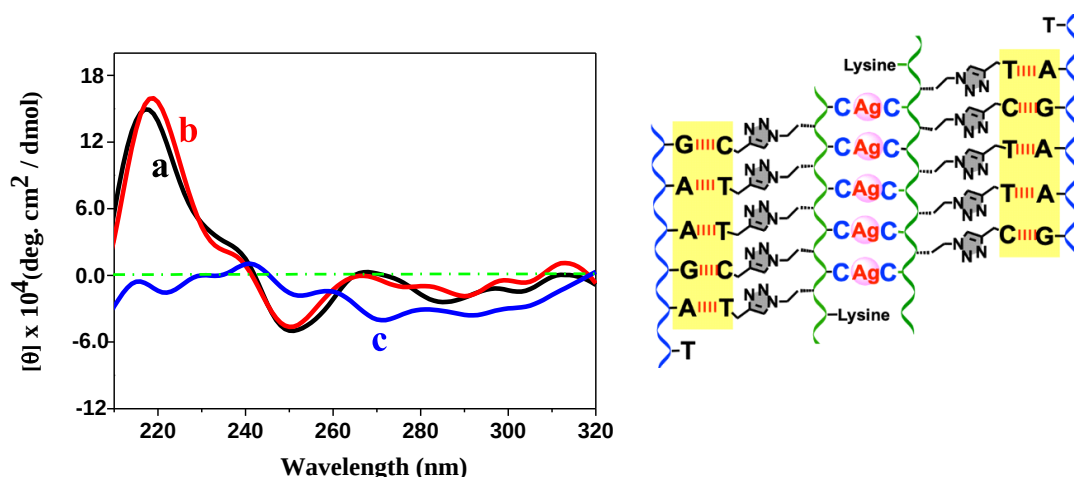


Figure 15. CD spectra of *bm*-PNA:DNA complexes, single stranded DNA **2** (a), duplex DNA **2**:*bm*-PNA **2** (b) and its complex with silver (c).

Morphological study by FESEM and elemental composition by Energy Dispersive X-ray Analysis (EDX)

The *bm*-PNA **1** alone showed well defined homo spherical nanoparticles of size 100 nm-200 nm (Figure 16A). When hybridized duplex DNA **1**: *bm*-PNA **1** the sample showed an irregular large sticky mass (Figure 16B). Upon complexation of *bm*-PNA **1** with Ag^+ , the resulting [*bm*-PNA **1**]₂: Ag^+ duplex exhibited alignment of spherical nanoparticles into a linear arrangement (Figure 16C). Upon hybridization of this silver complex with DNA **1**, the resulting complex [DNA **1**: *bm*-PNA **1**]₂: Ag^+ transformed into a larger conglomerate mass networked held through a high density fibrous network (Figure 15D) of spherical nanoparticles (Figure 16D). The presence of Ag^+ in the tetra-stranded complex was confirmed by EDAX data (Figures 16E and 16F).

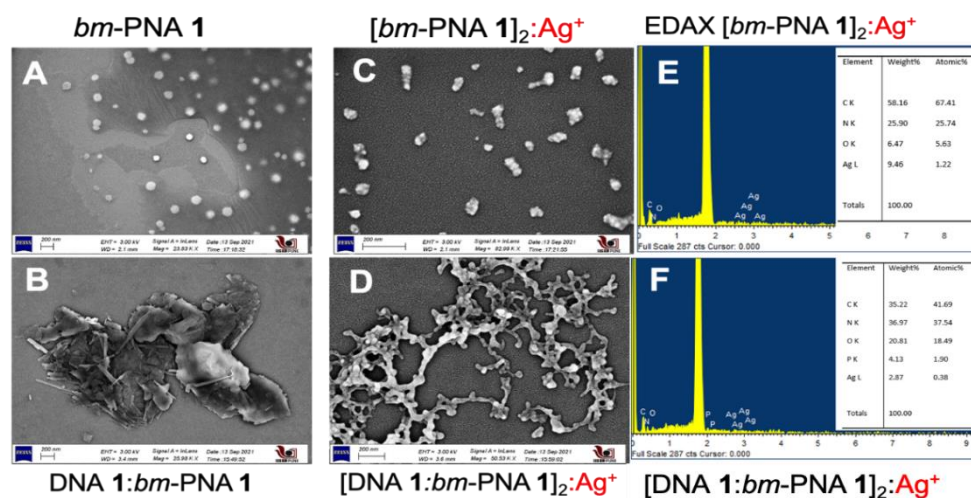


Figure 16. FESEM and EDAX of *bm*-PNA **1** complexes

Section B: Mercury mediated base pairing

Thermal stability of mercury mediated triple duplexes

The preformed WC duplex on t-amide face i.e DNA **1**: *bm*-PNA **1** (Figure 17a) was mixed with desired concentration of HgCl₂ solution. The UV-T plot of the resulting complex interestingly showed double sigmoidal transition with T_{ms} at 45.5 °C and 75.9 °C. This indicates formation of the double duplex of DNA **1**: *bm*-PNA **1**:Hg⁺⁺:*bm*-PNA **1**:DNA **1** (Figure 17b).

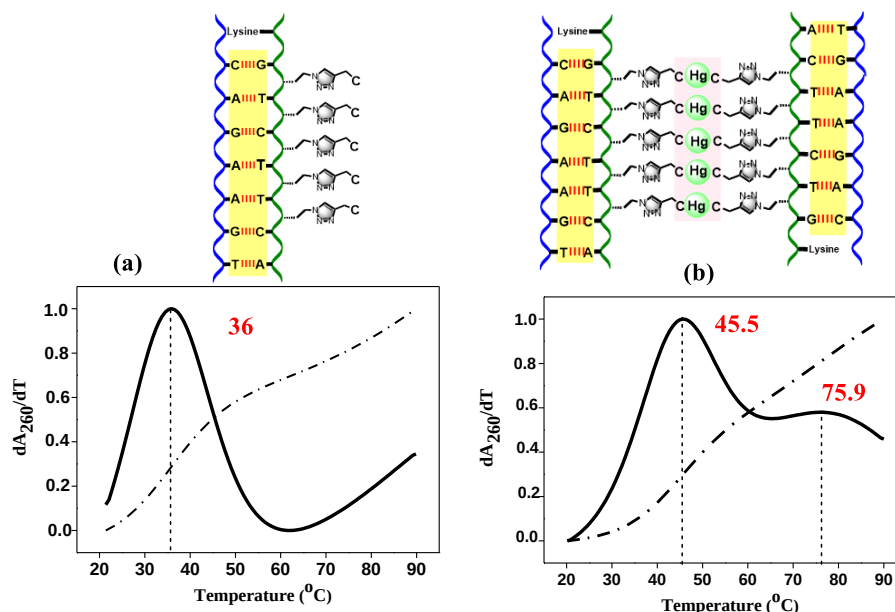


Figure 17 UV-T plots of *bm*-PNA **1** with all- C on triazole face (a) duplex DNA **1**:*bm*-PNA **1** (b) mercury complex of double duplex [DNA **1**:*bm*-PNA **1**]₂:Hg⁺⁺.

The triazole face mixed base sequence of *bm*-PNA **2** formed duplex with complementary DNA **2** of T_m 45.6 (Figure 18a) The amide face has free cytosines available for base pairing. After mixing with HgCl₂ solution, the resulting complex interestingly showed double sigmoidal transition in temperature dependent UV-T_m with T_{ms} at 46.1 °C and 78.6 °C. This indicates formation of the double duplex of DNA **2**: *bm*-PNA **2**:Hg⁺⁺: *bm*-PNA **2**:DNA **2** (Figure 18b).

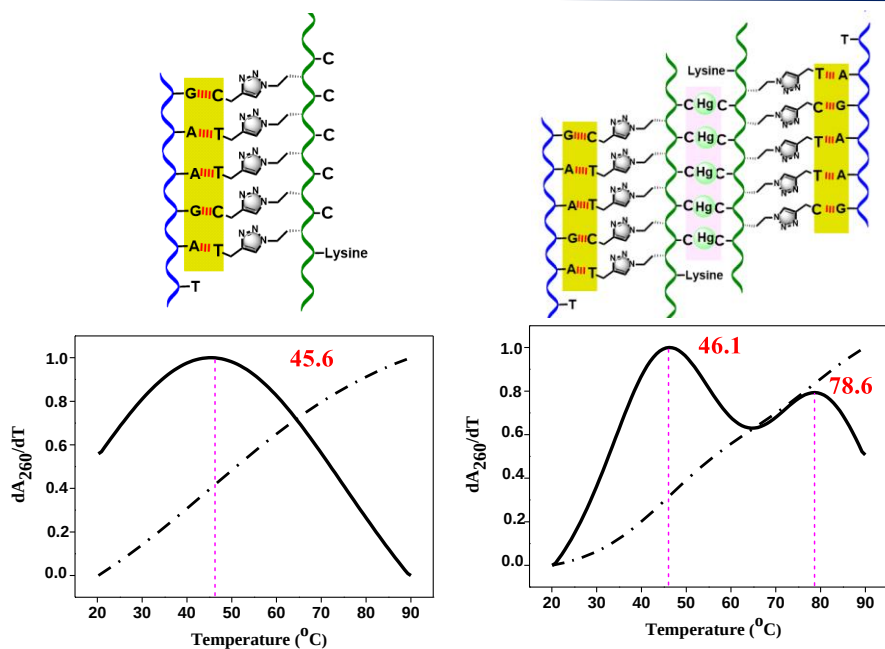


Figure 18 UV-T plots of *bm*-PNA 2 with all-C on t-amide face (a) duplex DNA 2: *bm*-PNA 2 (b) mercury complex of duplex [DNA 2: *bm*-PNA 2]₂: Hg⁺⁺.

Morphological study by FESEM

The duplex DNA 1: *bm*-PNA 1 the sample showed an irregular large sticky mass (Figure 19A). Upon addition of Hg⁺⁺ to the duplex, the resulting complex [DNA 1: *bm*-PNA 1]₂: Hg⁺⁺ transformed into a uniform particles (Figure 19B)

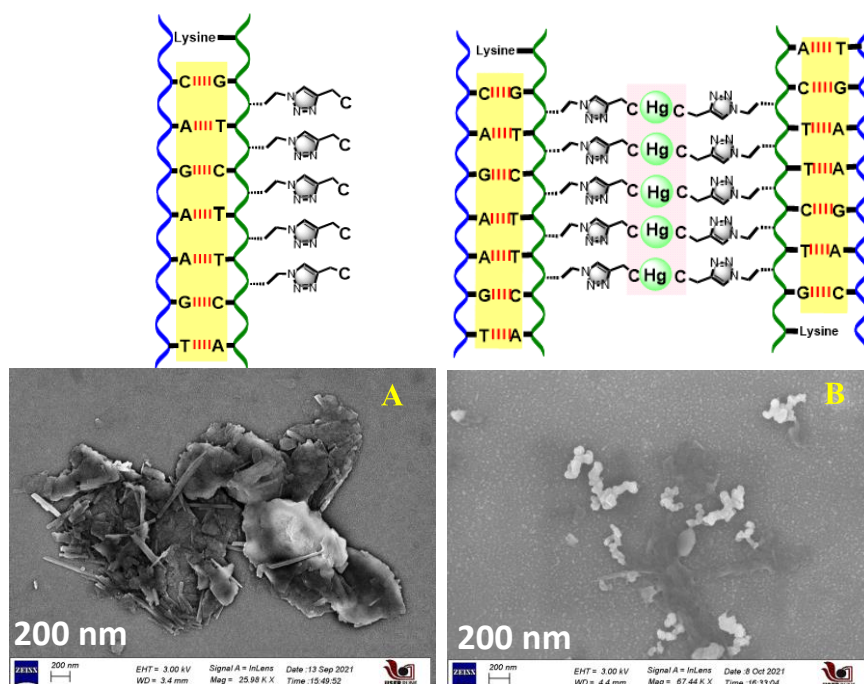


Figure 19. FESEM of DNA1: *bm*-PNA 1 complexes

This chapter conclusively demonstrated that *bm*-PNAs with all-C on triazole or t-amide face form metal mediated ($\text{Ag}^+/\text{Hg}^{++}$) duplexes, which in turn form duplexes with complementary DNA binding to mixed sequence on the other side to form triple duplexes.

Chapter 6: Synthesis and characterization of C5-Uracil substituted PNA monomers, their oligomerization and biophysical study

This chapter studies the effects of C5(F/CF₃/NH₂)-Uracil substituted PNA oligomers in the formation of derived PNA:DNA/RNA/PNA hybrids. In DNA, C5-modified deoxy uridine derivatives have been studied to improve the properties of nucleic acids such as the affinity to their complementary RNAs and the nuclease resistance. Similarly, to improve solubility, hybridisation properties, and cell membrane permeability PNA presents a versatile scaffold for chemical modifications on base, backbone and side chain (Figure 20).

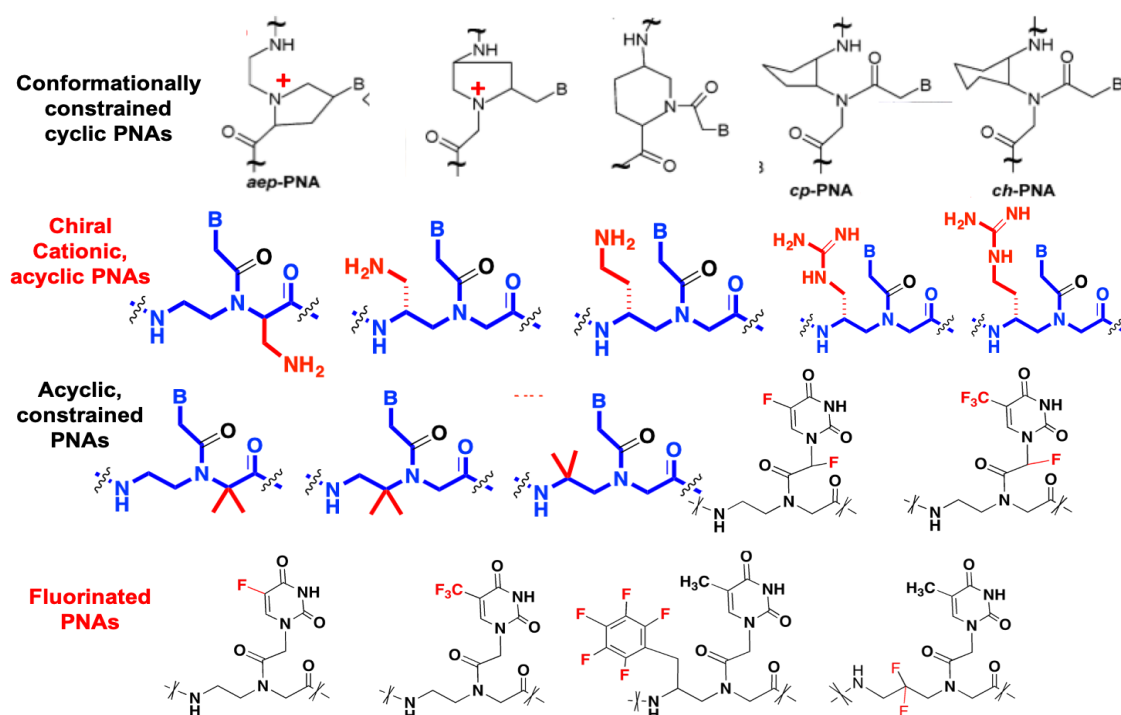


Figure 20. Some representative chemical modifications of PNA at backbone, sidechain and base from Ganesh *et al*¹⁸⁻²⁵

The C5-H/Me substitutions in pyrimidines Cytosine (C) and thymine (T) respectively are projected into the major groove of DNA in DNA:DNA duplexes and DNA:RNA duplexes and thereby alter the major groove characteristics and the stability of duplexes. While the effect of various C5 substitutions on DNA:DNA duplexes is well known, such is not the case with

PNA:DNA duplexes. In this context, the aim of this chapter is to investigate the effect of various, hydrophobic (H), hydrophilic (NH₂) polar hydrophobic (CF₃) substitutions on PNA:DNA/RNA duplex stability. This chapter presents synthesis of C5-modified uracil PNA monomers, their incorporation into PNA oligomers and complementation studies with DNA/RNA and PNA.

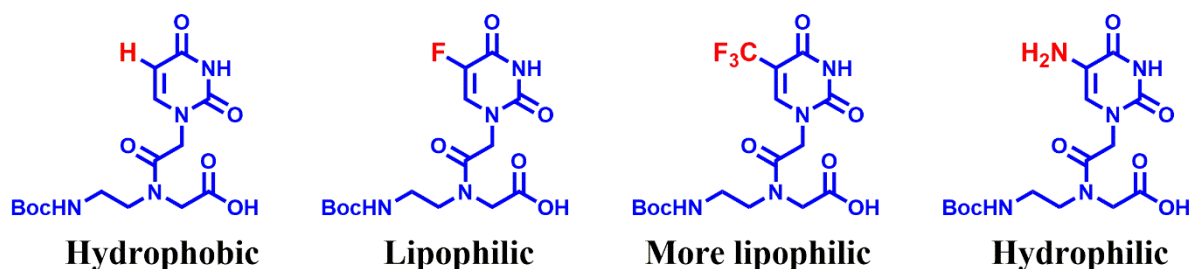
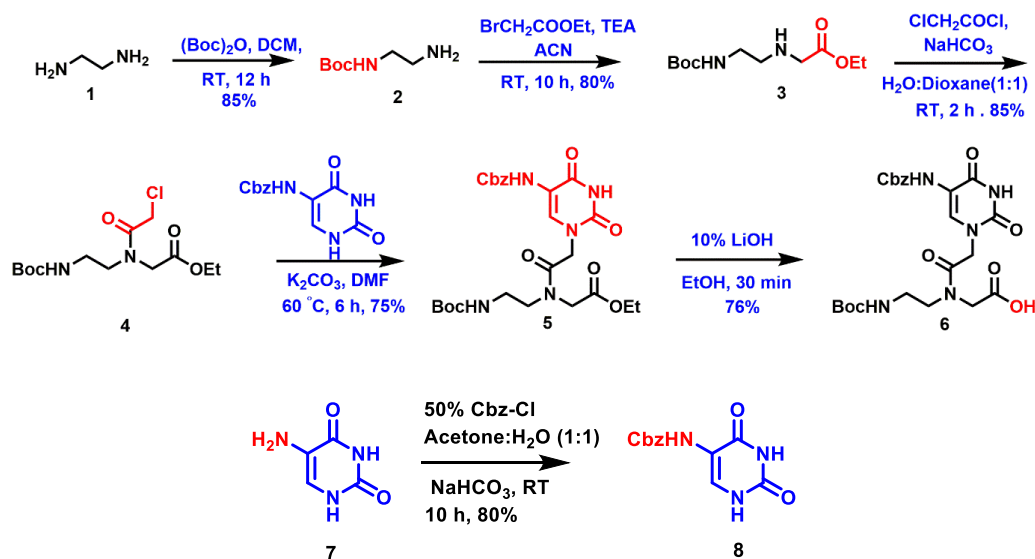


Figure 21 Various C5-substituted Uracil PNA monomers

Synthesis of ethyl N1-Boc-aminoethyl-5-NHCbz uracil glycine (5NHCbzU-*aeg*)²⁶

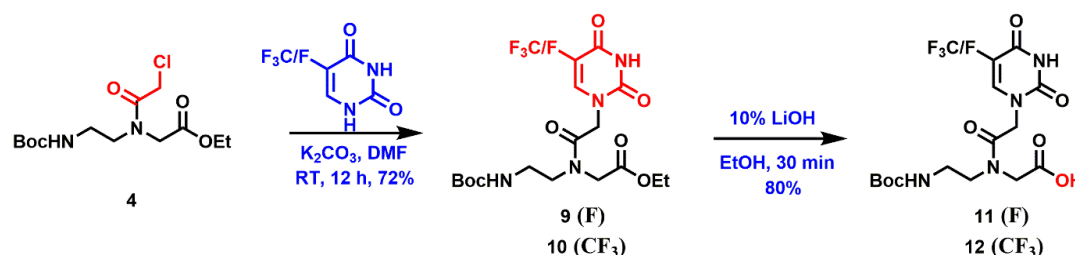
Ethelene diamine was treated with t-Boc anhydride in DCM to obtain mono NHBoc protected ethylenediamine (**2**) (Scheme 2.3). This compound was reacted with the ethyl bromoacetate to get N²-alkylated compound **3** which was acylated at N² with chloroacetyl chloride to obtain compound **4**. This compound was coupled with N⁵Cbz protected 5-aminouracil (**8**) which was synthesised from the commercially available 5-aminouracil (**7**) by treatment with 50% benzyl chloroformate (Scheme 6.2). The resulting *aeg* monomer ester **5** was hydrolysed with 10% aq. LiOH gave the desired acid monomer **6**.



Scheme 2.3. Synthesis of N5-Cbz-*aeg*-Uracil monomer **6**

Synthesis of N1(Boc)-N2(carbonylmethyl-N¹(5F/5CF₃ uracil) aminoethyl glycine (10, *aeg*-5F/CF₃-U)^{18, 19}

The ethyl [N1(Boc)-N2(chloroacetamide)] aminoethyl glycine **4** was reacted with commercially available 5F/5CF₃ uracil (Scheme 2.4) to give the ester compound **9,10**, which upon hydrolysis with 10% aq. LiOH gave the corresponding *aeg*-PNA acid monomer **11, 12**.



Scheme 2.4. Synthesis of C5-F/CF₃-uracil-aeg monomer 11/12

The synthesized PNA monomers were incorporated into the desired *aeg*-PNA oligomers (Table 3) by employing standard solid phase peptide synthesis protocol using *Boc* strategy.

Table 3 Synthesised PNA oligomers

PNA 1	CGACTTTCAG LysNH ₂
PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂
PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂
PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂
PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂
PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂
PNA 7	CTGAAAGTCG LysNH ₂

The thermal stability T_m of synthesized PNA oligomers with complementary DNA/RNA/PNA duplexes was examined by temperature dependent UV-spectroscopy and shown in Figure 22. From comparison of data (Table 4), it was observed that PNA:PNA duplexes are more stable as compared to PNA:DNA and PNA:RNA duplexes.

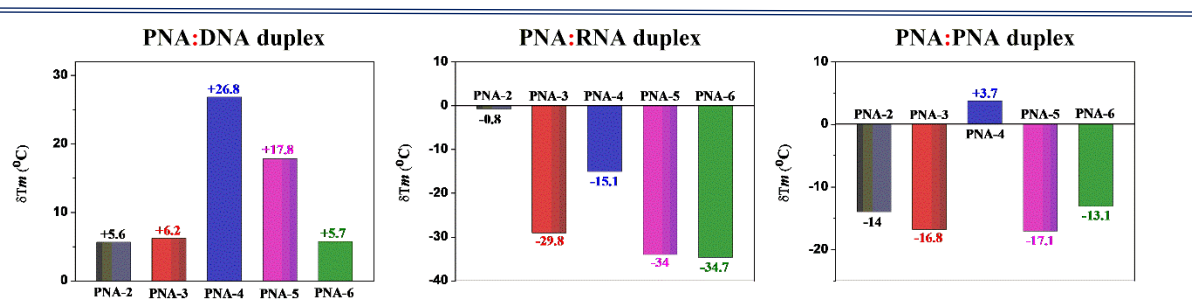


Figure 22. Comparison of thermal stabilities of various duplexes.

Table 4 Comparative UV T_m of PNA:DNA/RNA/PNA duplexes

Sr.No.	PNA code	C5 substitution	PNA:DNA	PNA:RNA	PNA:PNA
1	PNA 1	TTT	47.5	61.1	72.9
2	PNA 2	TU ^{NH₂} T	53.1	60.3	58.9
3	PNA 3	TU ^{NH₂} U ^F	53.7	32	56.1
4	PNA 4	U ^F U ^{NH₂} U ^F	74.3	46	76.6
5	PNA 5	TU ^{NH₂} U ^{CF₃}	65.3	27.1	55.8
6	PNA 6	U ^{CF₃} TU ^{NH₂} U ^{CF₃}	53.2	26.4	49.8

The overall results clearly suggested that the C5-U substituents incorporated PNA oligomers stabilise the derived PNA:DNA hybrids while destabilised the corresponding PNA:RAA/PNA duplexes.

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Chapter 1

Introduction to *bimodal* Peptide Nucleic Acids

1.1 Introduction

DNA is the basic hereditary material present in almost all living organisms and contains all information necessary to make proteins.¹ It has been 68 years since Watson and Crick elucidated the double-helical structure of DNA (Figure 1.1). DNA is a polymer made up of repeating units of nucleotides. Each nucleotide unit consists of a nitrogenous base, a deoxyribose sugar, and a phosphate backbone. The main chemical constituents of DNA are the (i) sugar-phosphate unit present on the outer side of the helix which constitutes the backbone of each strand and (ii) the nitrogenous bases adenine (A), thymine (T), guanine (G) and cytosine (C) which are pointed towards the centre of the helix. Each base is connected to a sugar via a β -glycosyl linkage. Hydrogen bonds between complementary base pairs (A:T; G:C) holds the two strands together (Figure 1.1). The molecular architecture of DNA consists of a double stranded helix of uniform diameter, with right-handed twist. RNA contains ribose rather than deoxyribose sugar with the base composition adenine (A), uracil (U), guanine (G) and cytosine (C).

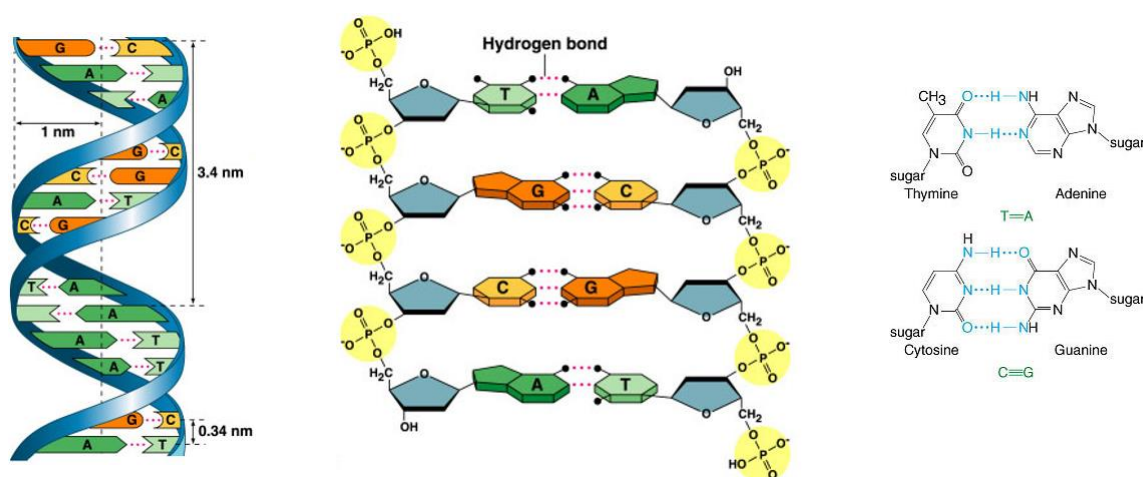


Figure 1.1 Structure of DNA double helix. (<https://plantlet.org/the-discovery-of-dna-double-helix-structure-watson-cricks-model/>)

The DNA double helix is nature's simple and elegant solution to the problem of storing, retrieving, and communicating the genetic information of living organism.² The specificity and the reversibility of the hydrogen bond formation between the complementary nucleobases is one of the most important characteristic features of DNA double helix. It allows the strands of the double helix to unwind and rewind in exactly the same configuration. Chemists are fascinated by DNA after the elucidation of its double helix and remarkable properties, governed by its structure. Tremendous efforts have been made to understand its structure, function and applications in biology as well as physicochemical and material sciences.³ The construction of DNA and design of its analogues for use in the recognition of specific DNA and RNA sequences has emerged as intellectual and practical assignment for synthetic and structural organic chemists. The recognition of DNA and RNA sequences by complementary oligonucleotides is a central feature of biotechnology and is vital for hybridization based biological applications. The study of such complementary recognition is possible with widely used experimental techniques and diagnostic protocols.

1.2 Base pairing in nucleic acids

The nucleobases are the key components that converse chemical variability to DNA/RNA. Watson and Crick recognized the complementarity in hydrogen-bonding capability of A:T and G:C base pairs during DNA model-building studies in 1952. The nucleobases have complementary hydrogen bond donor and acceptor sites that generate

specific associations between bases. Association via base pairing is normally seen between purines (Guanine, Adenine) and pyrimidine bases (Thymine, Cytosine, and Uracil), utilizing the basic Watson–Crick base pairing motif.¹ The G:C base pairing, with its three hydrogen bonds is more stable than the A:T/A:U base pairing with only two hydrogen bonds. The mutual recognition of A by T and of C by G using hydrogen bonds establishes the fidelity of DNA transcription and translation. The N-H groups of the bases are potent hydrogen donors (D), while the sp²-hybridized electron pairs on the oxygen of the base C=O groups and nitrogen on the ring, are much better hydrogen bonding acceptors (A) than the oxygen of either the phosphate or the pentose. The acceptor (A): donor (D) hydrogen bonds so formed have partial ionic character.

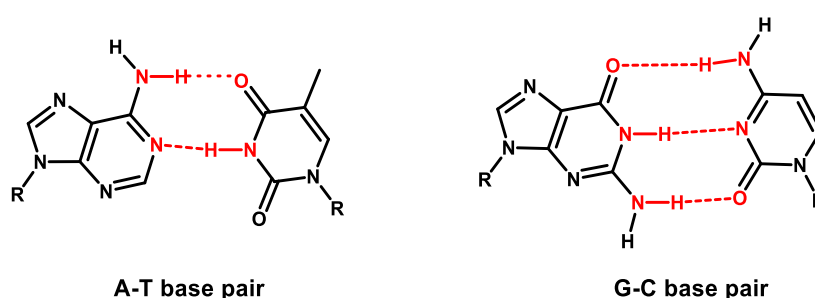


Figure 1.2. Watson-Crick base pairin through hydrogen bonding.

While Watson–Crick base pairing is found to be the dominant pattern, other base pairings also have been suggested of which the most significant to be identified are Hoogsteen base pairs⁴ and Crick ‘wobble’ base pairs.⁵ Hoogsteen base pairing allows binding of pyrimidine third strand in the major groove of purine: pyrimidine duplexes to form triple helical structures in 1:2 stoichiometry of [poly(dA):poly(dT)₂] and [poly(rG): poly(rC)₂]. The triplexes do occur in DNA and RNA in polypurine:polypyrimidine stretches in many regulatory sequence regions.

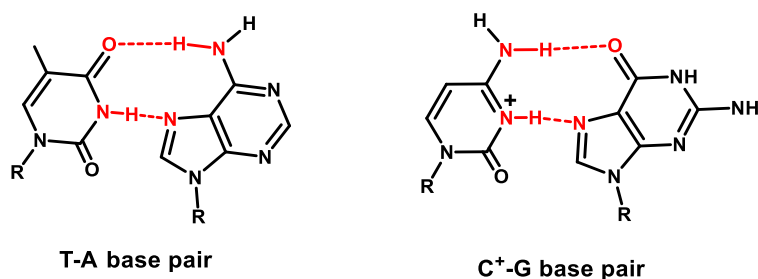


Figure 1.3 Hoogsteen hydrogen bonding

Francis Crick proposed the hypothesis for the existence of ‘wobble’ base pairings to explain the degeneracy of the genetic code.⁵ This hypothesis explains that only the first two

bases of the codon of tRNA have a precise pairing with the bases of the anticodon of mRNA, whereas the pairing between the third bases of codon and anticodon may Wobble (non-specific). In Wobble base pairing (Figure 1.4), a single purine base is able to recognize different pyrimidines (e.g. I:U and I:A where I = inosine, U = uracil, A = adenine). This has importance in the interaction of messenger RNA (*m*-RNA) with transfer RNA (*t*-RNA) on the ribosome during protein synthesis (codon-anticodon interactions). Several mismatched base pairs and anomalous hydrogen bonding patterns have been seen in X-ray studies of synthetic oligodeoxynucleotides.⁶

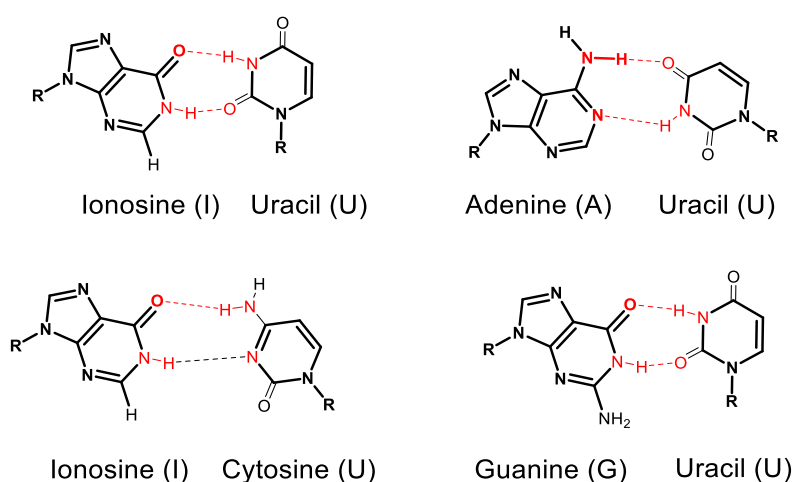


Figure 1.4 Wobble base pairs through hydrogen bonding

In addition to biological functions, nucleic acids ability to function as the carrier for storage and transfer of information has been extensively utilized recently for the creation of nanostructures and molecular electronics.⁷ In this context, nucleic acids and particularly DNA provides a promising tool for bottom-up fabrication of inorganic and bioorganic molecular devices. The DNA sequences can be designed to self-assemble into different types of architectures such as duplexes, triplexes, quadruplexes and nanostructures. Several attempts have been made to exploit and extend these properties of DNA by modifying backbone and nucleobases for various applications in both biological and nanomaterials chemistry. Keeping the fundamental design of the DNA double helix intact, several groups⁸ have studied replacement of the natural base pairs of DNA molecule with ligands that show pronounced affinity to bind to metal ions.

1.3 Metal mediated base pairing

It is well known that hydrogen bonding contributes significantly to folding and assembly of biomolecules through the intra- and intermolecular recognition. Metal coordination also plays vital role in maintaining and stabilizing biologically relevant structures in terms of molecular recognition. In this context, it has been of interest to see if natural base pairing can be replaced by metal mediated base pairing, which may provide valuable insights into the origin of chemical evolution that might involve both inorganic and organic compounds.⁹

Metal mediated base pairs have an advantage in comparison with unnatural bases since metal ions can be incorporated based on their unique physical and chemical properties, altered hydrogen bonding pattern and shape complementarity.¹⁰ Accordingly, rational selection of metal ions would enable appropriate adjustment of the coordination properties of metallo-base pairs, such as their selectivity and structures as well as thermodynamic stability. Simplest idea behind metal coordinate base pairing was to replace hydrogen atom within hydrogen bonded base pairs by a metal ion since it can accept lone pair of electrons on heteroatom (Figure 1.5). Lee et al.¹¹ in 1993 found that Zn^{2+} ion stabilizes the DNA duplexes at elevated pH at which hydrogen atoms involved in hydrogen bonding were released and metal ions were bound to the nucleobases. Another idea was to convert natural mismatched pair into a metal-coordinated base pair. It is well known that T-T mismatch is converted to a stable metallo-base pair in the presence of Hg^{2+} ions as originally proposed by Katz¹² and further studied by Marzilli et al.¹³ Such metal mediated base pairing is being used for metal responsive functional DNA molecules including allosteric DNazymes¹⁴ and logic gates.¹⁵

Although the idea of replacing the hydrogen atom of H-bond by metal ions is simple, it would inconveniently disorder the complementarity rule of natural base pairing. Thus, such metal coordinated base pairs could not become orthogonal to natural base pairs. This drawback tempted researchers to develop a novel metal mediated base pairing system by using synthetic ligand type nucleobases. The artificial metal coordinated base pairing is designed to match the structural requirement like size and shape of the canonical base pairs. Most important thing for ideal metallo-base pairing is that the central metal ion should be chosen in such a way that not to disturb the duplex structure to great extent.¹⁶

A strategy for metal ion incorporation into nucleic acid duplexes first proposed by Tanaka and Shionoya¹⁷ involves the replacement of the natural nucleobases by ligands that have a higher affinity for metal ions than the natural nucleobases. The metal complexes of

ligand-modified nucleic acids can mimic as alternative base pairs. If the ligands are aromatic and the geometry of the metal complex is planar, the complexes can participate in π -stacking interactions with adjacent nucleobase pairs. Sequences can be designed in a way to assemble into different types of induced architectures such as duplexes, triplexes, and a variety of branched nanostructures.^{18, 19} In the past decade, numerous ligands have been introduced into DNA oligomers.¹⁶

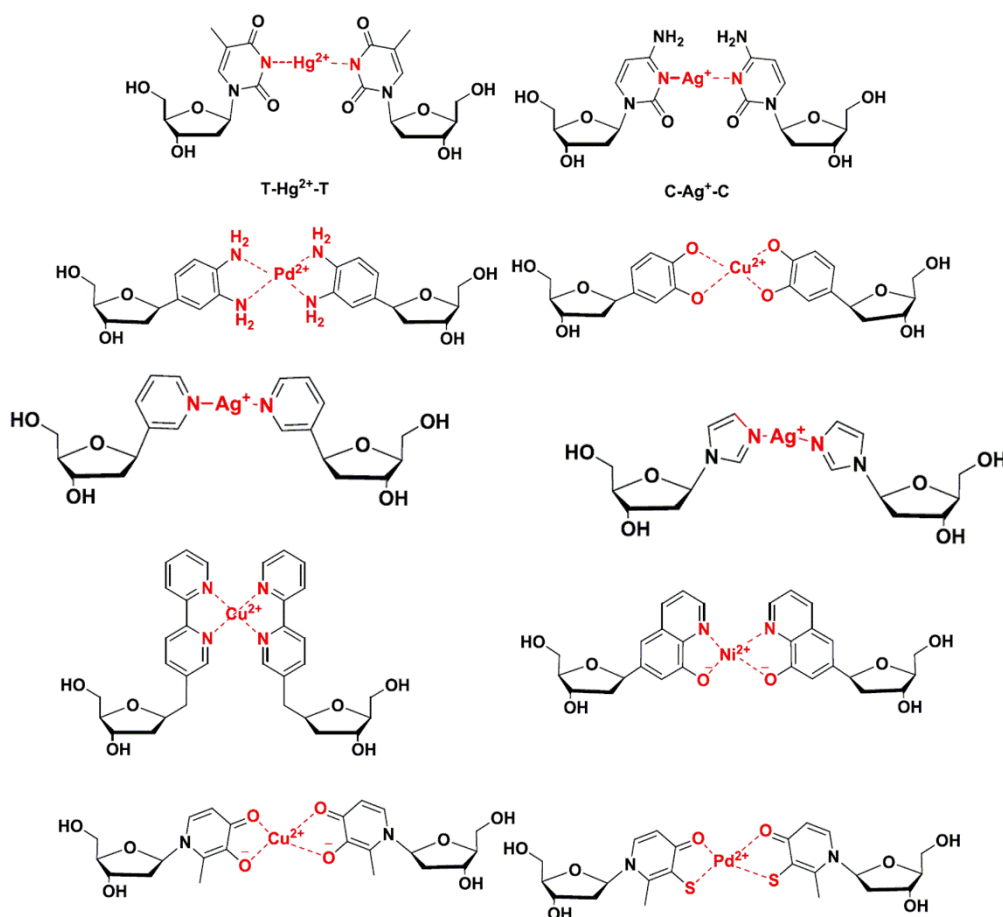


Figure 1.5 Metal mediated base pairs¹⁶

1.4 Secondary structures of nucleic acids

DNA exists in various possible conformations like A-DNA, B-DNA, and Z-DNA (Figure 1.6) although, the most common is the B-DNA which is a right-handed double helix.^{20, 21} It has a wide and deep major groove with a narrow and shallow minor groove wherein the bases lie perpendicular to the helical axis. A-DNA also forms a right-handed helix where the major groove is deep and narrow while the minor groove is broad and shallow. In both A and

B forms of DNA, the Watson-Crick base pairing is maintained along with *anti*-glycosidic conformation. Z-DNA is a left-handed double helix and most favoured in alternating G-C sequences. The left-handed helix for Z-DNA is a result of a switch in the glycoside bond with a *syn* conformation (Figure 1.7). The sugar conformation is however different in both forms, with the B-DNA form showing C2'-*endo* puckering and the A-DNA form exhibiting C3'-*endo* sugar-pucker (Figure 1.7).

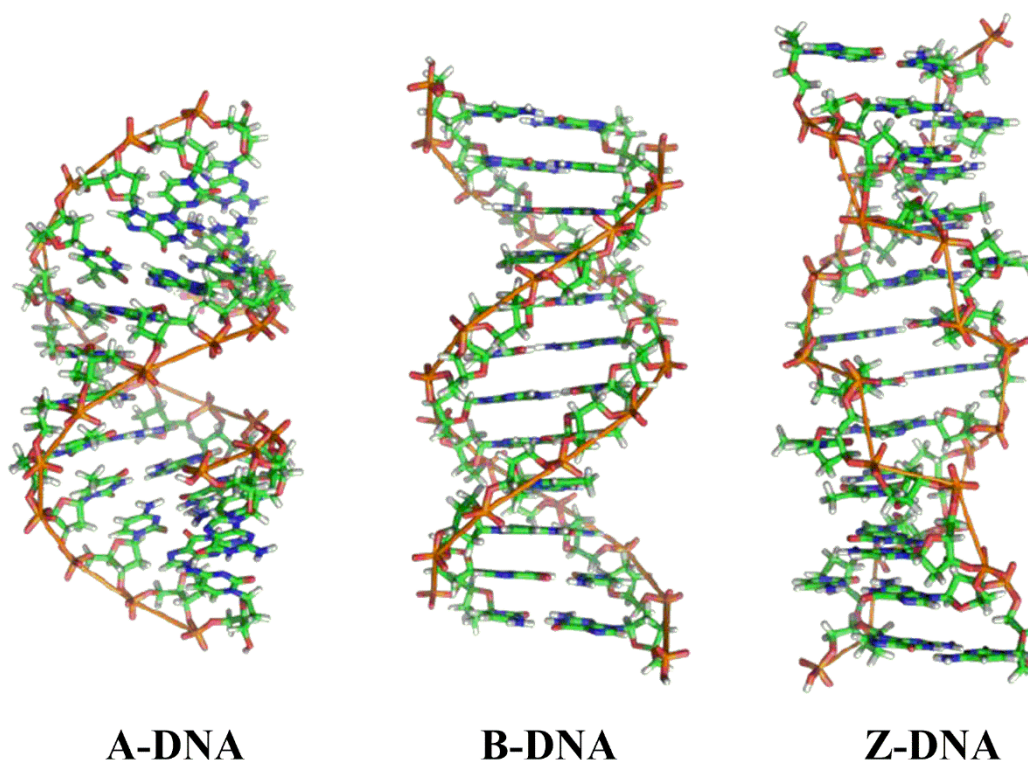


Figure 1.6 Molecular models of A-DNA, B-DNA and Z-DNA^{20, 21}

RNA has greater structural versatility than DNA in the variety of its structure, resulting in diversity of conformations and chemical recognition. The presence of the 2'-hydroxyl group in RNA hinders the formation of a B-form helix and it acquires the A-type helix showing C3'-*endo* sugar puckering. More commonly, RNA is single-stranded, can form complex and unusual shapes such as stem and bubble structures, which occur due to the intrachain base pairing. An example is *t*-RNA, the key molecule involved in the translation of genetic information to proteins.

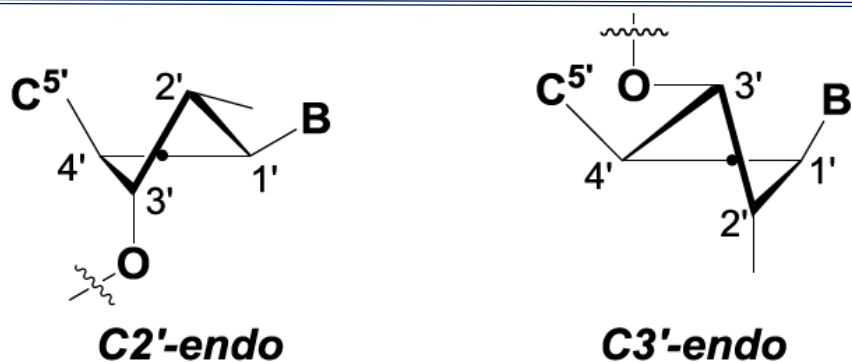


Figure 1.7 Structures of *C2'-endo* and *C3'-endo* sugar pucker²¹

1.4.1. DNA G-quadruplex

Guanine-rich DNA sequences can fold into stable intramolecular and intermolecular four-stranded non-B DNA structures called G-quadruplexes (G4s) (Figure 1.8). G4s arise via cyclic interaction at their Hoogsteen hydrogen sites of four guanines arranged within a planar quartet (guanine quartet; G-quartet). G-quadruplex structures that is further stabilized by monovalent cations, with a strength of stabilization in the order ($K^+ > Na^+ > NH_4^+ > Li^+$).²² G4 formation has been observed in synthetic oligonucleotide sequences derived from the human genome, particularly those from gene promoters and telomeres. Such studies, together with chemical biology approaches using G4-targeting small molecules or antibodies and computational predictions, suggest that G4s may be important in gene regulation and telomere biology.

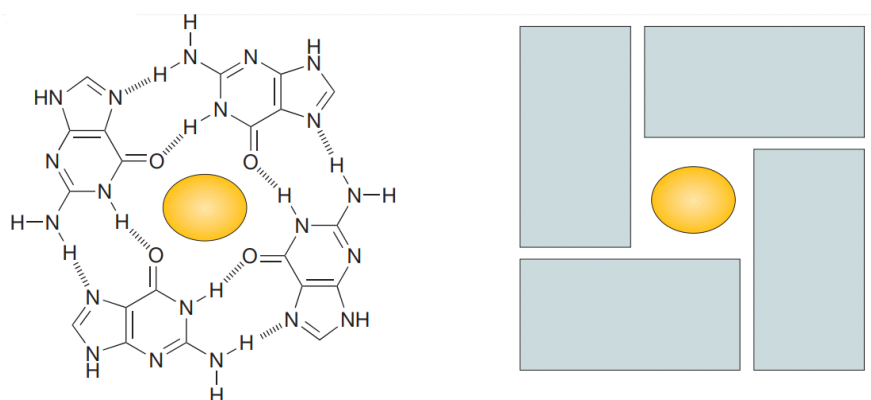


Figure 1.8 G-quartet structure

Classification of G-quadruplexes:

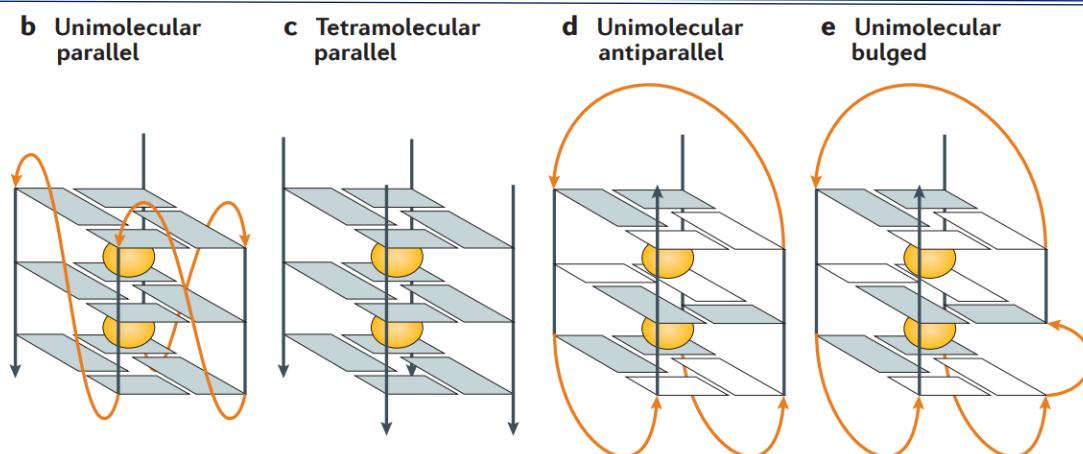


Figure 1.9 Types of G-quadruplex structures²²

1.4.2. DNA *i*-motif

DNA having poly cytosine sequences form hemi-protonated base pairs at acidic pH. Gueron et.al.²³ first discovered the DNA oligomer 5' -d(TCCCCC) at acidic pH forms interdigitated four stranded *i*-motif structures. Under acidic pH condition protonation of N3 takes place in cytidine (Figure 1.10). Due to three hydrogen bonds in C:C⁺ base pair the stability of *i*-motif is higher than the neutral C:C base pair.

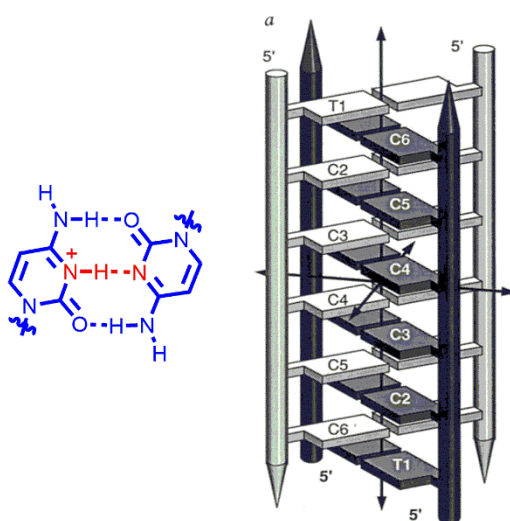


Figure 1.10 *i*-motif structure of DNA dTC₅²³

1.5 Applications of nucleic acids

Sequence-specific binding of oligonucleotides to single-stranded RNA or duplex DNA through triple helix formation provides a way to modulate the gene expression. The central feature of biology is the recognition of DNA and RNA sequences by complementary oligonucleotides and it is important for hybridization based biological applications. The study

of such complementary recognition is possible with widely used experimental techniques and diagnostic protocols. The conjugation or attachment of reactive functional groups to oligonucleotides has been investigated to cleave nucleic acids in a sequence specific way which is described as artificial nucleases.^{24, 25} The combination of specificity and simplicity makes oligonucleotides highly attractive as diagnostic and research tools.

1.5.1 Oligonucleotide analogue as therapeutic agents

The classical approach of designing drug molecules against protein target requires a deep understanding of 3D structure of the protein, binding site and the binding forces. Since very little is known about the process of protein folding and the lack of generality, this approach of drug discovery has various drawbacks. On the other hand, the nucleotide sequence in RNA and DNA is universal and the understanding of their structures is to design better drugs for nucleic acid targets. In principle, one can design drugs that are like nucleic acids, repetitive in its primary structure and bind sequence specifically to the nucleic acid drug targets. In order for the sequence specific recognition to happen, the drug should contain nucleobases which are fundamental units of nucleic acid recognition. In short, small pieces of oligonucleotides containing 15-25 nucleotides can act as a drug which would bind to target nucleic acid and stop further processing of protein production. Two innovative strategies are being tested for inhibiting the production of disease related proteins using such sequence specific DNA fragments as gene expression inhibitors.

The first strategy known as *antisense strategy*²⁶ aims to selectively impede translation by inhibiting the protein synthesis. Antisense oligonucleotides (modified DNA / RNA) recognize a complementary sequence on target mRNA through Watson-Crick base pairing and form a RNA:RNA/DNA duplex which is not recognized for further processing by protein synthesis machinery. Thus, it will retard the expression of corresponding disease related proteins (Figure 1.11). In the second strategy which is known as *antigene strategy*, the production of unwanted proteins can be stopped by selectively inhibiting the transcription process. In this strategy, oligonucleotides target the major groove of DNA where it winds around the double helical DNA to form triplex involving Hoogsteen hydrogen bonds (Figure 1.11).

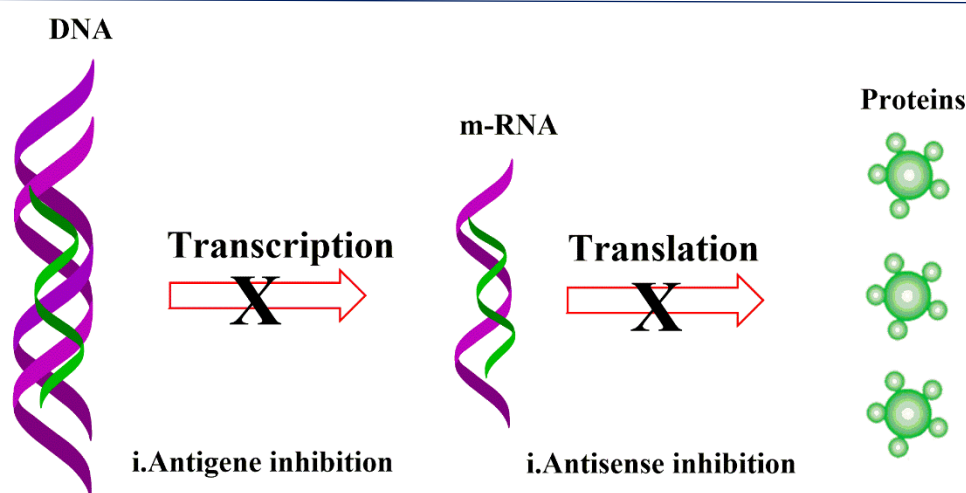


Figure 1.11 Principle of antisense and antigene oligonucleotides^{26, 27}

1.5.2 Antisense oligonucleotides

The principle of antisense drugs is based on the direct utility of sequence information and sequence-specific recognition of nucleic acids and hence such drugs have the potential for the treatment of several viral and bacterial infections, cancer and inflammatory or genetic disorders. Various cellular processes can be inhibited depending on the site at which the antisense oligonucleotide hybridizes to the target nucleic acid. Zamecnik and Stephenson²⁸ discovered that viral replication in cell culture was inhibited by oligonucleotides (ONs) and these ONs have the potential to act as antisense agents for therapeutics. There are several pre-requisites for oligonucleotides to serve the purpose of inhibiting translation and thereby act as antisense therapeutic agents.²⁹ These include

- i) Ease of synthesis in bulk quantities
- ii) *In-vivo* stability to cellular degrading enzymes
- iii) Ability to penetrate the cell membrane
- iv) Retention by the target cells
- v) Ability to interact with their cellular targets (DNA/RNA)
- vi) No non-specific interaction with other macromolecules.

Although the requirements for specificity and binding affinity are satisfactorily met by the unmodified oligonucleotides, their susceptibility to hydrolytic enzymes (nucleases) and poor cell permeability are the major obstacles for their use as nucleic acid therapeutic agents. In

order to meet all the above-mentioned requirements, it is necessary for ONs to be chemically modified in a suitable manner.

1.6 Chemical modifications of DNA

To improve the rate, affinity, and specificity of oligonucleotide recognition, while enhancing the cell membrane permeability and resistance to nuclease degradation, several chemical modifications of DNA have been attempted³⁰ in literature (Figure 1.12).

In general, three types of chemical modifications of DNA can be distinguished as

- Modified phosphate linkages
- Modified sugars and
- Altered sugar-phosphate backbone.

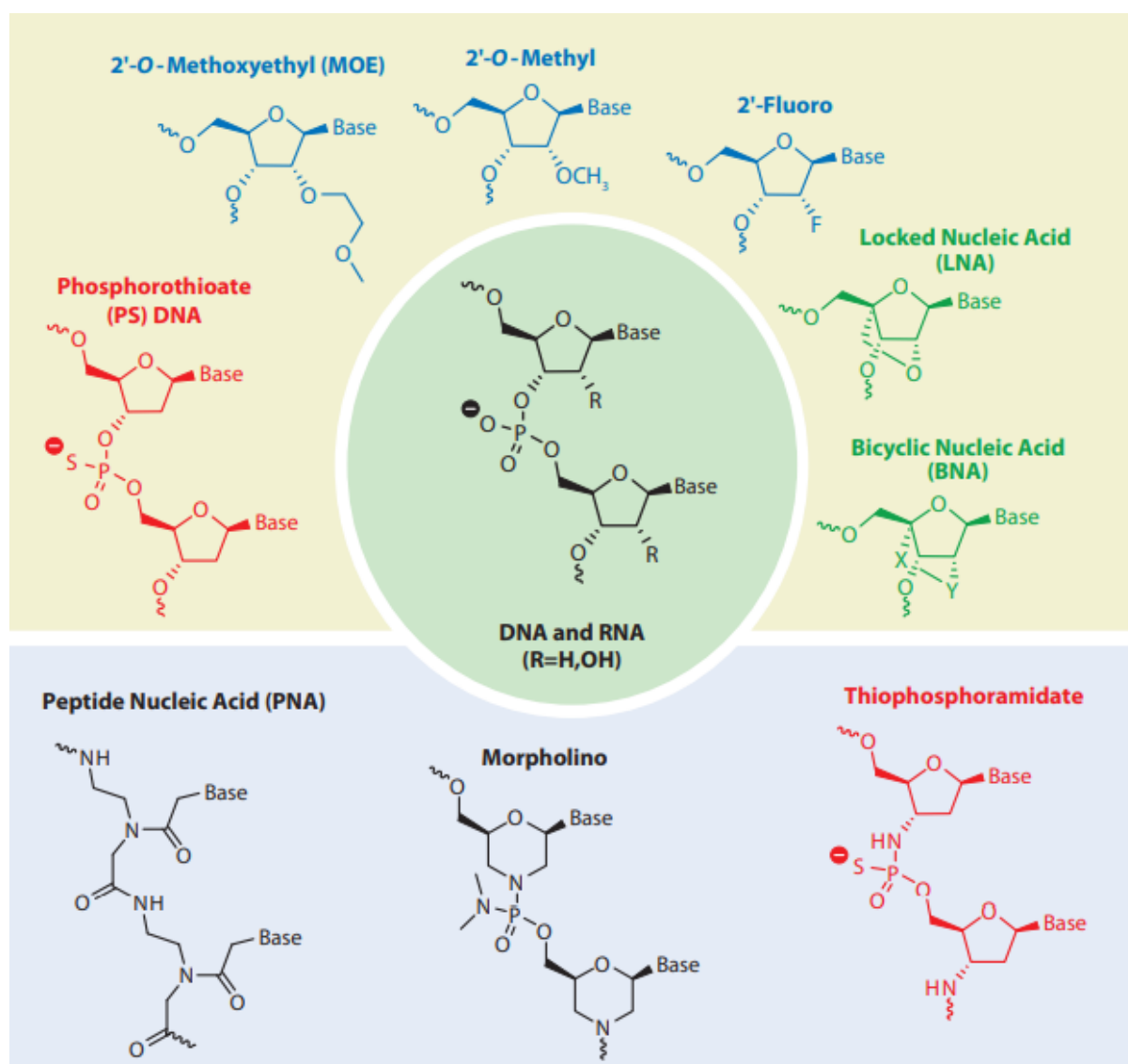


Figure 1.12 Examples of chemical modifications of oligonucleotides³⁰

1.6.1 Phosphate backbone analogues

The modifications of phosphate moiety resulted in the development of (i) phosphorothioates³¹ (ii) methyl phosphonates³² (iii) phosphoroamidates³³ (iv) phosphotriesters³⁴ and (iv) borano phosphonates³⁵⁻³⁷ (Figure 1.13). Phosphorothioate (PS)-containing oligonucleotides differ from natural nucleic acids in that one of the nonbridging phosphate oxygen atoms is replaced with a sulphur atom. Phosphorothioate oligonucleotides are one of the earliest and most widely used backbone modifications for antisense drugs. The substitution of sulphur for oxygen in PS oligos greatly increases stability to nucleolytic degradation. PS-oligos are able to efficiently elicit the RNase H cleavage of target mRNA, which is critical in the mechanism of action of antisense oligonucleotides.²⁹ In comparison to the corresponding chiral phosphorothioates, the achiral phosphorodithioate can be easily synthesized on a commercial DNA synthesizer,^{26,38,39} but the avidity of this analogue for the complementary DNA was lower than that of natural oligonucleotides. The non-stereospecific synthesis of chiral (*R/S*) phosphorothioates results in the formation of a mixture of diastereomers, with the conformational heterogeneity leading to lower melting temperature. As a consequence, the search for a stereocontrolled synthesis has been undertaken towards obtaining all-*S* or all-*R* analogues.^{40,41} The synthesized all-*R* chiral analogue of PS-oligos were used for the biological studies of antisense targets inside living cells. The PS-oligos complexes with RNA are substrates for RNase H degradation and these first-generation antisense agents have been extensively tested in human clinical trials. The first antisense agent approved by FDA (Vitravene) is based on PS-oligos. However, PS-oligos have tendency to induce non-specific effects, through binding to cellular and extracellular proteins⁴² as well as cleavage of non-target *m*-RNAs that are only partially complementary by RNaseH,⁴³ Replacement of both non-bridging phosphodiester oxygens leads to non-chiral phosphorodithioate which exhibit less non-specific protein binding and more resistance to nuclease. The binding of PS-oligos to certain proteins, particularly those which interact with polyanions such as hairpin-binding proteins, prove to be their major drawback.⁴⁴⁻⁴⁶

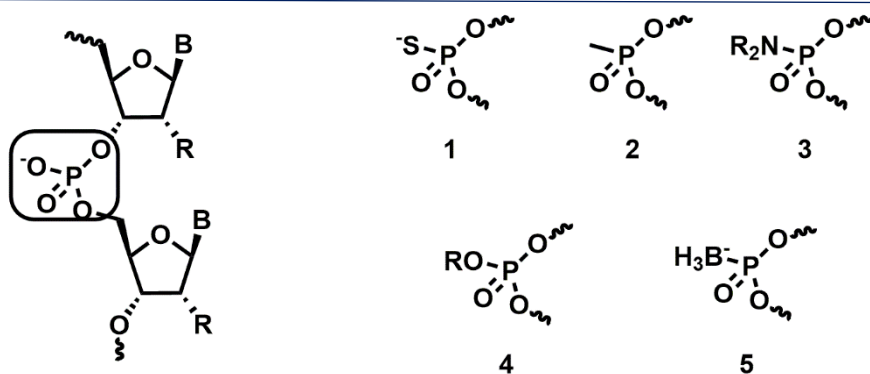


Figure 1.13 Structure of alternative phosphate linkages

Other backbone modifications (methyl phosphonates, phosphoramidites etc.) of oligonucleotides have been less successful at improving the oligonucleotides properties. In methyl phosphonates, one of the nonbridging oxygen atoms is replaced with a methyl group and is neutral in charge.³⁰ Although it provides high nuclease resistance, it does not induce RNase H activity. Additionally, increasing the number of methyl phosphonate units in an oligomer leads to loss of affinity towards the target mRNA and to poor water solubility.

1.6.2 Sugar modifications

This class of ONs is represented by nucleotides with alkyl substitutions at the 2'-position of ribose sugar (Figure 1.13). Organization of the sugar into RNA-like *C3'-endo* pucker increases the binding affinity of these ONs towards the complementary RNA.⁴⁷ Furthermore, the 2'-substituent in an oligonucleotide increases the steric bulk near the 3'-phosphate and makes it difficult for degrading enzymes to cleave the phosphodiester bond. The increase in binding affinity of 2'-modified ONs is energetically driven by the electronegative substituent at the 2'- position. Among all 2'- modified ONs, 2'-fluoro modification (Figure 1.14a) imparts the highest binding affinity towards the target RNA. 2'-O-Methyl (Figure 1.14b) group enhances the binding affinity to a lesser extent than do the 2'-fluoro modification but impart a substantial degree of nuclease resistance to the corresponding oligonucleotide. 2'-O-Methoxyethyl⁴⁸ (MOE) (Figure 1.14c) is currently the most advanced in the 2'-modified series and many antisense drugs having this modification have entered clinical trials. Unfortunately, the lack of RNase H activity restricts the use of 2'-modified oligonucleotides for antisense purpose. To overcome this drawback, the gapmer strategy has been used where regions of 2'-modified residues flank a central DNA region of the oligonucleotide.³⁰

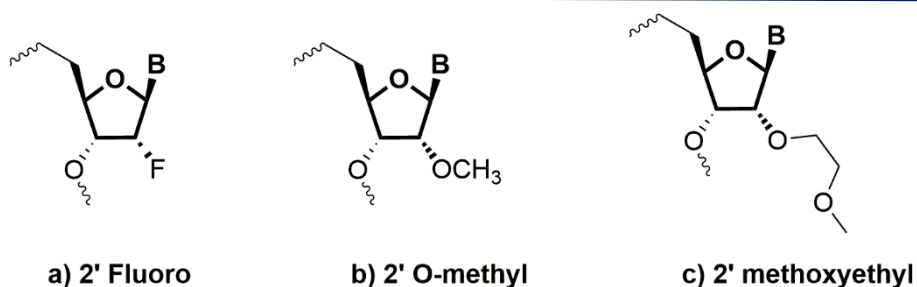


Figure 1.14 2'-modified oligonucleotides

1.6.3 Sugar-phosphate backbone modifications

In addition to phosphodiester and sugar modifications, replacement of the sugar phosphate backbone with isosteric structures has been devised. The concept of conformational restriction has been used widely to enhance binding affinity and biostability. Some of the DNA and RNA analogues developed with modified sugar phosphate backbones are described below.

1.6.3a Locked nucleic acid (LNA). These oligonucleotides developed by Wengel's group and Imnishi's group,^{49, 50} in 1998 are one of the most promising class of chemically modified ONs. These analogs are bicyclic systems that contain a methylene bridge that connects the 2'O- of the ribose with the 4'C, locking the ribose in *C3'-endo* conformation (Figure 1.15). Introduction of LNA into a DNA sequence induces a conformational change of the DNA:RNA duplex towards the A-type helix but prevents the RNase H cleavage of the target RNA. LNAs and LNA:DNA chimeras have been shown to efficiently inhibit gene expression when targeted to a variety of regions within the luciferase mRNA.⁵¹ LNA shows remarkably increased hybridization properties relative to a DNA:RNA duplex and improves nuclease resistance. Few analogues of LNA have been reported, which have improved activity and/or toxicity profiles in animals.^{52, 53} Thus, LNA offers attractive properties, such as nuclease stability, high target affinity, potent biological activity and lack of acute toxicity.

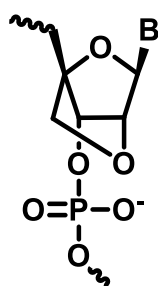


Figure 1.15 Locked nucleic acid (LNA)

1.6.3b Morpholino oligonucleotides (MF). Phosphonodiamidite morpholino oligonucleotide has morpholine ring replacing the furanose ring in DNA/RNA. It has the phosphonodiamidite linkage which connects the morpholine nitrogen atom with the hydroxyl group of the 3'-side residue (Figure 1.16).³⁰ Morpholino ONs are non-ionic, and therefore unlikely to have unwanted electrostatic interactions with nucleic acid binding proteins. These ONs are stable to nucleases and have a similar affinity as in DNA:DNA duplexes. However, morpholino ONs do not activate the RNase H and are primarily used in translation arrest or in other steric blocking mechanisms, such as alteration of splicing.^{54, 55} Eteplirsen (EXONDYS 51) is the first morpholino oligo-based antisense drug approved by FDA for treatment some forms of Duchenne muscular dystrophy (DMD).

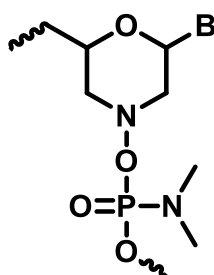


Figure 1.16 Morpholino oligonucleotide (MF)

1.6.3c N3'-P5' phosphoroamidates (NPs). N3'-P5' phosphoroamidate (NPs) is a modified phosphate backbone, in which the 3'-oxygen of the deoxyribose ring is substituted with an amino group (Figure 1.17). NPs are resistant to nucleases and have high affinity towards a complementary RNA strand but do not activate the RNase H.⁵⁶ The sequence specificity of phosphoroamidate-mediated antisense effects by steric blocking of translation initiation has been demonstrated in cell culture and *in vivo*.⁵⁷

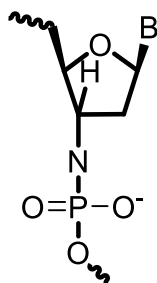


Figure 1.17 N3'-P5' phosphoroamidates (NPs)

1.7 Peptide nucleic acids (PNA)

Peptide nucleic acids (PNAs) are a radically different class of oligonucleotide modifications that contain a peptide link instead of phosphate link. PNA was first introduced by Peter Nielsen *et al*⁵⁸ in 1991. PNAs are highly resistant to degrading enzymes like proteases and nucleases, exhibit high binding affinity towards target DNA/RNA, but do not activate RNase H and, as such, have been used primarily in translation inhibition and splicing modulation antisense mechanisms. PNAs seem to be non-toxic, as they are uncharged molecules with low affinity for proteins that normally bind nucleic acids. PNAs are DNA analogs where the sugar-phosphate backbone is replaced with a pseudopeptide backbone in the form of 2-aminoethyl-glycine linkage. Nucleobases are attached through a methylene carbonyl linker to this backbone at the amino nitrogen. The PNA backbone is constituted by six atoms for each repeating unit and a two-atom spacer between the backbone and the nucleobase, similar to the natural DNA (Figure 1.18).⁵⁹ PNA was originally designed and developed as a mimic of a DNA recognizing, major groove binding, triplex-forming oligonucleotide.^{59, 60} However, the polyamide backbone of PNA has proven to be a surprisingly good structural mimic of the sugar-phosphate backbone of nucleic acids.

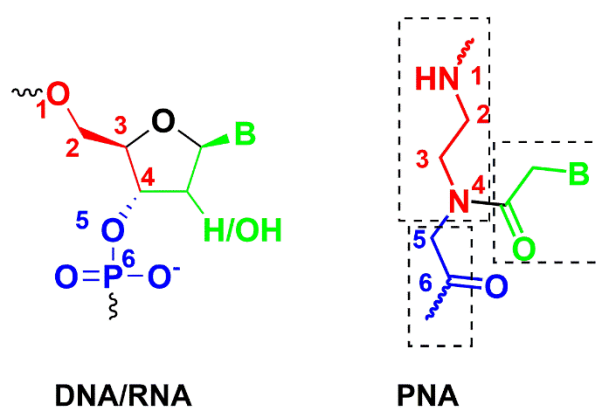


Figure 1.18 Chemical structures of DNA/RNA and PNA

The advantages of PNA over the conventional antisense oligonucleotides are numerous, partially due to the high flexibility and the absence of charge in the backbone. PNAs being neither peptide nor nucleic acids are resistant to both proteases and nucleases and consequently have a longer life span in the cellular environment. PNA hybridizes with complementary DNA/RNA with thermal stability superior to DNA:DNA or DNA:RNA duplex. This results from a decrease in electrostatic repulsion between the two negatively charged strands in DNA/RNA duplexes. They have higher mismatch discrimination and form selective duplexes upon binding to complementary DNA or RNA sequences. Therefore, PNA has attracted wide

attention in medicinal chemistry for the development of gene therapeutics, especially antisense or antigene drugs.

1.7.1 PNA structure

PNA binds to a complementary DNA/RNA through classical Watson-crick base-pairing mechanism. The PNA bases form a helical π -stack similar to DNA but the smaller twist of the PNA double helix and the larger π -overlap between the neighbouring bases makes it different from the DNA. NMR methods and X-ray crystallography have been used to derive the three dimensional structures of the major families of PNA complexes including PNA:RNA,⁴⁴ PNA:PNA,⁶¹ PNA:DNA duplexes^{62, 63} and PNA₂:DNA triplex⁶⁴ (Figure 1.19). Although PNA oligomer, to some extent is able to structurally adapt to the oligonucleotide complement, it also has a preferred structure of its own, termed the '*P-form*' helix.

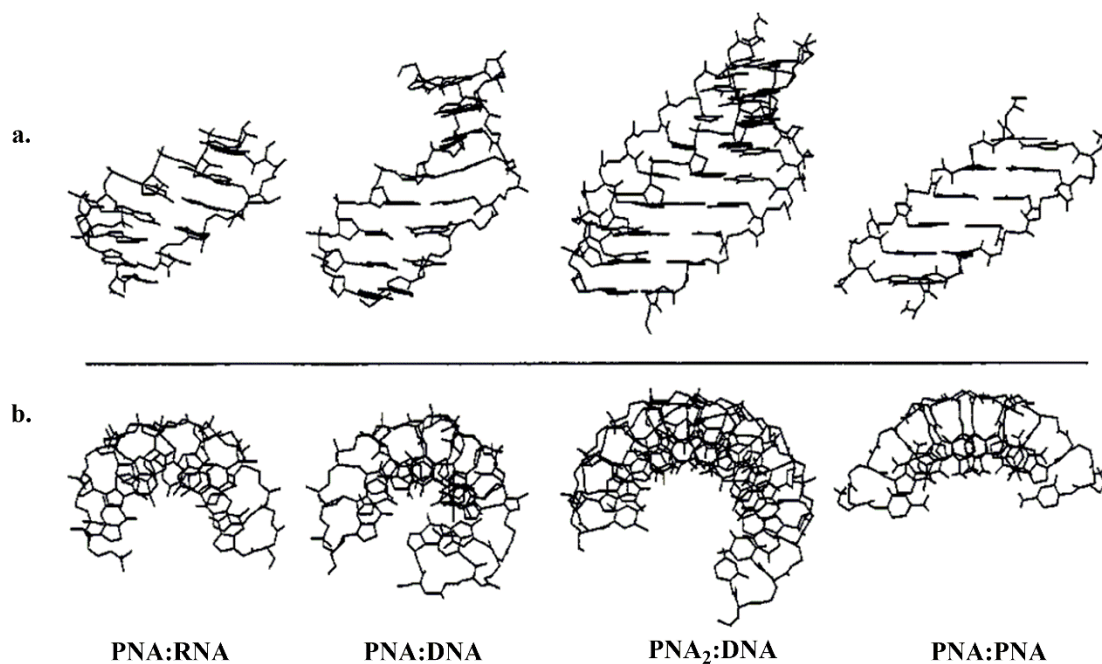


Figure 1.19 Structures of various PNA complexes shown in a) side view and b) top view^{65, 66}

The *P-form* helix constitutes a very wide PNA duplex (28 Å diameter) with an accordingly large base pair helical displacement and a very large pitch (18 bp). A canonical *B form* helix which is typical for DNA duplexes has a diameter of 20 Å and a pitch of 10 bp per turn. A canonical *A-form* helix, typical of RNA duplexes, also has a diameter of 20 Å but a pitch of 11 bp per turn, and the base pairs are tilted 20° relative to the helix axis. Also, the base pairs are displaced away from the helix leaving a central 'tunnel' in the helix, analogous to that seen in the *P-form*. These structures suggest that PNA can adapt well to its nucleic acid partner,

as the RNA strand in the PNA:RNA duplex is essentially in A-form conformation, whereas PNA:DNA duplex adopts a B-form conformation.

Ganesh *et al.*⁶⁷⁻⁶⁹ used the fluorescent probe strategy to measure the polarity of the major groove of PNA:DNA/RNA/PNA duplexes (Figure 1.20). They have revealed that in PNA:DNA/RNA/PNA duplexes, major grooves are more nonpolar (lower dielectric constant, ϵ) compared to those DNA:DNA duplexes.

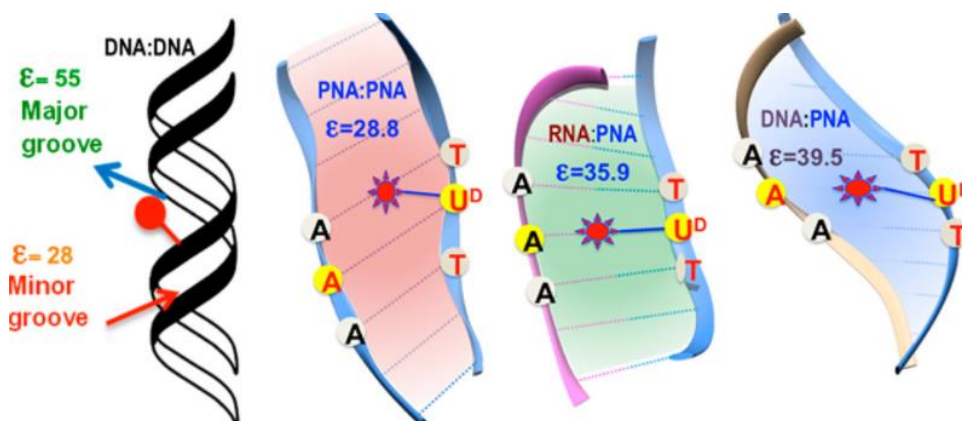


Figure 1.20 Representation of the DNA:DNA, PNA:PNA, RNA:PNA and DNA:PNA duplexes, where one strand is modified with fluorophore.⁶⁹ ϵ indicates dielectric constant.

1.7.2 Triple helix formation of PNA

Polypyrimidine PNAs are able to form stable triplexes with complementary polypurine DNA, through the formation of PNA₂:DNA triplexes.⁵⁸ The base pairing in these triplexes occurs through Watson-Crick and Hoogsteen hydrogen bonds as with DNA triplexes. When same sequence PNA strand is used to form a PNA₂:DNA triplex, both strands are necessarily either antiparallel or parallel to DNA strand. When two different homopyrimidine PNA sequences are used, the Watson-Crick PNA strand binds to the homopurine strand of DNA in antiparallel orientation, but the Hoogsteen strand binds to the DNA strand in parallel orientation to form a stable triplex. The sequence specificity of triplexes is based on the selectivity of formation of the intermediate PNA:DNA duplex, whereas binding of the third strand contributes only slightly to selectivity. The stability of these structures enables PNA to perform strand invasion,^{58, 70} a property which is unique to PNAs.

1.8 Applications of PNA in Biological and material science

1.8.1 PNA as antisense / antigene therapeutic agents

PNAs can be used as a gene therapeutic agent because of their unique strand invasion properties, binding affinity towards the complementary DNA/RNA and their chemical as well as biological stability. Therefore, two strategies involved in using PNAs as therapeutic drugs are antisense and antigene methods. Moreover, no sign of any general toxicity of PNA has been observed so far.

1.8.1a Inhibition of transcription

PNAs are capable of stopping transcriptional processes by virtue of their ability to form a stable triplex structures, by strand invasion or by forming strand displacement complexes with DNA. If the template DNA were to be structurally obstructed, the polymerase could be stopped, halting transcription (Figure 1.21).⁷¹ PNA targeted against the promoter region can form a stable PNA/DNA duplex that stops the DNA access of the corresponding polymerase. Even an 8-mer length of PNA (T8) is able to block phage T3 polymerase activity.⁷²

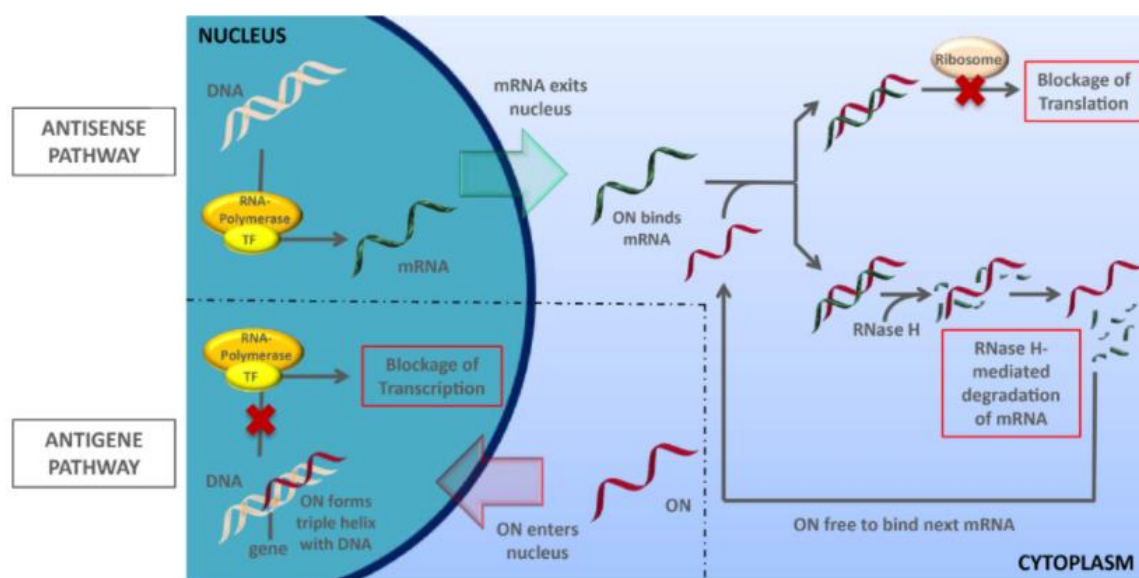


Figure 1.21 Antigene and antisense inhibition strategy⁷³

1.8.1b Inhibition of translation. Antisense strategy, the nucleic acid analogs can be designed to recognize and hybridize to complementary sequences in mRNA and so, inhibit its translation (Figure 1.22). Normally, the PNA antisense activity depends on the steric blocking of mRNA during translation. It has been shown in *in vitro* translation experiments involving rabbit

reticulocyte lysates that both duplex-forming and triplex-forming PNAs are able to inhibiting translation at targets overlapping the AUG start codon.⁷⁴

1.8.1c Inhibition of replication. PNA can also inhibit the elongation of DNA primers by DNA polymerase. Further, the inhibition of DNA replication should be possible if the DNA duplex is subjected to strand invasion by PNA under physiological conditions, or if the DNA is single stranded during the replication process. Taylor *et al.*⁷⁵ has reported efficient inhibition of extra-chromosomal mitochondrial DNA. this is largely single-stranded during replication.

1.8.2 Tools for molecular biology

Molecular genetics applications such as amplification of VNTR (variable number tandem repeat) loci for genetic typing make wide use of PCR (polymerase chain reaction), but preferential amplification of small allelic products relative to large allelic products presents a problem. This may result in incorrect typing in a heterozygous sample. Enhanced amplification of a specific VNTR product is possible by PNA. This has been done in the case of VNTR locus D1S80.⁷⁶ For PCR amplification, the template is stopped by a small PNA and becomes unavailable for intra- and inter-strand interactions during the re-association step. Although PNA blocks re-association, primer extension can occur. During extension the polymerase displaces the PNA molecules from the template, and the primer is extended towards completion of the reaction. This approach has the potential of PNA application for PCR amplification where fragments of various sizes are required to be more accurately and evenly amplified.

1.8.2a Artificial restriction enzyme Strömberg *et. al.*⁷⁷ have synthesized chelating ligand 5-amino-2,9-dimethyl-1,10-phenantroline attached to a diaminopropionic acid unit and incorporated it in the middle of a PNA oligomer. This PNA was used for complementary RNA cleavage in the presence of Zn^{2+} and Cu^{2+} (Figure 1.22). A remarkable improvement in the catalytic activity was seen in the case of Cu^{2+} ; it showed site-specific cleavage and in less time. In this PNA modification, rate of RNA cleavage was the highest among the artificial nucleases till date. After cleavage of RNA in the presence of Cu^{2+} , the HPLC chromatogram showed fragments A and B eluted at 25 min and 32 min respectively, which are represented by peak A and peak B. (Figure 1.22)

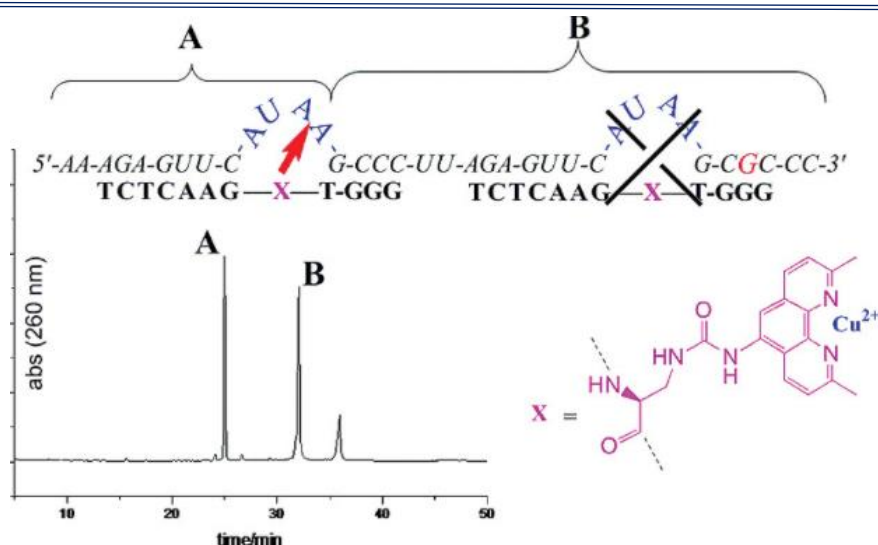


Figure 1.22 PNA as Artificial restriction enzyme.⁷⁷ The chromatogram shown is the HPLC profile of the cleavage products.

1.8.3 Single base pair mutation analysis using PNA-directed PC clamping

Single-base-pair mutation or single-nucleotide polymorphism (SNP) analysis is possible by the PCR technique, if PNA sequence targets the primer binding site (Figure 1.23).⁷⁸ Basically, in the PNA directed PCR clamping technique, at the annealing step the PNA is targeted against one of the PCR primer sites. The temperature set for this step is more than that for normal PCR primer annealing. In this condition the PNA is selectively bound to the DNA molecule. The PNA binds to the primer binding site instead of the primer. This PCR product formation is then stopped by PNA effectively. PNA is able to discriminate between single mismatch and fully complementary targets (mutations) in a mixed target PCR. Hence, out-competing PNA annealing will be favoured over binding of primer. Consequently, mutated sequences will be favourably amplified.

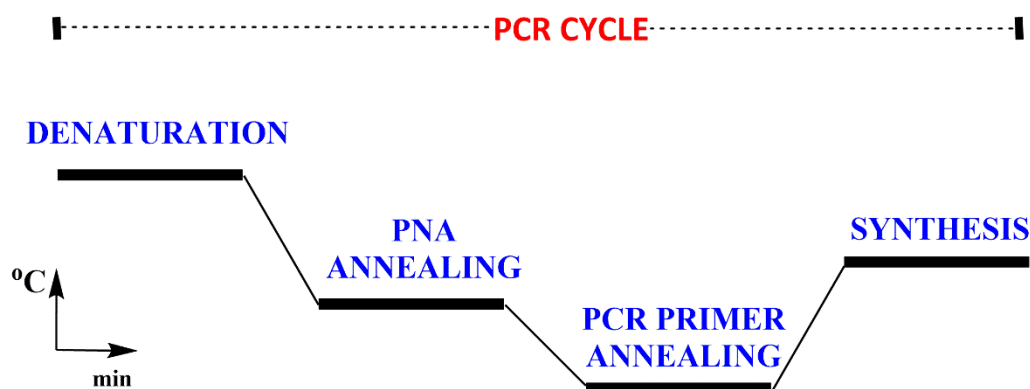


Figure 1.23 representation of cyclic profile used in PNA-directing clamping⁷⁸

1.8.3 PNA based Self-Assembly

The control of supramolecular assembly of a greater number of molecules is possible with high degree of precision and predictability, when the oligonucleotides are programmed using simple rules of Watson-Crick base pairing and duplex geometry. As PNA shares its informational blocks, the nucleobases with DNA, PNA can also be used in the same manner. With an amide backbone, the PNA offers versatility, flexibility and structural complexity. Similar to peptides, PNA were also attached to alkyl chains to induce self-assembly in aqueous solvents. Schneider *et. al.*⁷⁹ prepared amphiphiles of 10-mer PNA and reported that it still retained its DNA hybridizing capacity. Stupp *et. al.*⁸⁰ showed the self-assembly of peptide PNA conjugate into nanofibers. Ganesh *et. al.*⁸¹ reported that fluorinated chain in the PNA structure thus caused compaction of the assembled particles. PNA strands attached to a perfluoroalkyl formed particles that were smaller by 2-3fold than the PNA amphiphiles attached to hydrocarbon tail.

1.8.2a G-Quadruplex and i-motif formation by PNA. DNA and RNA oligomers that contain multiple stretches of consecutive guanine (G) nucleotides are able to fold into a stable secondary structure known as G-quadruplex^{82, 83} that is gaining increasing attention due to its implication in regulation of gene expression. Balasubramanian *et al.*⁸⁴ have shown the formation of quadruplex composed entirely of PNA (Q-PNA) (Figure 1.24C). A homologous PNA (i.e. a PNA having the same sequence as the target) forms a stable PNA₂:DNA₂/RNA₂ hybrid quadruplex by disrupting a bimolecular DNA/RNA G-quadruplex (Figure 1.24 A,B).⁸⁵

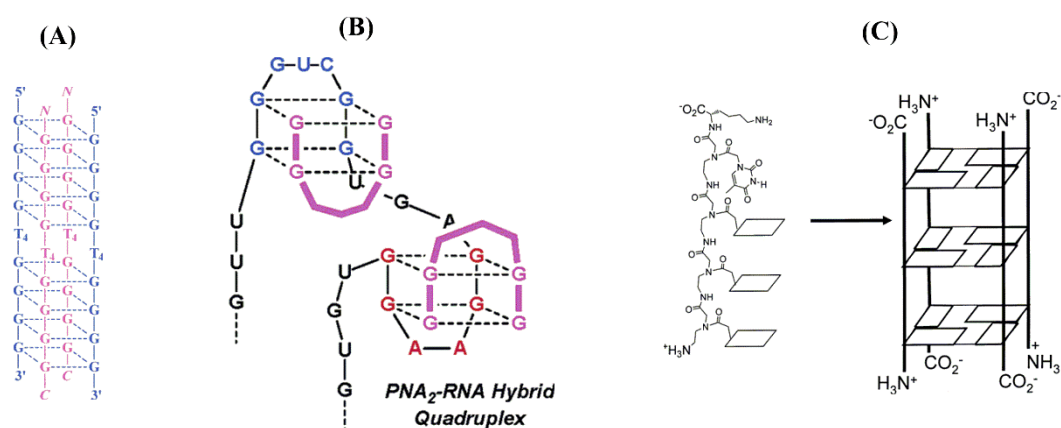


Figure 1.24 (A) Schematic drawing for PNA:DNA quadruplex (B) RNA homologous quadruplex⁸⁵ (C) Schematic diagram for PNA-Q4 quadruplex⁸⁴

Ganesh *et al.*⁸⁶ demonstrated the formation of C:C⁺ tetraplex (i-motif) with unmodified PNAs TC₄ and TC₈ in acidic pH. The tetraplex formation was monitored using UV-thermal

melting at 295 nm, show a reverse sigmoidal pattern, which is characteristic of C:C⁺ tetraplex formation (Figure 1.25C). DNA having polycytidine stretches able form stable hybrid *i*-motif structure with *aeg*-PNA shown by Krishnan *et.al.*⁸⁷ Also, PNA having proline ring on the backbone i.e *aep*-PNA increases the stability of hybrid *aep*-PNA:DNA *i*-motif.⁸⁸ compared to *aeg*-PNA:DNA hybrid *i*-motif.(Figure 1.25 A,B)

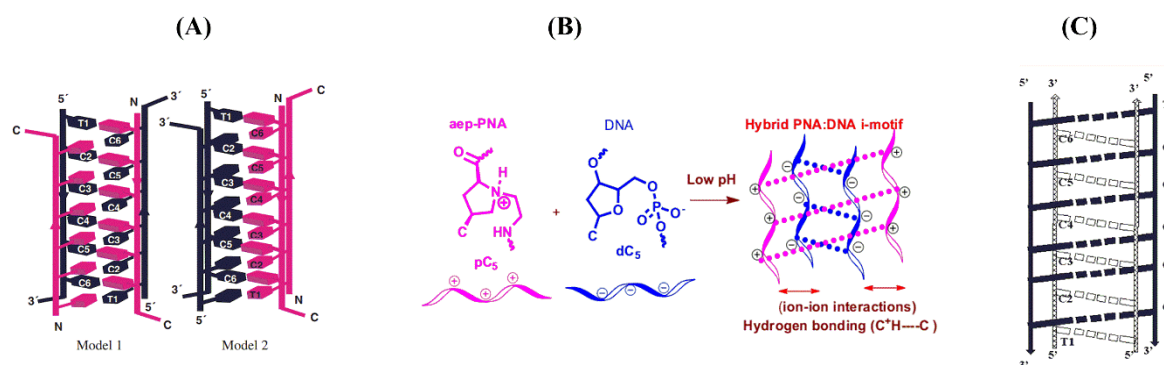


Figure 1.25 (A) Two possible models for *aeg*-PNA:DNA hybrid *i*-motif⁸⁷ (B) Schematic diagram for *aep*-PNA:DNA hybrid *i*-motif⁸⁸ (c) Schematic diagram of *i*-motif formation in the *aeg*-PNA⁸⁶

1.9 Chemical modifications of PNA

The antisense/antigen potential of PNAs can be improved by enhancing the binding affinity for DNA/RNA and improve the cell permeation by chemical modifications. One approach for improving DNA binding affinity is the design and synthesis of preorganized PNAs that prefer a right-handed helical conformation which can be achieved by adding of substituents to the backbone or by cyclization of the PNA backbone (Figure 1.26).^{89, 90}

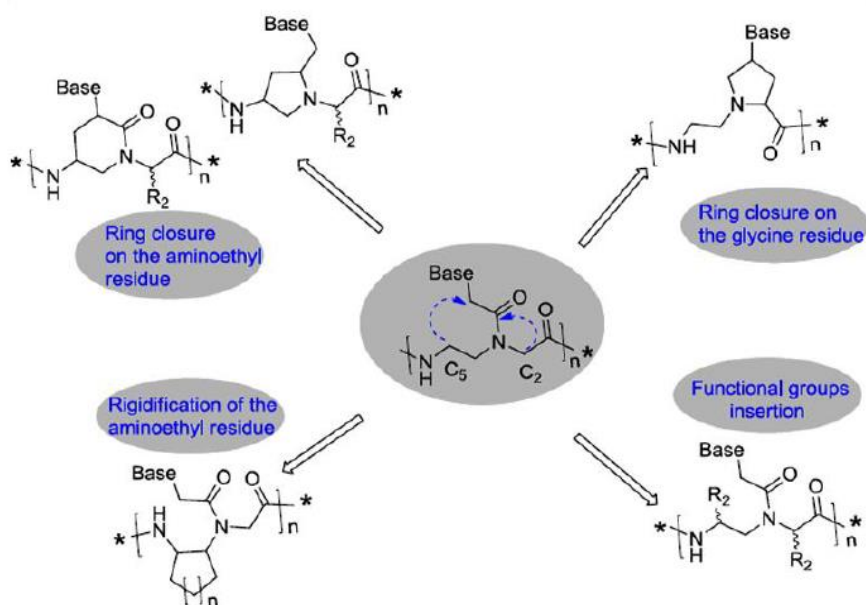


Figure 1.26 Strategies for inducing preorganization in the PNA structure

1.9.1a Substitutions on acyclic backbone of PNAs. The N-(2-aminoethyl) glycine unit in PNA backbone is a versatile motif for the modification of the PNA. Replacement of glycine by other α -substituted amino acids and substitution in ethylenediamine results in chiral PNAs that bear substituents at different positions (α , β , γ) (Figure 1.27)

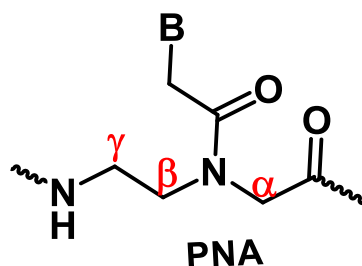


Figure 1.27 Structures of achiral PNA, B = A/T/G/C. (A = Adenine, T = Thymine, G = Guanine and C = Cytosine)

$C\alpha$ -PNAs: The first $C\alpha$ -PNAs were reported by Nielsen *et al.*⁹¹ in 1994, derived from both L and D alanine (Figure 1.24a). PNA:DNA duplex stability derived from D-alanine in PNA is almost equal to unmodified PNA whereas $C\alpha$ -substituted PNA derived from L-alanine drastically destabilised the duplex stability. PNAs derived from D-lysine (Figure 1.28b) in place of glycine exhibited higher T_m than that from the corresponding PNA derived from L-lysine, with T_m more than that with unmodified PNAs.⁹² $C\alpha$ -substituted PNAs derived from D-amino acids prefer P helix formation and mismatch discrimination ability is more than those of unmodified PNAs.⁹³ PNA oligomer containing three units of Lys-D-PNA and its peptide conjugate tested for cellular uptake ability, were found to be taken in most of the tested cells.⁹⁴ D-Arginine in place of glycine leads to α -guanidinium PNAs (GPNAs, Figure 1.28c) that showed destabilization of the derived PNA:DNA duplex. However, incorporation of multiple α -guanidinium PNA units (α -GPNA) in a PNA decamer improved the binding affinity, partly from electrostatic interactions between guanidinium group and phosphates.⁹⁵ $C\alpha$ -PNAs with negatively charged side chains (derived from glutamic acid and aspartic acid) destabilized the PNA:DNA duplex due to electrostatic repulsions between negatively charged PNA backbone and polyanionic DNA backbone.⁹⁶ $C\alpha$ -substituted PNAs derived from α -amino acids having bulky side chains such as tyrosine, histidine, tryptophan, phenylalanine and valine showed lower T_m values due to steric hindrance.⁹⁷ 1-(2,2-Dimethylcyclobutyl) ethanone containing α -PNA (Figure 1.28e) showed almost equal binding affinity with DNA and RNA.⁹⁸ Gem-dimethyl substitution at $C\alpha$ increases the T_m of derived PNA:DNA duplexes,⁹⁹ and the homologous aminopropyl-(α,α -dimethyl)glycine (*apdmg*) PNAs improved the binding affinity

to DNA. These gem-dimethyl substituted PNAs have showed preferential binding to DNA than to RNA.

C β -PNAs: In 2011, Sugiyama *et al.*¹⁰⁰ reported the first C β -substituted PNA bearing a methyl group at β -position using D and L enantiomers of alanine (Figure 1.28f). C β -PNA oligomer derived from L-alanine showed similar binding affinity as that of unmodified *aeg* PNAs, whereas the corresponding C β -PNA derived from D-alanine did not bind to DNA. Circular dichroism revealed that C β -S-PNA exhibited a right-handed helical structure whereas C β -R-PNA exhibited left-handed helical structure.¹⁰⁰

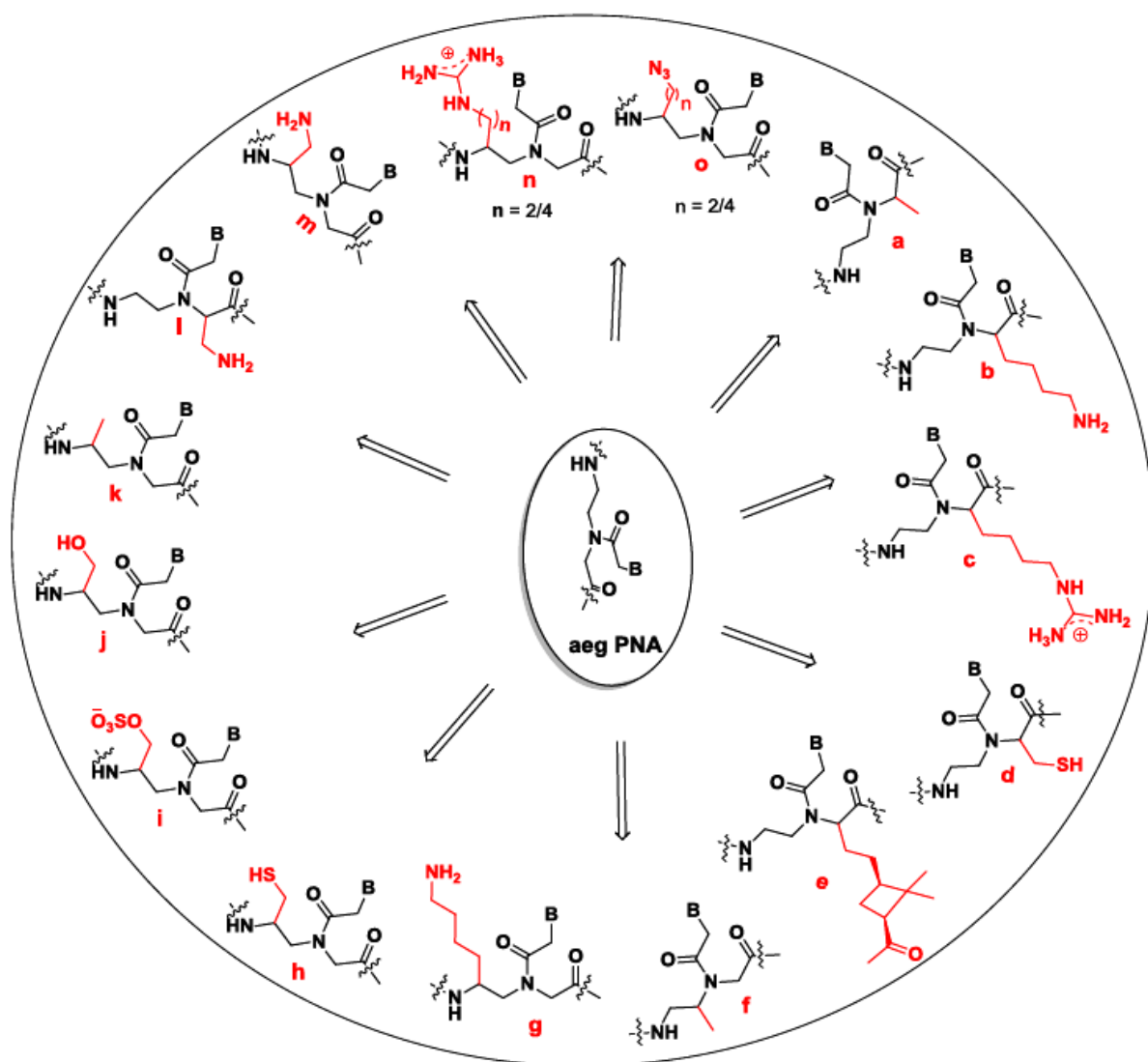


Figure 1.28 Modified acyclic PNAs.

C γ -PNAs: Although the first chiral γ -PNA was reported in 1994, γ -PNA oligomers did not appear in literature until 2005.¹⁰¹⁻¹⁰⁵ C γ -(L)-Lys-modified PNAs (Figure 1.28g) stabilized PNA:DNA duplexes and showed good mismatch discrimination relative to unmodified PNAs,

whereas γ -D-Lys-PNAs markedly destabilized the duplex.¹⁰⁶ Cysteine based PNA substitution (Figure 1.28h) at the N-terminus of a PNA oligomer can be used for the native chemical ligation with PNAs carrying thioesters to yield long chain PNAs. The effect of stereochemistry on DNA binding was more effective in $C\gamma$ -PNAs than that of $C\alpha$ -PNAs.¹⁰⁷ Homo pyrimidines incorporated with $C\gamma$ -PNA bearing a sulfate group (Figure 1.28i) formed a triplex with lower stability which arises from the repulsions between negatively charged phosphate DNA and sulfate of modified PNAs. The modified PNA could be lipofected into human breast cancer (SKB3) cells and exhibited antigene activity against ErbBr gene.¹⁰⁸

Serine or alanine based γ -PNAs (Figure 1.28j and 1.28k) revealed that single-stranded γ -backbone modifications preorganized to right-handed helix, similar to that in PNA:DNA duplex.^{109, 110} Ly *et al.*¹¹¹ have reported a variety of γ -PNAs and demonstrated that $C\gamma$ -position can accommodate hindered side chains unlike $C\alpha$ -PNAs without affecting the hybridization properties considerably. The cellular uptake of a fully modified alternate $C\gamma$ -GPNA decamer by HeLa cells is comparable to that of TAT transduction domain. Recently, Manicardi *et al.*¹¹² reported the inhibition of micro-RNA by GPNAs carrying an arginine side chain. Anti-miR-210 activity of PNAs in leukemic K562 cells was examined using a series of modified 18-mer PNAs (α or γ , PNAs conjugated with arginine octamer and unmodified PNAs). The best anti-miR-210 activity was observed with γ -GPNAs with consecutive placement.

Ganesh *et al.*¹¹³ have reported the design and synthesis of chiral $C\alpha$ - and $C\gamma$ - aminoethyl PNAs (*am*-PNAs) with substitutions in the PNA backbone (Figure 1.28l and 1.28m). The *am*-PNAs formed stable PNA:DNA duplexes and the order of stabilization was, γ -(S)- *am*-PNA > α -(R)- *am*-PNA > α -(S)-*am*-PNA. The *am*-PNAs are taken up by HeLa cells, with decreasing order of uptake efficiency as γ -(S)-*am*-PNA > α -(R)-*am*-PNA > α -(S)-*am*-PNA. Recently⁸² it was shown in γ -GPNAs (Figure 1.28n, n = 2) and γ -azido PNAs (Figure 1.28o) that side chain lengths is important for hybridization with DNA. The guanidinium PNAs with shorter spacer chain increased the PNA:DNA duplex stability. These PNAs taken up in 3T3 and HeLa cells were visualized by confocal microscopy and quantified using fluorescence assisted cell sorter (FACS).¹¹⁴ The azido functionality of $C\gamma$ -substituted methylene/butylene azido PNAs enable the attachment of multisite labelling and introduction of fluorophores in a single step through click reaction without any protection/deprotection steps. The azido fluorescent PNA oligomers have been shown to accumulate around the nuclear membrane in 3T3 cells.¹¹⁵

Employing a combination of molecular dynamics with metadynamics, Corradini et al¹¹⁶ reported that single strand γ -modified PNA undergoes better conformational pre-organization than single strand PNA, otherwise unrevealed through only classical molecular dynamics. Their simulations driven on backbone modified PNAs lead to the conclusion that this γ -functionalization affects the single strand preorganization and targeting properties to the DNA/RNA, in agreement with circular dichroism (CD) spectra obtained for this class of compounds. In the γ modified PNA with the CH_2OH as γ substituent, the torsional angle of H-N-C-H bond is almost planar i.e., 173° favoring polar interactions with the solvent. In comparison, the torsional angle of H-N-C-H in the unmodified ssPNA is -90.7° , suggesting a higher flexibility and propensity of γ -ssPNA. Thus properly preorganized γ -ssPNA strands require significantly less free energy to associate in the duplex compared to the unmodified ssPNA.¹¹⁶

1.9.2b Conformational preorganization through cyclic PNAs. The PNA being acyclic, is conformationally flexible and formation of ordered PNA:DNA/RNA complexes is accompanied by enthalpic advantage through hydrogen bonding and base stacking interactions but has an undesirable loss in entropy of the PNA strand.¹¹⁷ A high rotation barrier is encountered in the interconversion of *cis* and *trans* rotamers around the tertiary amide linkage in PNA and leads to different PNA:DNA/RNA hybridization kinetics in parallel and antiparallel hybrids.^{118, 119} Ganesh *et al.*¹²⁰ have synthesized many modified cyclic PNAs that can potentially preorganize PNA to form PNA:DNA/RNA complexes and achieved hybridization preferences (decreased loss in entropy) for either DNA or RNA complementation and parallel or antiparallel orientation. Many strategies have been developed to enrich populations of single-stranded PNAs that have the favourable, pre-organised conformation for binding to complementary DNA/RNA. The preorganized conformers could trigger a shift in equilibrium toward the desired complexation because of the net reduced entropy loss upon complexation. The strategies to conformational preorganization are based on the introduction of methylene/ethylene groups to bridge the aminoethyl-glycyl backbone and methylene carbonyl side chain to generate diverse five- or six membered nitrogen heterocyclic analogues. Some of these strategies are described below in detail.

Aminoprollyl PNA (ap-PNA): 4(R/S)-Aminoprollyl PNA synthesized by the introduction of a methylene bridge between β -carbon atom of the aminoethyl segment and the α'' -carbon of the glycine segment on *aeg*-PNA backbone (Figure 1.29).¹²¹ None of the homochiral aminoprollyl

thyminyl PNAs corresponding to any of the diastereomers bound to target DNA sequences¹²² and this might be attributed to high rigidity in the backbone resulting in structural incompatibility.¹²³

Aminoethylprolyl PNA (*aep*-PNA): Chimeric aminoethylprolyl PNA (*aep*-PNA) analogues arise from connecting the glycine on backbone with side chain to give 4(*S*)-2(*S/R*)-*aep*-T oligomers (Figure 1.29) that showed strong and specific binding properties toward target DNA sequences.¹²⁴ The homooligomer *aep*-PNA-T₈ with (2*R*,4*R*) stereochemistry displayed significant stabilization of the complexes with poly rA,¹²⁵ while the adenine A₈ homooligomer with (2*S*,4*S*) stereochemistry showed improved binding to the target DNA.¹²⁶ Nucleobase dependent binding and orientation selectivity were observed with these analysis.

Aminoethylpyrrolidinone PNA (*aepon*-PNA): The aminoethylprolyl-5-one thymine (*aepon*) monomers contain cyclic amide and were incorporated into *aeg*-PNA-T₈ backbone at different positions.¹²⁷ The *aepon*-PNAs (Figure 1.29) showed remarkable stabilization of derived PNA₂:DNA triplexes compared to *aeg*-PNA.¹²⁸ The homo oligomers of *aepon*-PNA-T₈ bound to the complementary 12-mer DNA more strongly than that of cRNA. This suggested that structurally preorganized PNAs can discriminate their binding selectivity to DNA or RNA.

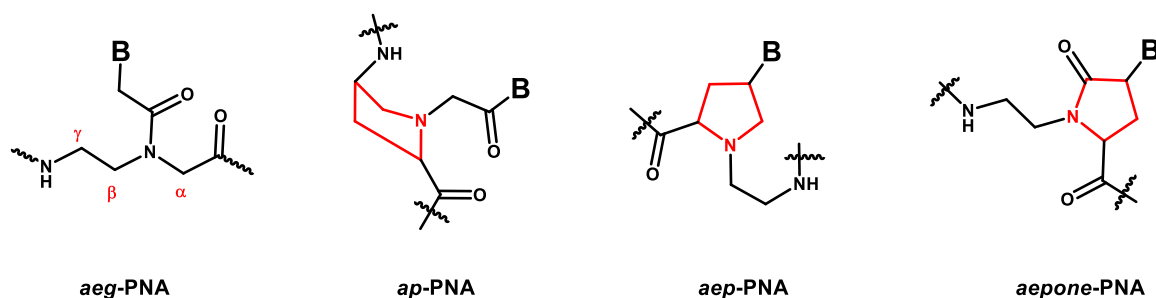


Figure 1.29 Cyclic PNAs: *ap*-PNA, *aep*-PNA and *aepon*-PNA

Pyrrolidine PNAs: Deletion of the *endocyclic* carbonyl group in pyrrolidinone PNA gave the pyrrolidine PNA¹²⁹ (Figure 1.30a). The derived (2*R*,4*S*) stereomeric homo adenylate chimeric *aeg*-PNA oligomer formed a stable complex with both DNA and RNA.¹³⁰ The (2*R*,4*R*) version of this PNA analogue was shown to bind target DNA and RNA with high affinity and kinetic selectivity toward RNA.¹³¹ (2*S*,4*S*) pyrrolidine PNA analogue, and its thyminyl oligomer resulted in a decreased binding efficiency with target DNA and RNA sequences.¹³² PNA:DNA dimer prepared from (2*R*,4*R*) pyrrolidine PNA-T, which when placed in a PNA-DNA chimera lead to decreased DNA triplex stability.¹³² Introduction of the α' - β -methylene bridge led to another pyrrolidine-PNA (Figure 1.30b)¹³³ having the nucleobase away from the pyrrolidine ring by one carbon. The (2*R*,4*S*) pyrrolidine-T monomer when introduced into the middle of the *aeg*-PNA-T₈, bound to the target DNA better than the diastereomeric (2*S*,4*S*) PNA. This is

an example of stereochemical discrimination effects in PNA:DNA recognition. The 4*R* pyrrolidine-T PNA sequences stabilized the PNA:DNA duplex with a significant difference in parallel/antiparallel binding compared to *aeg*-PNA:DNA duplexes,¹⁰¹ while the PNAs with 4*S* modifications destabilized the duplexes without much parallel/antiparallel binding differences as compared to the unmodified hybrids. The enantiomeric pairs (2*S*,4*S*) and (2*R*,4*R*) formed antiparallel complexes with RNA much stronger than that of *aeg*-PNA or other diastereomers. Linking of the nucleobase to the backbone through a cyclopentane ring directly as in *aep*-PNA (Figure 1.29) or via a flexible ethylene linker as in pyrrolidene PNA (Figure 1.30 b), creates positive charge in the PNA backbone at the expense of the conformational rigidity of the tertiary amide linkage.¹²³ Such positive charges enhances the binding of the modified cationic PNA backbone with poly anionic DNA backbone by electrostatic effects.

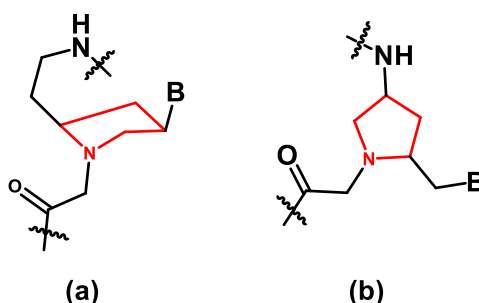


Figure 1.30 Pyrrolidine PNAs⁹⁶

Pipecolic and Piperidinyl PNAs: The pipecolyl PNA oligomers were designed by introducing a methylene, or an ethylene bridge between the C γ - or C β -carbon of the aminopropyl segment and the α '-carbon of the glycyl segment into an aminopropyl-glycyl PNA analogue (Figure 1.31).¹³⁴ The modified PNA-T10 oligomer (*pip*-PNA-1) carrying the *trans* (2*S*,4*S*) monomer destabilized the PNA2:DNA triplex formation. However, the homo thyminyll mixed *aeg*-PNA sequences incorporating the 5-aminopipecolyl unit (*pip*-PNA-2) form stable complexes with target DNA oligomers. The chiral six-membered analogues (2*S*,5*R*) *aepip*-PNA and the *trans* (3*S*,5*S*)-piperidinyl PNA-T upon incorporation into the *aeg*-PNA-T₈ homooligomer at different positions stabilized the corresponding PNA2:DNA triplexes.¹³⁵

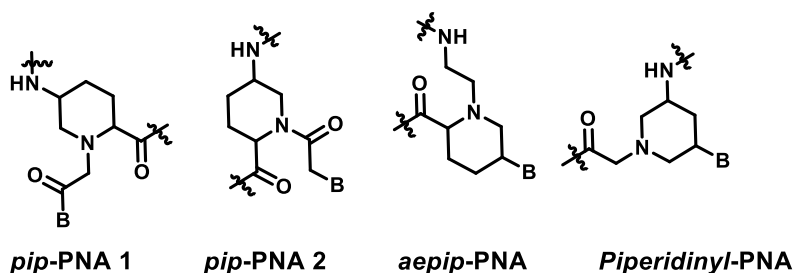


Figure 1.31 Pipecolic and Piperidinyl PNAs¹³⁴

Cyclohexanyl PNA: One of the earliest PNA modifications was to constrain the flexibility in the aminoethyl segment around a single C-C bond by introducing a cyclohexyl ring (Figure 1.32).¹³⁶ The derived *trans*-(*S,S*)-cyclohexyl PNA oligomer hybridized with complementary DNA as good as the unmodified *aeg*-PNA, while the enantiomeric *trans*-(*R,R*)-cyclohexyl PNA oligomers lacked such a property. Significant stereo differentiation was observed with *SR*- and *RS-ch*- PNAs, the *SR* isomer was more destabilizing than the *RS* isomer in *ch*-PNA:DNA complexes while In case of RNA complexes *RS* being more destabilizing than *SR*. From *T_m* values it was observed that (*R,S*)-*ch*-PNAs bound to RNA with higher affinity than to DNA. This is attributed to the inherent rigidity of the *cis-ch*-PNAs that forbids structural readjustments to bind to DNA (PNA:DNA $\beta \sim 140^\circ$) and prefers binding to RNA (PNA:RNA). This discrimination was achieved via the concept of preorganization.

Cyclopentanyl PNAs: Ganesh *et al.*¹³⁷ also synthesized *cis*-(1*S*,2*R*/1*R*,2*S*)-cyclopentyl PNA-T oligomers in which the characteristic *endo-exo* puckering that dictates the pseudoaxial/pseudoequatorial dispositions of substituents may allow better torsional adjustments to attain the necessary hybridization-competent conformations. The homothymine *aeg*-PNA-T10 oligomers with modifications at defined positions exhibited binding affinities with DNA and RNA. It was observed that the *RS-cp*-PNA enantiomer showed higher affinity for DNA as compared to *SR-cp*-PNA isomer and in case of RNA this was reversed. The all-modified homooligomers of both enantiomers exhibited significant stabilization of their triplexes with DNA and poly rA without sacrificing the base specificity. *ch*-PNA and *cp*-PNA-T monomers of both (*S,R*) and (*R,S*) enantiomers were introduced into mixed sequences, in all the cases PNA:RNA hybrids were more stable than the corresponding PNA:DNA hybrids, and significantly, *cp*-PNA oligomers showed much higher *T_m*s compared to *ch*-PNA

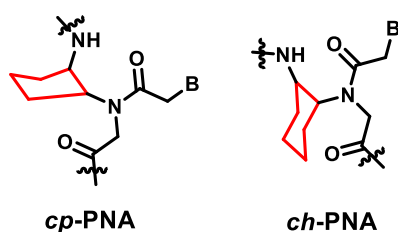


Figure 1.32 Cyclopentyl and cyclohexyl PNAs

1.10 Synthetic heterocycles as *Janus* nucleobases

The term Janus heterocycle, was first coined by Nobel laureate J.M. Lehn, after the twofaced Roman god.^{138, 139} The term Janus heterocycle is employed to describe heterocycles that are capable of forming hydrogen bonds on both faces of the heterocycle,¹³⁹ in contrast to natural nucleobases that typically present only one face for functional hydrogen bonding (not counting the potential for simultaneous formation of Hoogsteen pairs) (Figure 1.33).

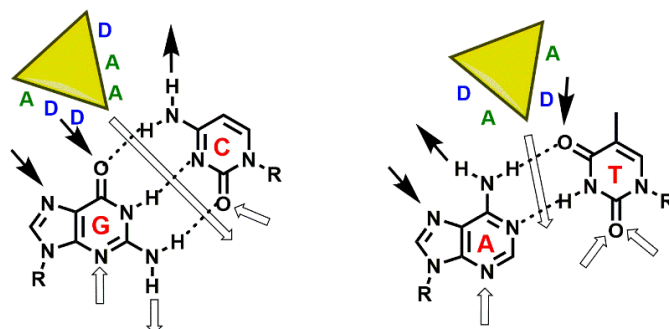


Figure 1.33 Janus-wedge recognizing regular DNA base pairs (A: hydrogen bond acceptors, D: hydrogen bond donors, R=deoxyribose)

A Janus heterocycle therefore represents one type of artificial molecule that can be rationally designed with predefined hydrogen bonding donors and acceptors relative to regular nucleobases. For example, if one side is complementary to A (which has the character of T) the other side can complement C (which has the character of G base), this Janus heterocycle is named Janus-TG. Typically, this Janus-TG heterocycle can form Watson-Crick base with A on one strand and C on another strand, as AC cannot form direct associate through base pairing. Taking this complementarity into account, there are two kinds of Janus heterocycles, one of which can form base pair with itself whereas the other cannot. So far, several Janus heterocycles have been reported, including the reported Janus-GA (Lehn *et al.*¹⁴⁰) and subsequently, the Janus-GC (Mascal *et al.*^{141, 142}) and Janus-AT (Perrin *et al.*¹⁴³) (Figure 1.34).

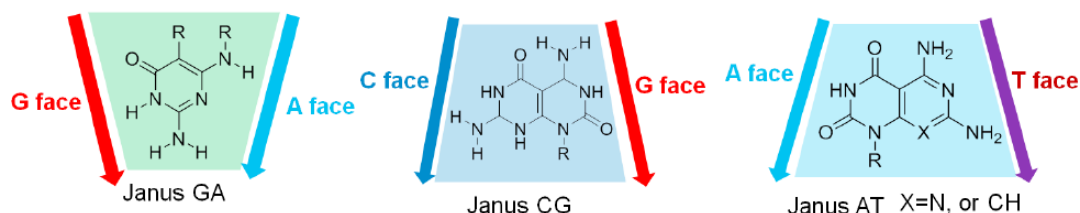


Figure 1.34 Janus heterocycles (R=alkyl groups in all three kinds of Janus heterocycles)

Due to self-complementary hydrogen-bonding donor and acceptor groups on two faces, Janus-GC and Janus-AT can aggregate to form interesting non-covalent supramolecules, the driving force being the formation of hydrogen bonding as well as stacking interactions that

favor creation of higher order structures. For example, Fenniri *et al*¹⁴⁴⁻¹⁴⁶ reported the occurrence of helical rosette nanotubes in water by employing Janus-GC heterocycle (Figure 1.35b).

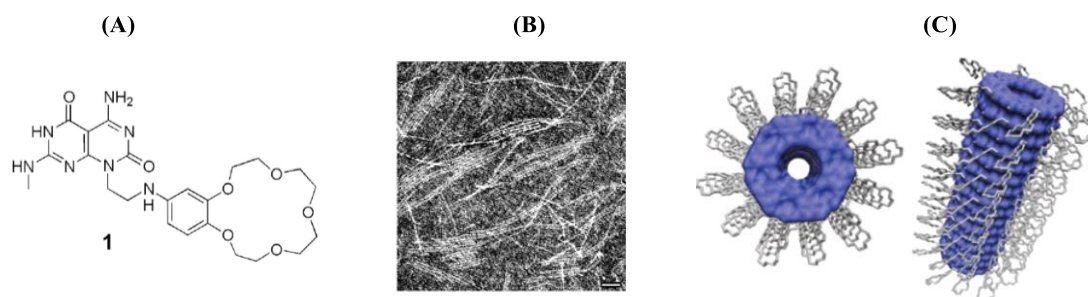


Figure 1.35 Self-assembly rosette nanotubes of Janus GC conjugate (A) GC conjugate 4-aminobenzo-18-crown-6-ether (B) TEM image of **A** (C) hierarchical self-assembly of **A**.

This group also reported that the property of helical rosette nanotubes could be adjusted by changing the side chain attached to the Janus-GC heterocycle. It was shown that chirality of rosette nanotubes could be induced with the addition of chiral amino acids when the side chain of Janus-GC was a crown ether. In addition, when Janus-GC heterocycle was combined with the ribose forming Janus-GC nucleosides, Janus-GC nucleosides also have the capacity to self-aggregate forming the aforementioned rosette nanotubes but with chiral properties owing to the presence of the ribose moiety.^{147, 148} When the Janus-AT nucleoside^{149, 150} was examined under similar conditions, instead of rosette cyclic structure, a linear sheet-like structure was observed. Although a given Janus-GC heterocycle or Janus-GC nucleoside can form the rosette nanotube superstructure, one significant limitation to the application of these kinds of rosette nanotubes is the lack of reproducibility of the identical nanotubes¹⁴³ In order to achieve pre-defined nanotubes, they hypothesized that a certain number of Janus heterocycles should be incorporated into oligonucleotides to form a Janus heterocycle oligonucleotide, where sequence specificity can be modulated through suitable length as well as the incorporation of standard nucleosides. This is now being pursued by several laboratories.¹⁵¹ Besides its utility in the construction of nanotubes, another potential application of a Janus heterocycle would be to act as a device by which one could recognize specific DNA sequences based on Watson-Crick base pairings.

1.10.1 Peptide Nucleic Acids: incorporation of Janus wedge motifs.

PNA recognizes single stranded and double-stranded nucleic acids through hydrogen bonding via Watson-Crick and Hoogsteen faces of the nucleobases. This approach works well

for triplexes T*A:T, C⁺*G:C, A*A:T, and G*G:C but does not cover the full scope of possibilities, such as mismatched nucleobases, due to difficulty in triplex formation with pyrimidine bases. McLaughlin *et al*¹⁵² successfully used an oligo-PNA with Janus wedge motifs (Figure 1.31) to target T-C mismatches in DNA. Using a heterocycle called W which presents hydrogen bonding motifs that would complement the Watson-Crick faces of thymine and cytosine, they were able to form stable triplexes with a duplex DNA having 8 consecutive T-C mismatches flanked by eleven canonical bps. In later work, heterocycles targeting A-T and G-C bps were able to successfully form triplexes when canonical base pairs were flanked by multiple mismatched bases and discriminate against non-complementary nucleobases.¹⁵³ Ganesh *et al*¹⁵⁴ utilized cyanuric acid and 8-amino adenine moiety displayed on a PNA backbone to strengthen triplex formation with oligo-A DNA. They found that a single cyanuric acid moiety in the middle of an oligo-T displaying PNA stabilized the triplex by a 12 °C in *T_m* measurements by providing a Watson-Crick competent binding face, without mattering which rotamer of the cyanuric acid presented to the DNA nucleobase.

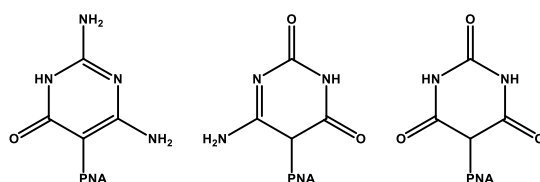


Figure 1.36 Janus wedge motifs used in PNA

1.10.2 Peptide Nucleic Acids: native peptide backbone

After Nielsen discovered PNA as a competent nucleic acid mimic, other groups have looked at native peptide backbones as a way of displaying genetic information. Lenzi *et al*¹⁵⁵ first attempted a native peptide backbone nucleic acid with a self-complementary sequence incorporating thymine and adenine nucleobases, but the hairpin had a modest *T_m* of 19 °C, possibly due to a lack of flexibility in the backbone. Diederichsen *et al*¹⁵⁶ synthesized alanyl peptide nucleic acids with alternating stereo centres to create nucleobase displaying beta sheets, again with modest success compared to PNA. Huang *et al*¹⁵⁷ introduced α -PNA in which the nucleobases are displayed on an alpha-helical peptide. Using a positively-charged helical structure, cooperative binding to the DNA sequence GGAGG was demonstrated as well as high melting temperatures in a possible triplex structure. Eschenmoser and Krishnamurthy,¹⁵⁸ interested in the primordial evolution of genetic material, studied α -peptides and peptoids displaying triazine heterocycles as potential precursors to modern nucleic acids (Figure 1.37). In their systems 2,4-diamino-1,3,5- triazine and 2,4- dioxo-1,3,5-triazine rings were displayed

on a glutamyl, aspartyl iminodiacetic acid, and ethylenediamine monomers with alternating aspartic acid or dialkyl-ammonium groups for solubility. Their findings showed that the binding of these small oligos, displaying as few as six nucleobase mimics, were robust in binding to RNA and DNA when containing the 2,4-diamino-1,3,5-triazine heterocycle displayed on the longest linker. They found that the 2,4-dioxo-1,3,5- triazine nucleobase mimic did not bind to nucleic acid targets due to deprotonation in the assay buffer. While they ruled out that the triazine heterocycles as possible nucleobase precursors, they did show that α -peptides are competent as nucleic acid analogues even with negatively charged backbones. Furthermore, while not a major finding of the paper, they found that the 2,4-diamino-1,3,5-triazine (Figure 1.37) is effective as a nucleobase mimic capable of recognizing uracil and thymine. In a follow up communication using α -peptides, the researchers showed that pK_a of the nucleobase is important.¹⁵⁹ Their findings yielded a relationship between pK_a of the base and pH of the solution, in that if the base becomes deprotonated or tautomerize in the buffer (at pK_a near or equal to the pH of the solution), then molecular recognition is weak.

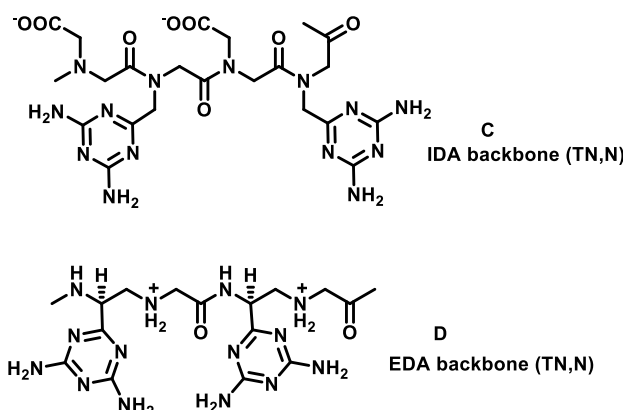


Figure 1.37 Structures of triazine-tagged oligomers (IDA=iminodiacetic acid; EDA=ethylenediamine;(TN,N)=(2,4-diamino)triazin-6-yl)¹⁵⁸

The purines (A and G) are capable of forming base pairing from both faces, through Watson-Crick / Hoogsteen H-bonding schemes, while pyrimidines (C and T) cannot achieve the same. In a way, A and G are “Janus” bases with two non-identical faces and are important in the formation of triplexes (A/T*A:T and C⁺/G*G:C) and G-quadruplexes. Mutual recognition of non-complementary bases has been achieved by insertion of wedge-like heterocyclic “Janus bases” having two faces designed with H-bonding sites, matching that of the target nucleobases. The versatility of synthetic “Janus” bases to simultaneously recognize two natural nucleobases and the geometric separation of adjacent bases in *aeg*-PNA attuned to that in natural DNA / RNA. Motivated from Janus nucleobases, recently Ganesh et al reported

various novel PNA analogues modified at C α and C γ positions of standard *aeg*-PNA backbone known as bimodal PNA.

1.11 Bimodal Peptide Nucleic Acid (*bm*-PNA)

Bimodal PNAs are novel PNA analogues which can target two different DNAs simultaneously to form double duplex. This new class of PNA synthesised from C α or C γ modified *aeg*-PNA oligomers. Ganesh *et.al*^{160, 161} reported modified C α or C γ face of bimodal PNA attached through two types of linkers. The C α modified *bimodal* PNA synthesized by triazole linker on solid phase using sequential coupling and click reaction to introduce a second base in each monomer.¹⁶⁰ The Amide linker was used for C γ modification.¹⁶¹ The resulting *bimodal* PNA:DNA complexes show stability higher than that of individual duplexes in both triazole and amide linker attached *bimodal* PNAs.

1.11.1 C α modified *bimodal*-PNA

1.11.1a Double duplex from bimodal-PNA. The standard *aeg*-PNA can form stable duplex with complementary DNA/RNA. (Figure 1.38.b). When *aeg* backbone extended at Ca position and nucleobases are only at triazole face (*iso*-PNA, Figure 1.38c) can also form stable duplex compared to isolated duplex.¹⁶⁰

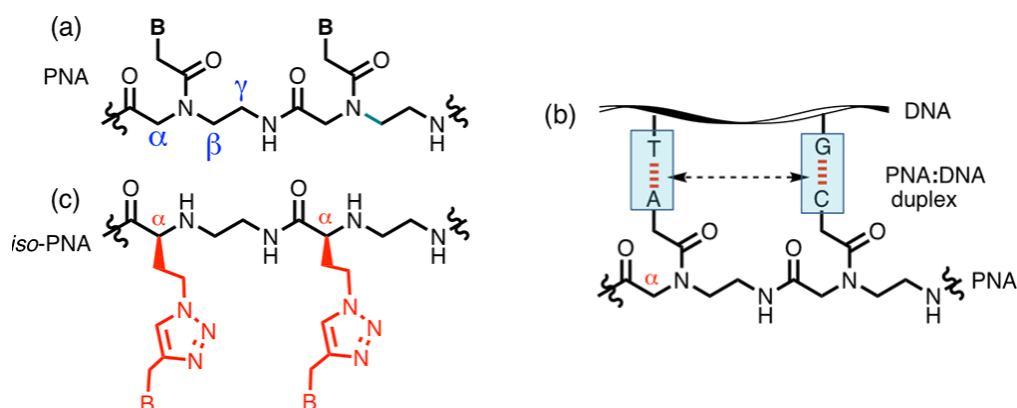


Figure 1.38. Structures of (a) *aeg*-PNA, (b) PNA:DNA duplex, (c) *iso*-PNA

In *bm*-PNA nucleobases are available on both t-amide and triazole face. The *bm*-PNA having mixed base sequences on both faces forms double duplexes by simultaneous binding of two complementary DNA/RNA strands or from long single strand. (Figure.1.39)

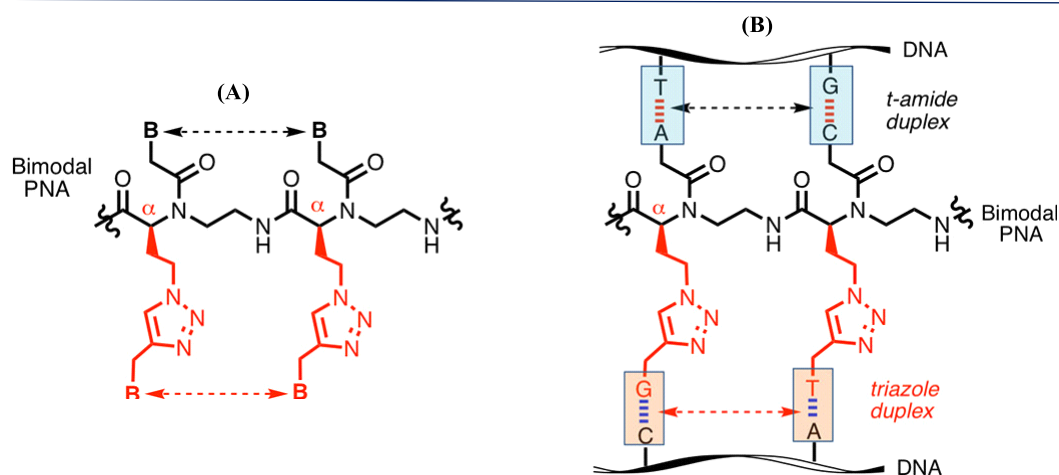


Figure 1.39. Structures of (A) *bimodal* PNA (*bm*-PNA), and (B) *bimodal* PNA double duplex. B = T/A/G/C

1.11.1b Double duplex of triplex (pentameric complex) bimodal-PNA. Polypurine or polypyrimidine sequences can form triple helix structure via Hoogsteen base pairs with a third strand that is complementary to purine strand. Here, The *bm*-C α -PNA with homothymine (T₇) on the *t*-amide face and homo cytosine (C₅) on triazole face can forms double duplex of triplex structure.¹⁶² In the presence of complementary DNAs dA₈ and dG₆ *bm*-C α -PNA forms a higher order pentameric double duplex of a triplex composed of two *bm*-C α -PNA-C₅:dG₅ duplexes built on a core (*bm*-C α -PNA-T₇)₂:dA₈ triplex.

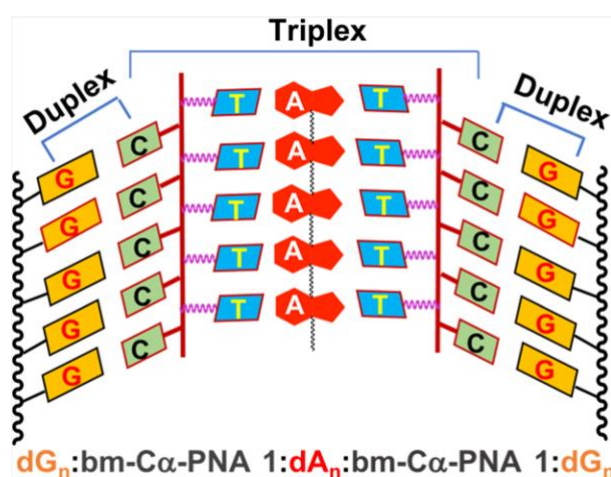


Figure 1.40 Pentameric double duplex of triplex complex of *bm*-PNA¹⁶²

1.11.2 C γ modified *bimodal*-PNA

The designed *bimodal* C γ (S/R)-*bm*-PNAs individually bind DNAs complementary on each side to yield the corresponding binary C γ -amide and tert-amide duplexes¹⁶¹ Together, they hybridize on both sides of *bimodal* PNA backbone to generate ternary complexes, which show

biphasic double transition with higher stability compared to independent duplexes. $C\gamma(S)$ -duplexes show significantly higher stability than $C\gamma(R)$ duplexes.

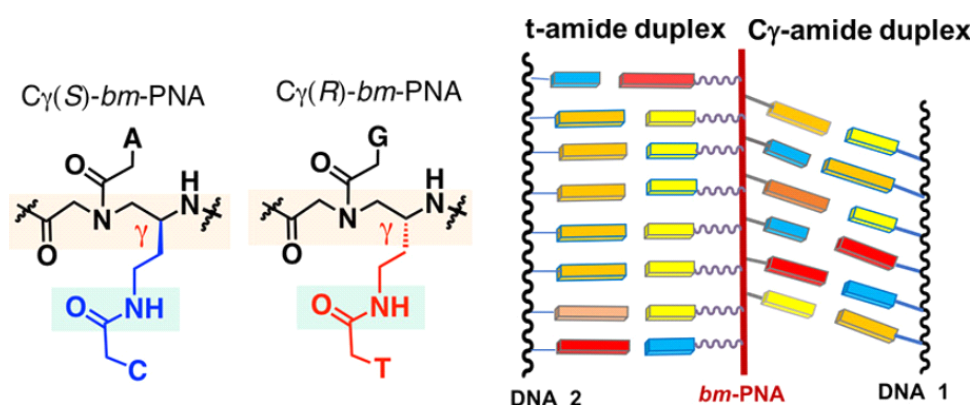


Figure 1.41 Double duplex from $C\gamma(S/R)$ -bm-PNAs¹⁶¹

1.12 Present work: Supramolecular assemblies of $C\alpha$ -bimodal PNAs

In *bm*-PNA, a homo oligomeric sequence poly T_n can form a triplex with dA_8 and duplex formation from the nucleobase linked to $C\alpha$ or $C\gamma$, results pentameric triplex of duplex complex.^{162, 163} *Bimodal* PNAs are thus good templates to organise nucleic acid assemblies. The present work illustrates that the possibilities of *bimodal* PNAs having poly C_n or Poly G_n sidechains can generate assemblies. Also, metal mediated base pairing in *bm*-PNA can assist to generate higher order assemblies which are applicable for nanoarchitecture

1.12.1 Rationale for present work

- ❖ The *bimodal* PNAs having C on $C\alpha$ sidechains to form an *i*-Motif scaffold to which DNA strands complementary to t-amide strand can bind to create tetraplex of tetra duplexes. They can also form hybrid tetraplexes with two strands of DNA / RNA binding to two *bm*-PNA strands, that leads to duplex of tetraplexes.
- ❖ Similarly, *bm*-PNA with poly G face can form derived G4-tetraplexes, at neutral pH in presence of metal ions (Na, K) that in turn can complex with complementary DNA to form supramolecular assemblies
- ❖ The base cytosine can also complex with metal ion like silver to give rise to metal mediated self-pairs. Thus, bimodal PNA having poly C on face can form $(bm-PNA)_2-Ag^+$ self-duplexes or with other poly C containing oligomers (dTC_5 or *aeg*-PNA- C_5) to

form mixed duplexes held by metal ions. These in turn can bind to complementary DNA to form corresponding duplex assemblies.

1.12.2 Chapter 2: Synthesis and Characterization of $C\alpha(S)$ -bimodal PNA monomers for supramolecular assemblies

This chapter describes, synthesis of $C\alpha$ -ethylazido substituted *aeg* PNA monomers (1-3) as rationally designed PNA analogues having side chains carrying azido function for conjugating different nucleobases by click reaction in resin. The propargyl nucleobases (4-6) can also be conjugated post assembly of PNA sequences by global click reaction.

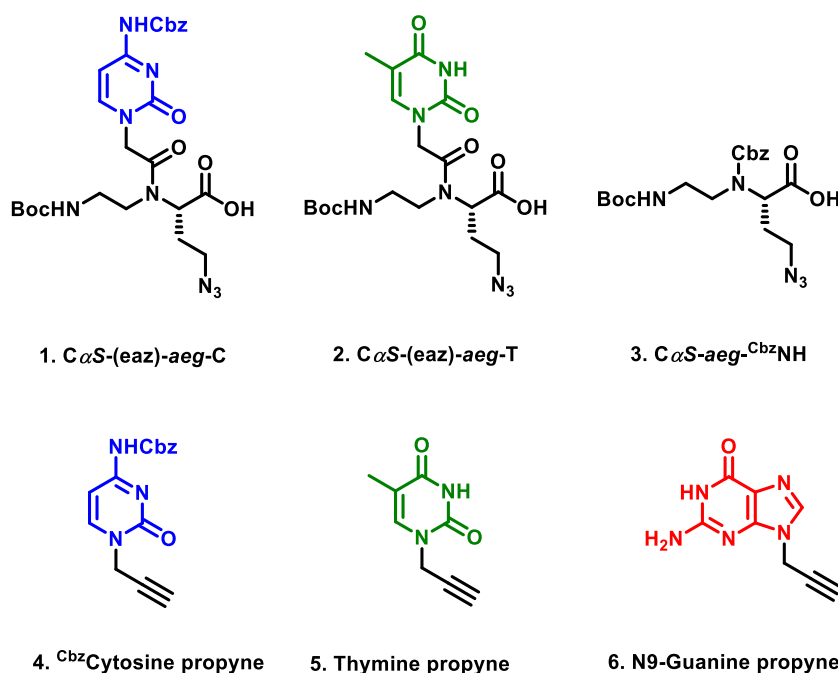


Figure 1.42 Monomer synthesised

1.12.3 Chapter 3: Solid phase synthesis, Purification and Characterization of bimodal PNA Oligomers

The above synthesised PNA monomers incorporated on solid phase followed by global click reaction to synthesise various types of *bm*-PNA oligomers. Synthesised oligomers cleaved from resin, purified by RP-HPLC and characterised by MALDI-TOF spectrometry.

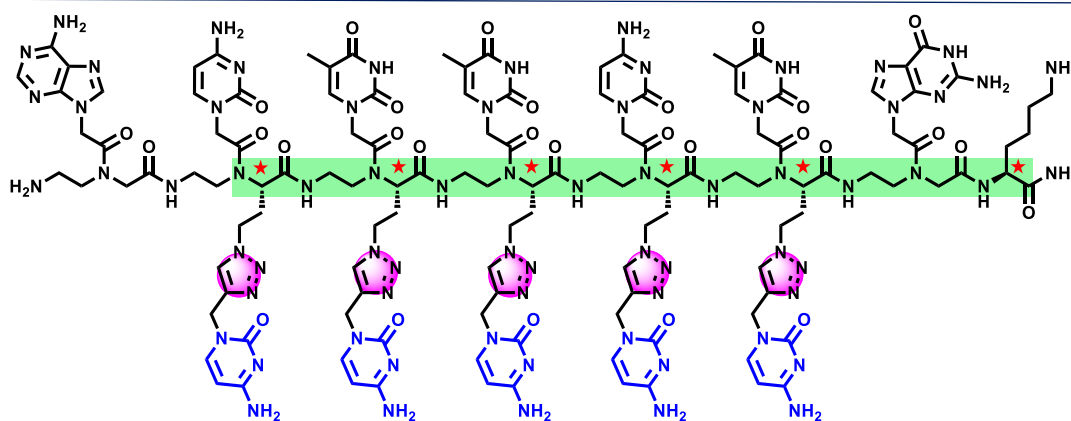


Figure 1.43 *bm*-PNA-C₅ oligomer

1.12.4 Chapter 4: Biophysical evaluation of *bm*-PNA i-motifs

This chapter deals with *i*-motif formation by *homo bimodal* PNA poly C oligomers analysed by pH dependent UV absorbance. Stability of these formed complexes studied by UV-*T_m* and CD spectroscopy. The stability of *i*-motif formed by *bm*-PNA with poly C side chains is compared with *i*-motifs formed by dTC₅, *aeg*-PNA-C₅, and *iso*-PNA-C₅. Similarly thermal stability of G quadruplex formed by *bimodal* PNA carrying poly G sidechain is compared with tetraplexes of dTG₅, *aeg*-PNA-G₅ and *iso*-PNA-G₅ at different salt concentrations. Further the possibilities of formation of multistranded assemblies by tetraplexes with complementary is explored.

1.12.5 Chapter 5: Silver and mercury assisted supramolecular assemblies of *bm*-PNA

This chapter investigates the silver and mercury assisted base pairing in the *bimodal*-PNA self-complexes as well as hybrid complexes such as *bm*-PNA:dTC₅, *bm*-PNA:*aeg*-PNA-C₅ and *iso*-PNA-C₅. Stability of these complexes analysed by temperature dependent UV absorbance, From CD spectroscopy conformation of complex determined. Morphological behaviour of these complexes monitored by FESEM and AFM technique. Finally, metal composition of these complexes investigated from Edax analysis.

1.12.6 Chapter 6: Synthesis of C5-substituted-Uracil *aeg*-PNA monomers, their oligomerization and biophysical studies

Chapter 6 reports on the synthesis of C5(H/ F / CF₃/NHR)-substituted uracil *aeg*-PNA monomers and incorporation into oligomers. Such modified PNA oligomers were hybridised with DNA and biophysical studies were studied to examine the effect of multiple base

modifications on the stability of derived PNA: DNA/RNA/PNA hybrids, using UV-T, CD and fluorescence spectroscopy.

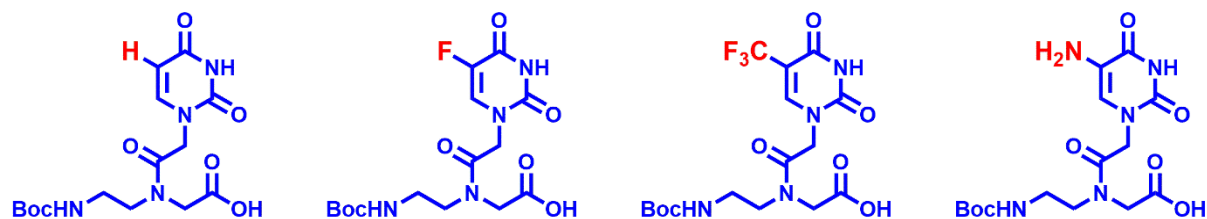


Figure 1.44 Modified PNA monomers

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162. Gupta, M. K.; Madhanagopal, B. R.; Ganesh, K. N., Peptide Nucleic Acid with Double Face: Homothymine–Homocytosine Bimodal C α -PNA (bm-C α -PNA) Forms a Double Duplex of the bm-PNA₂:DNA Triplex. *J. Org. Chem.* **2021**, 86, 414-428.
163. Bhingardev, P.; Jain, P.; Ganesh, K. N., Molecular Assembly of Triplex of Duplexes from Homothyminy–Homocytosiny C γ (S/R)-Bimodal Peptide Nucleic Acids with dA₈/dG₆ and the Cell Permeability of Bimodal Peptide Nucleic Acids. *ACS Omega* **2021**, 6, 19757-19770.

Chapter 2

Synthesis and characterization of C α (S)-ethyl azido PNA monomers: precursor for bimodal PNA oligomers

2.1. Introduction

Peptide nucleic acid (PNA) is an interesting scaffold developed by Nielsen and co-workers. It is a DNA mimic, with a neutral pseudo peptide backbone and capable of storing sequence-specific nucleotide code.¹ Unmodified PNA has some disadvantages such as poor cell permeability, low aqueous solubility and ambiguity in orientation of DNA binding. Several modifications of the PNA backbone have been attempted to overcome these limitations. Apart from a number of acyclic substitutions on *aeg*-backbone of PNA, conformationally constrained cyclic PNA analogues have been designed towards structural preorganization of backbone to address favorable attributes of PNA for selective hybridization with DNA/RNA.² Specifically, chiral PNA monomers have been developed to include modifications at the α -, β - and γ -sites on backbone, with unique synthetic routes and properties of the derived oligomers.³

Replacement of achiral glycine component of *aeg*-backbone with chiral amino acid having L- or D- form leads to C α -chiral PNAs. The oligomers synthesized from D-amino acids (C α S) show higher affinity towards complementary DNA than the L-form (C α R).⁴ Gem dimethyl substitution at C α - position of PNA monomer not only achiral but also increases binding stability towards cDNA than cRNA⁵.

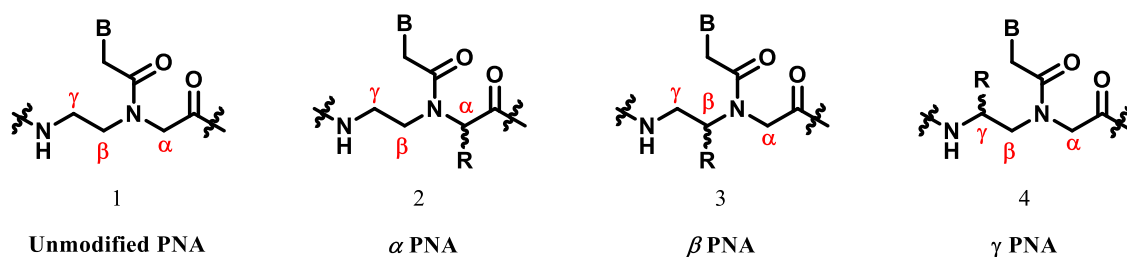


Figure 2.1 Chemical structures of Achiral and α , β , γ - modified PNA with amino acid side chain (B = nucleobase)

In 2011, Sugiyama et al.⁶ have introduced C β - modified PNA (Figure. 2.1). The C β modified *S*-form monomer derived PNA formed a superior hybrid with complementary DNA, than PNA oligomer from the *R*-form. The configuration of the stereogenic centers at C α , C β and C γ influences the preference of the PNA to form left-handed or right-handed helices. (Figure 2.1). This, in turn affects the stability of the PNA: DNA duplex through the control of helical handedness.⁷ Nielsen and co-workers⁸ were the first one to introduce PNA oligomers modified at C α - and Liang and co-workers⁹ reported C γ -modified PNA backbones. Following these leads, Danith Ly group^{10,11} showed that C γ -substituted guanidine PNA has more affinity towards complementary DNA and RNA than C α -guanidine modification. Similar results on DNA binding was observed by Ganesh *et al.* in the case of C α - and C γ -methylene PNA.¹²

In contrast to unmodified PNA, backbone modified PNAs have significant potential in antisense applications. There are several PNA derivatives of C α - or C γ - modifications on *aeg*-PNA backbone, such as cationic side chain guanidine, aminobutyl (lysine), and many other functional groups from amino acid side chains.¹³ However, the stereochemistry of the side chain substituents at C α - and C γ - seems to be important for the derived PNA oligomers in exhibiting efficient hybridization properties with *R* configuration at C α and *S* configuration at C γ .^{14, 15} This chapter describes synthesis and characterization of various C α -ethyl azido substituted PNA monomers (Figure 2.6) that are precursors for assembling *bimodal* PNA oligomers (Figure 2.3) through the coupling of pre-synthesized *bimodal* triazolyl monomers by post-assembly click reaction for *bimodal* homo-oligomers.¹⁶

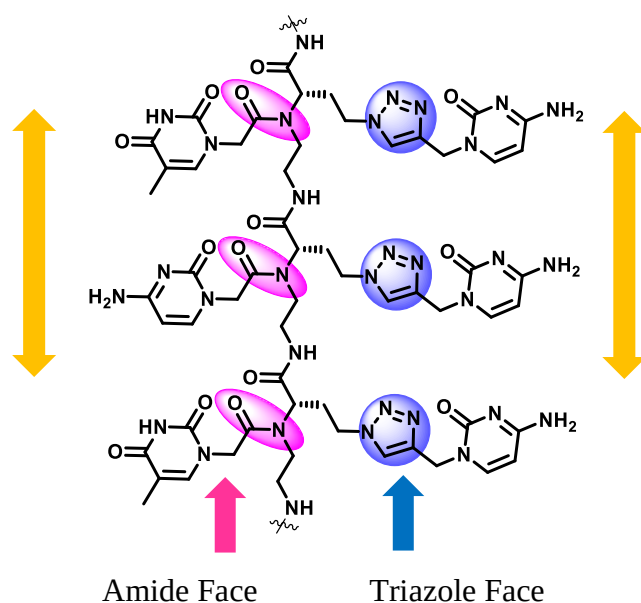


Figure 2.2 Representation of *bm*-PNA oligomers having an amide and a triazole face

2.2 Rationale of the present study

As far as the regio and stereospecificity of the chiral monomer is concerned, PNAs having monomers derived from D-amino acids ($C\alpha$ -*R*) in the glylcyl fragment of *aeg* backbone bind to the complementary DNA with greater thermal stability than those derived from the corresponding L-amino acids ($C\alpha$ -*S*) at glycine site.¹² Substitutions in *eda* fragment of *aeg* backbone derived from L-amino acids translate into $C\gamma$ -*S* PNA oligomers which bind to complementary DNA/RNA with higher affinity.¹¹ This situation turns out to be the reverse for substitutions of chiral amino acids in glycine segment in *aeg* backbone. The direct correspondence between D/L amino acids with *R/S* configuration in the derived PNA backbone should be interpreted with care since for certain amino acids (e.g. for serine and cysteine), the L-amino acid stereochemistry translates into *R*-stereochemistry in the PNA backbone.

Recently, Ganesh *et al* introduced new class of PNA structures termed as *bimodal* PNA.^{16, 17} This *bimodal* PNA has nucleobases on two faces: t-amide face as in standard PNA and another newly created face at $C\alpha$ or $C\gamma$. The interbase distances between two adjacent bases on both faces in *bimodal* PNAs are exactly similar to the DNA/RNA. This novel *bimodal* PNA targets two different DNAs/RNAs from two faces. These modified PNAs are synthesized from a $C\alpha/C\gamma$ -substituted PNA monomers. These *S*-ethyl azido monomers incorporates substitution at $C\alpha$ in the *aeg* PNA backbone to result α -(*S*-ethyl azido) aminoethyl glycy C/T PNA monomers (**1-2**) required for solid phase synthesis of *bimodal* PNA homo oligomers through click reaction. The propyne nucleobases (**4,5,6**) monomers can be clicked on resin with $C\alpha$ azido monomer to obtain desired oligomers (Figure 2.3, 2.4 and 2.5) in which the second nucleobase is linked by triazole linker and can be directly used to assemble mixed sequence *bimodal* PNA oligomers. In order to examine the potential of $C\alpha$ -triazolyl linker for complementation of DNA, control oligomer as *iso*-PNA with nucleobases linked only by triazole spacer, without any bases on t-amide was synthesized using the precursor monomers (**3**) by post solid phase click reaction with the propargyl nucleobases (**4,5,6**)

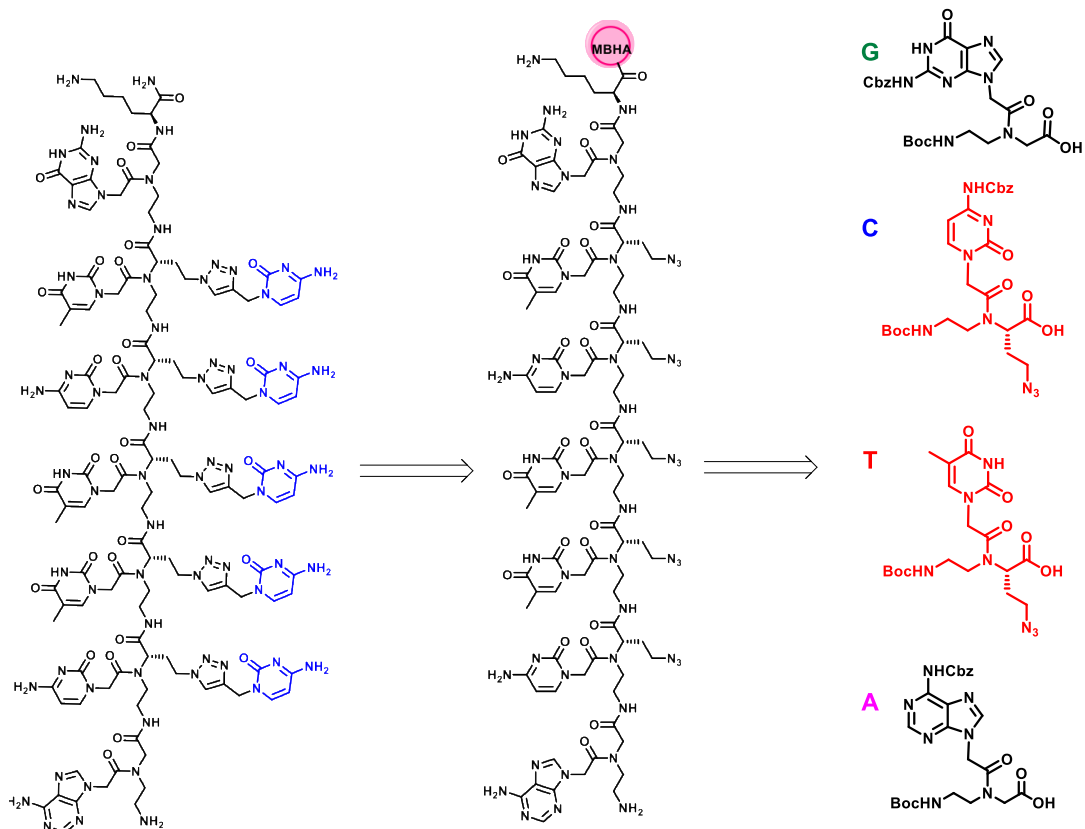


Figure 2.3 Retrosynthetic strategy of *bm*-PNA 1 oligomer

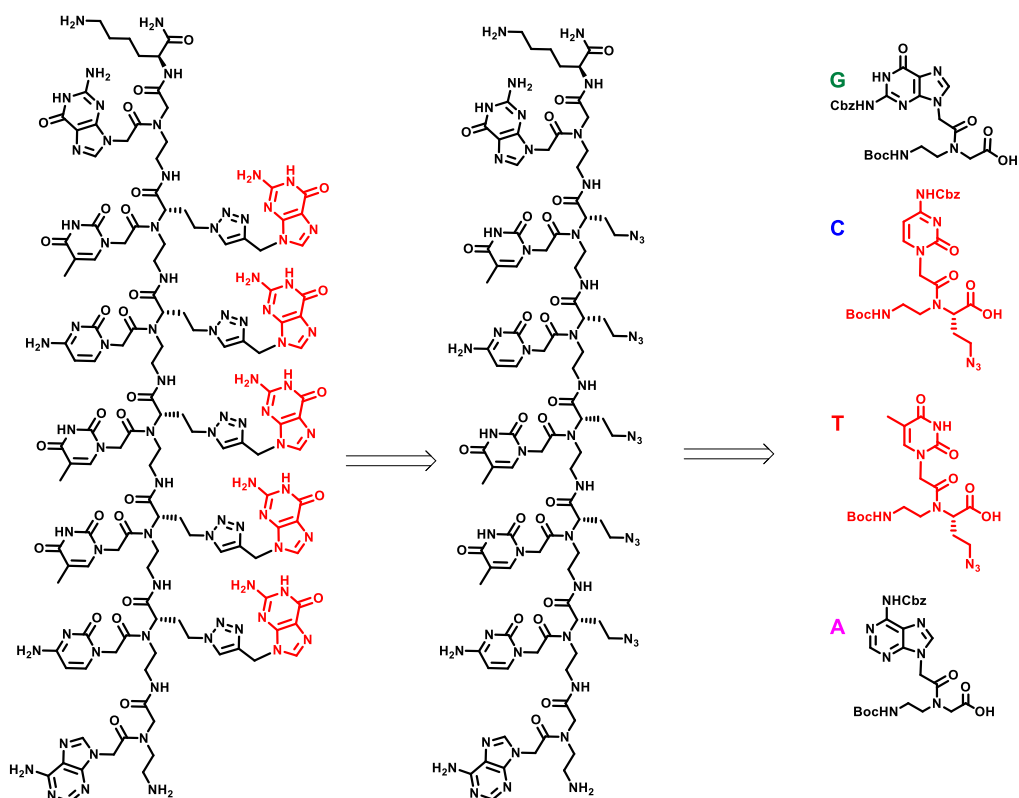


Figure 2.4 Retrosynthetic strategy of *bm*-PNA 3 oligomer

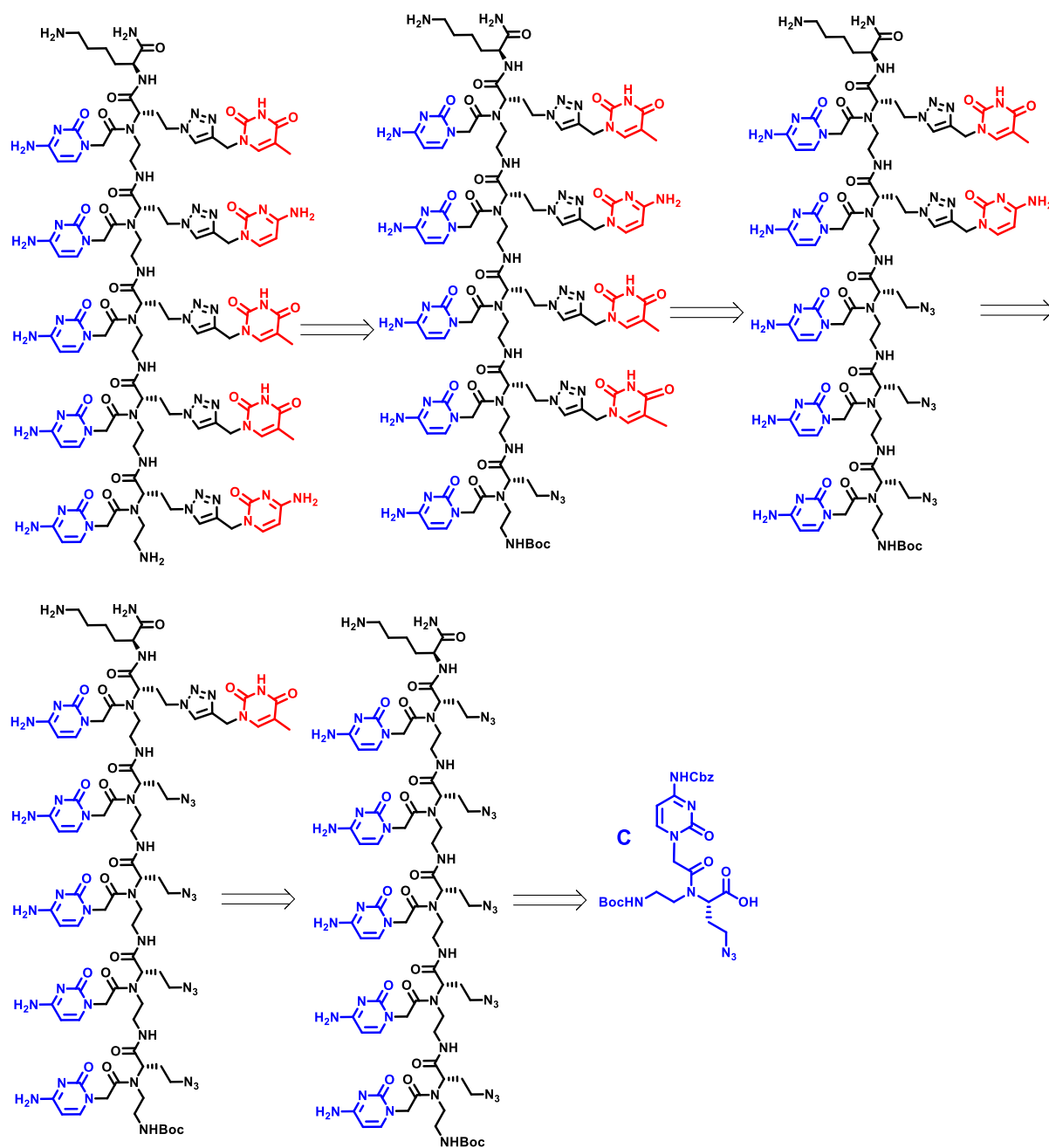


Figure 2.5 Retrosynthetic strategy of *bm*-PNA 2 oligomer

The precursors for *bm*-PNA oligomer (*bm*-PNA 1 and *bm*-PNA 2) and *bm*-PNA 3 are synthesized from ethyl azido T/C monomers. The azide side chain at C α can be conjugated with G/C/T base propynes via click reaction.¹⁶ The synthesized *bm*-PNA oligomers were used for studying i-Motif, and metal mediated complexation to generate multistranded complexes.

2.3 Objectives of the present work:

- Synthesis of $C\alpha$ (S-ethylazido) *bm*-PNA Thymine, Cytosine monomers (**1, 2**)
- Synthesis of $C\alpha$ (S-ethylazido) *iso*-PNA monomer (**3**)
- Synthesis of Propyne derivatives of ^{Cbz}Cytosine, Thymine and Guanine (**4, 5 and 6**)

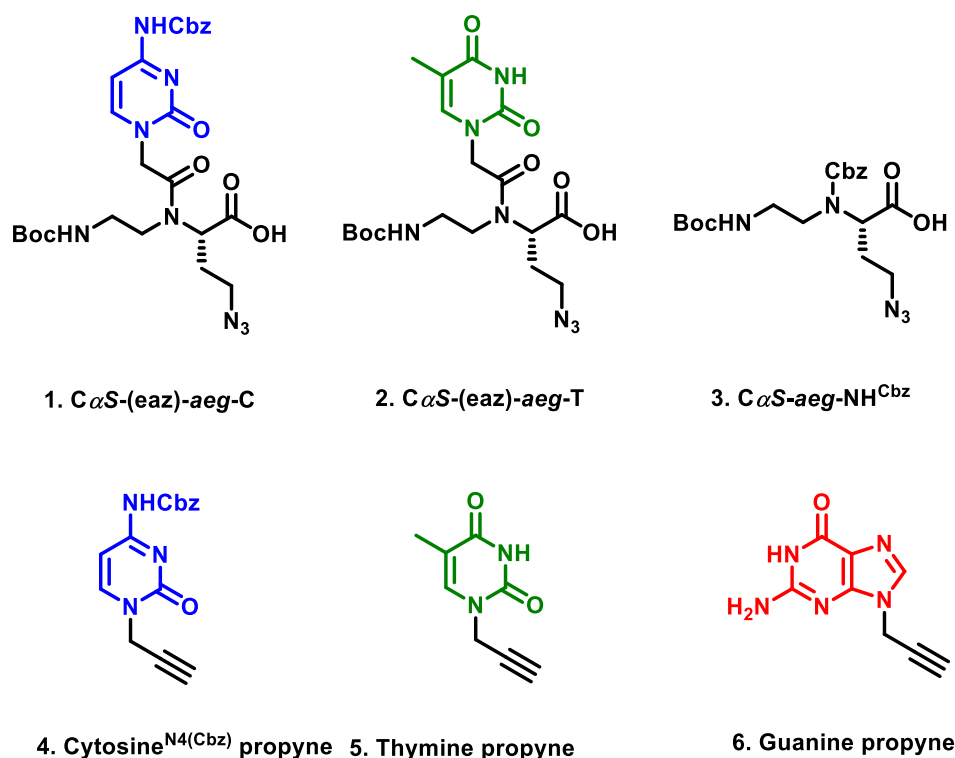


Figure 2.6 Target $C\alpha$ modified *bm*-PNA monomers and propyne derivatives

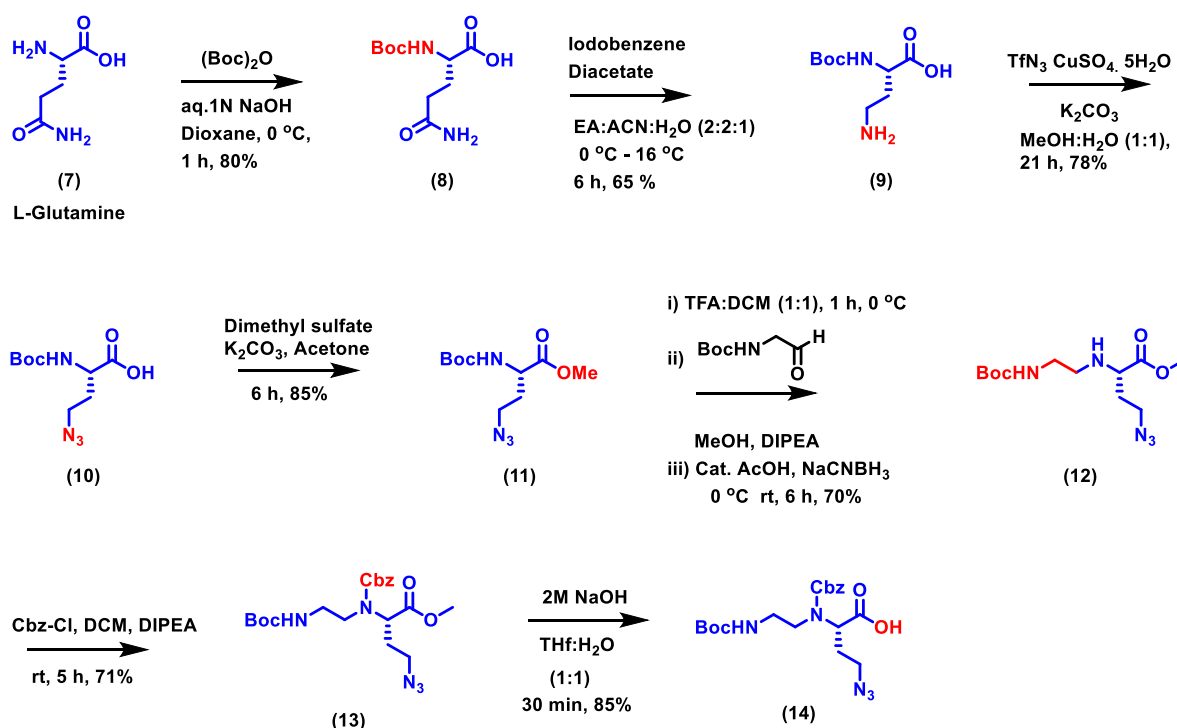
2.4 Synthesis of $C\alpha$ modified *bimodal* PNA monomers¹⁶

This section describes the synthesis of rationally designed $C\alpha$ -S-(eaz)-aeg-T and $C\alpha$ -S-(eaz)-aeg-C, $C\alpha$ -S-(eaz)-aeg-NH^{Cbz} monomer and N1-propyne-Thymine, N1-propyne Cytosine^{N4(Cbz)} and N9-propyne Guanine (**1-6**).

2.4.1 Synthesis of $C\alpha$ (S-eaz)-aeg-NH^{Cbz} monomer (**14**)

The synthesis of desired compound **8** was started from commercially available L-glutamine **7** (Scheme 2.1). After treatment with di-*t*-butyl decarbonate [(Boc)₂O] it gave protected NHBoc-L-Glutamine **8**. The amido group in **8** was converted to amino function, by reaction with iodobenzenediacetate (IBA) to obtain 4-amino-2-(N-tert-butoxycarbonyl)amino-

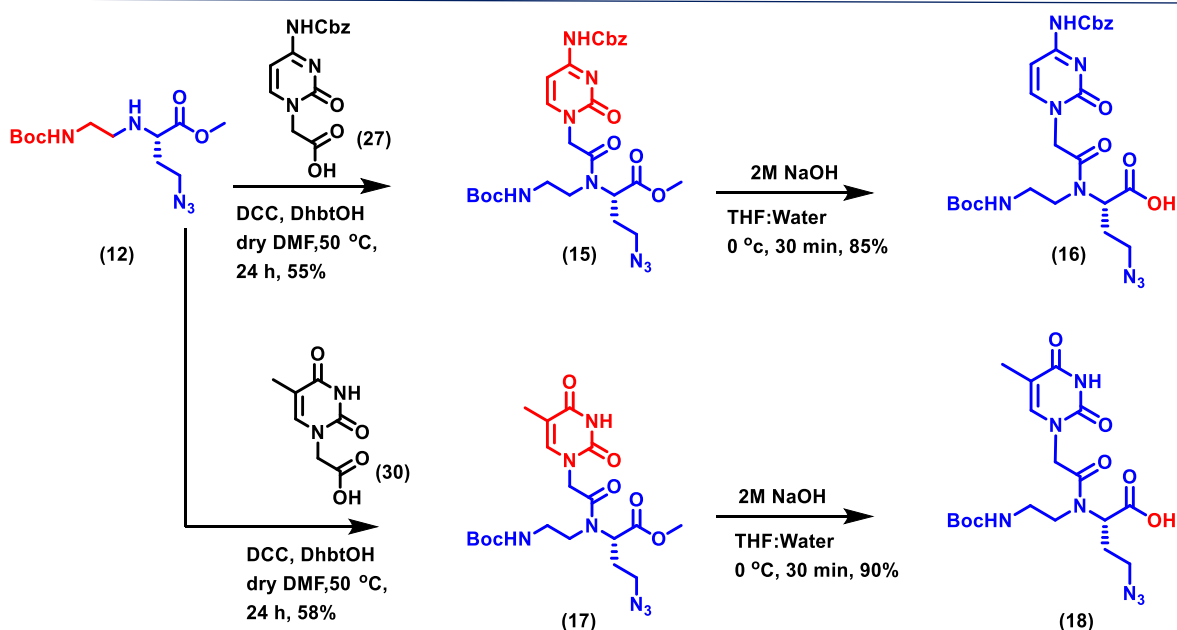
butanoic acid **9**. This was followed by transformation of the primary amine in **9** to azide **10** through diazo transfer reaction by treatment with triflic anhydride and sodium azide (in-situ generation of triflyl azide) in presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ¹⁸ Then resultant acid treated with dimethyl sulfate to forms methyl ester derivative **11**. This compound reacted with NHBoc aminoacetaldehyde to yield C α -substituted aminoethyl glycyl derivative **12** in good yield. The secondary amino group in **12** was protected as Cbz group to get compound **13** that was hydrolysed under basic conditions to afford the desired N^{Boc},N^{Cbz} aminoethyl (C α -eaz) glycine **14** in very good yield. This monomer was used for solid phase synthesis of *iso*-PNA which has nucleobases only on the triazole face and for further synthesis of other target *bimodal* PNA monomers.



Scheme 2.1. Synthesis of N^{Boc},N^{Cbz} aminoethyl (C α -eaz) glycine **14**

2.4.2 Synthesis of N^{Boc},N[′](N1-acetamido-T) aminoethyl (C α -eaz) glycine methy ester (**11**) and N^{Boc},N[′](N⁴Cbz-N1- acetamido C) aminoethyl (C α -eaz) glycine methy ester (**16**)

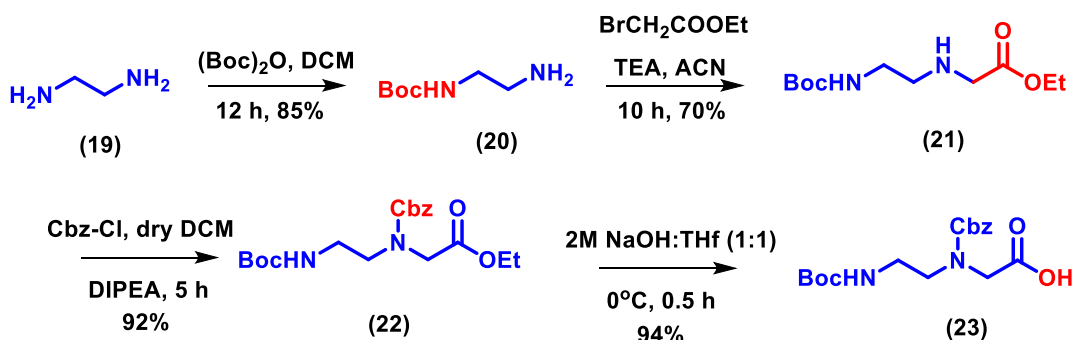
The compound **12** was coupled with N⁴(Cbz)-Cytosine N-acetic acid (**27**) and Thymine N1-acetic acid (**30**) at high temperature using DhBtOH and DCC as coupling agent (Scheme 2.2)¹⁹ to obtain the esters **15** and **17** These were hydrolyzed under basic conditions to afford the C α -S-(ethylazido)-C (**16**) and C α -S-(ethylazido)-T (**18**) acid monomers.



Scheme 2.2 synthesis of $C\alpha$ -S-(eaz)-aeg- N^{Cbz} Cytosine and Thymine monomers

2.4.3 Synthesis of (N^{Boc} , NH^{Cbz}) aminoethyl glycine (23)

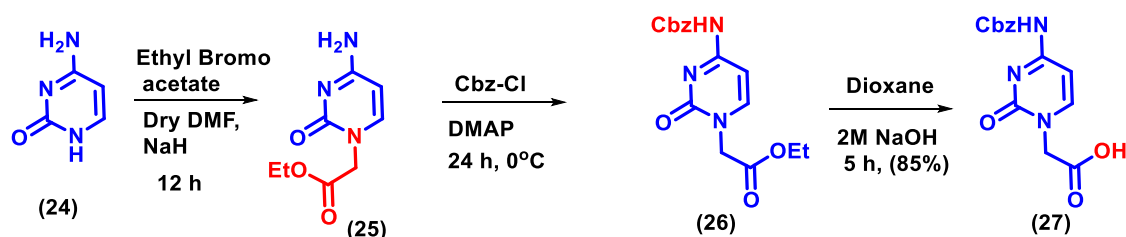
The commercially available ethylene diamine **19** was treated with di-*t*-butyl decarbonate [(Boc)₂O] to get mono NH-Boc-protected ethylenediamine **20** (Scheme 2.3). This was N-alkylated by the reaction with ethyl bromoacetate, triethylamine in acetonitrile as solvent to yield compound **21**.¹⁶ This was then protected with Cbz group in presence of DIPEA in dry DCM as reported in to obtain Compound **22** which was further hydrolyzed under basic conditions to afford the desired NHBoc protected monomer **23** in very good yield for synthesis of *iso*-PNA-tz-B₅ oligomers.



Scheme 2.3 Synthesis of N1(Boc), N2(Cbz) aminoethyl glycine (22)

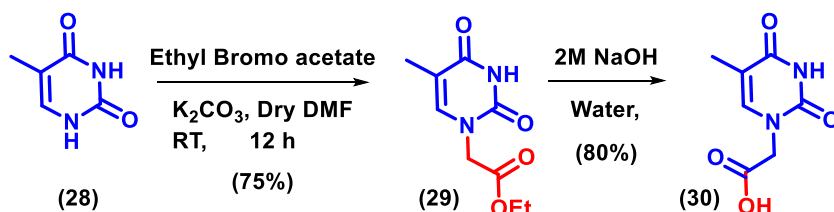
2.4.4 Synthesis of nucleobase acetic acids

2.4.4a Synthesis of (N⁴-benzyloxycarbonyl,N1-cytidinyl)-acetic acid. Cytosine **24** was alkylated with ethylbromo acetate in the presence of NaH in dry DMF to obtain compound **25**. The N⁴-amino group of N-alkylated Cytosine **25** was protected by reaction with benzyloxycarbonyl chloride in pyridine (Scheme 2.4). The ester compound **26** hydrolysis in basic medium to afford cytosine acetic acid **27** in 85% overall yield.²⁰



Scheme 2.4 Synthesis of (N⁴Cbz) cytidinyl N1-acetic acid (**27**).

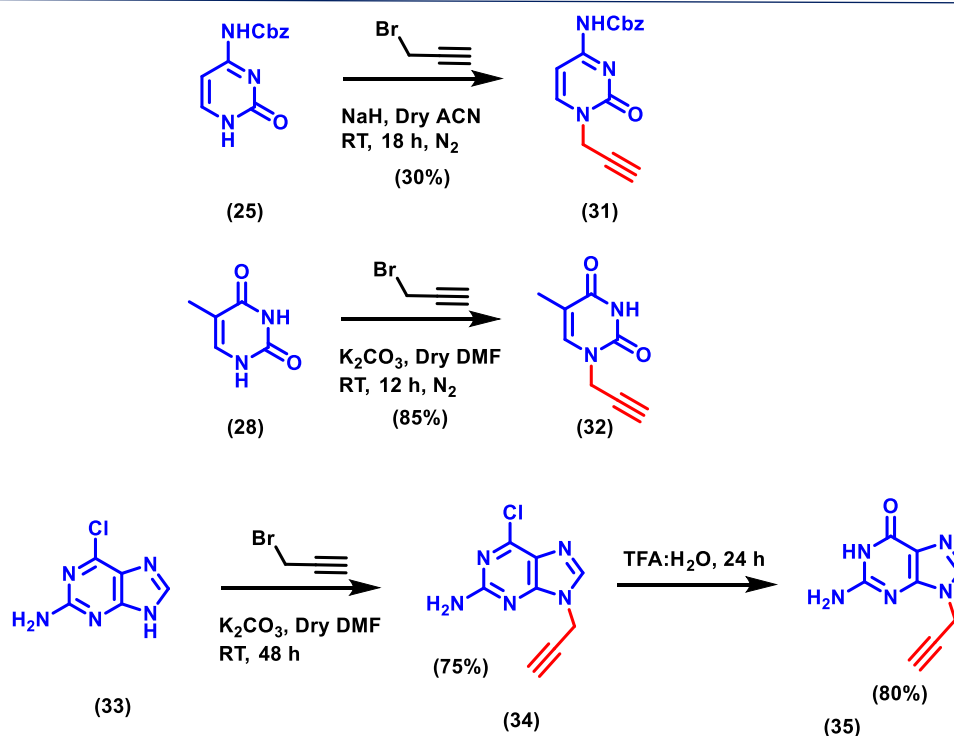
2.4.4b Synthesis of Thymine-N1-yl acetic acid. The commercially available Thymine **28** on alkylation in presence of ethylbromo acetate formed the ester **29**. This was hydrolyzed with alkali to get the compound **30**. (Scheme 2.5)²¹



Scheme 2.5 Synthesis of -Thymine N1-acetic acid (**30**).

2.4.5 Synthesis of N1-propargyl C/T and N9-propargyl G

The N⁴-Cbz protected cytosine **25** was subjected to propargylation at N1 in the presence of NaH in Dry ACN to afford the compound **31** (Scheme 2.6)²². A similar reaction with thymine **33** gave the compound **32** in good yields²³. 6-Amino-9-Chloro-purine on treatment with propargyl bromide in K₂CO₃ for 48 h gave **34** which further treated with TFA:water yielded N9-propargyl G²⁴



Scheme 2.6: Synthesis of N1-propargyl (N^4 CBz) cytosine (31), (N1-propargyl) thymine (32) and (N9-propargyl) Guanine (35)

2.5 Experimental section

This section describes the detailed synthetic procedures and spectral characterization of the rationally designed PNA monomers.

2.5.1 General procedures

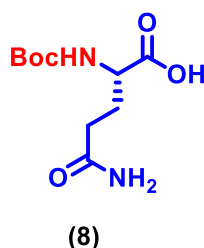
All reactions were conducted in oven-dried glassware under argon atmosphere. Low temperature experiments were carried out by cooling down the flasks with ice bath. The flasks were equipped with septum caps. All commercial solvents were distilled prior to use. THF was distilled over sodium/ benzophenone under nitrogen atmosphere, CH_2Cl_2 over CaH_2 and DMF over P_2O_5 . Analytical thin layer chromatography (TLC) was performed on precoated 250 μm layer thickness silica gel GF254 (Merck 5554) plates. Visualization was performed by ultraviolet light and staining with a solution of ninhydrin. Column chromatography was performed using 100-200 μm silica gel. ^1H NMR and ^{13}C NMR, were recorded using JEOL and Bruker 400 MHz NMR spectrometer at 100 MHz respectively. ^{13}C NMR were recorded under broadband decoupling conditions. The delta (δ) values for chemical shifts are reported in ppm and are referred to internal standard TMS or deuterated NMR grade solvents. The optical rotation values were obtained on Rudolph Research Analytical Autopol V polarimeter.

Mass spectra for all reaction products were recorded using Synapt G2 high-definition mass spectrometry.

2.5.2 Experimental procedures and spectral data

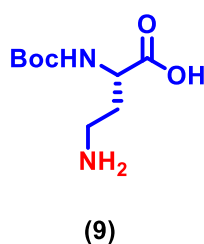
Each intermediate and monomers that were synthesized, and purified according to standard reported protocol.¹⁶ The characterization data for all known compounds were consistent with the previously reported data.¹⁶

4-Carboxyamido-2(*S*)-amino(NH-*t*-butoxycarbonyl) pentanoic acid (**8**)



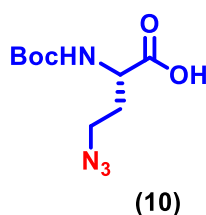
A solution of di-*tert*-butyl dicarbonate [(Boc)₂O] (16.4 g, 17.3 ml, 75 mmol) in dioxane (100 ml) was added in portion to an ice-cold solution of L-glutamine **7** (10 g, 68.5 mmol) in aq. NaOH (1 N, 100 ml). The reaction mixture was further stirred at 0 °C for 1 h. After completion of the reaction, dioxane was removed completely under vacuum from the reaction mixture. The aqueous layer was washed with diethyl ether to remove excess (Boc)₂O. The aqueous layer was cooled in an ice-water bath, acidified to pH 2-3 by slow addition of saturated KHSO₄ solution and then extracted with ethyl acetate (3×150 ml). The combined organic extracts were dried over anhydrous Na₂SO₄, filtered and concentrated to give compound **8** as a white powder, which was used without further purification (14.5 g, 86% yield). M.p = 117-120 °C; R_f = 0.40 in EtOAc/MeOH (50:50); [α]²⁵_D 3.1 (c 0.5, Methanol); MS (HRMS-ESI) *m/z* calcd. for C₁₀H₁₈N₂O₅ [M+H]⁺ 247.1294, Observed: 247.1292.

4-Amino-2(*S*)-amino(NH-*t*-butoxycarbonyl) butanoic acid (**9**)



A slurry of compound **8** (5 g, 20 mmol) in ethyl acetate:acetonitrile (1:1, 48 ml) and water (12 ml) was treated with iodobenzene diacetate (7.9 g, 25 mmol) and cooled with stirring at 16 °C for 30 min. The temperature was allowed to reach 20 °C and the reaction mixture was stirred until completion (approximately 4 h). as followed by TLC. The reaction mixture was then cooled to 0 °C and filtered under vacuum. The filter cake was washed with ethyl acetate and dried in vacuum to obtain compound **9** (2.7 g, 59% yield). M_p = 200-201 °C; R_f = 0.2 EtOAc/MeOH (50:50); [α]_D²⁵ +13.6 (c 0.5, Methanol); HRMS (ESI) *m/z* calcd. for C₉H₁₉N₂O₄ [M+H]⁺ 219.1345, obsd. [219.1346.

4-Azido-2(S)-amino(NH-*t*-butoxycarbonyl) methyl butanoate (**10**)

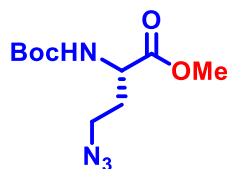


Triflic azide preparation: Sodium azide (2.7 g, 41.3 mmol) was dissolved in DCM (16 ml) and water (8 mL) and solution cooled to 0 °C. Triflic anhydride (1.4 mL, 8.3 mmol) was added slowly while stirring and left to stir for 2 h. After phase separation, the aq. phase was extracted with DCM (2x20 mL). The combined organic phase was washed with saturated sodium carbonate solution. The triflic azide prepared was used directly for next step reaction without purification.

Caution: Triflic anhydride is hazardous and explosive and appropriate care was taken during handling the reagent.

Diazo transfer reaction: A solution of Boc-(L) protected amine (0.9 g, 4.13 mmol), CuSO₄·H₂O (10 mg, 0.04 mmol) and K₂CO₃ (0.9 g, 6.2 mmol) in H₂O (16 mL) and MeOH (24 mL) was treated with triflic azide solution while stirring by dropping funnel. After overnight reaction at RT, the organics were removed by evaporation, and the remaining aqueous fraction was extracted with DCM (2x40 ml). The aqueous layer was diluted with water (20 ml) and acidified to pH 2.0 with aq. HCl (1M) and extracted with DCM (3x40 ml). The combined organic layers were washed with aq. (sat) brine (50 ml), dried (MgSO₄), filtered and concentrated *in vacuo* to afford the product of 2(S)-amino(NH-*t*-butoxycarbonyl)-azidobutanoic acid **10** (65% yield) as a colourless oil. HRMS (ESI) *m/z* calcd. for C₉H₁₇N₄O₄ [M+H]⁺ 245.1250, obsd. 245.1243.

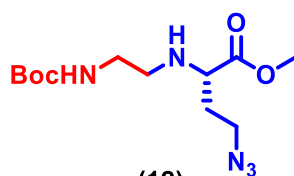
4-Azido-2(S)-amino(NH-*t*-butoxycarbonyl) methyl butanoate (**11**)



(11)

To a stirred solution of compound **10** (5.9 g, 24.2 mmol) and K₂CO₃ (8.35 g, 60 mmol) in dry acetone (100 ml) was added dimethyl sulfate (2.7 ml, 21.8 mmol, *dimethyl sulphate is carcinogenic, safety gears such as goggles, mask and gloves used while setting reaction*) and the reaction mixture was heated to 55 °C for 4 h under reflux condenser. Acetone was evaporated completely and water (100 ml) was added to the concentrate, which was then extracted with ethyl acetate (EtOAc) (3x80 ml). The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated. The residue was purified on silica gel (60-120 mesh) using petroleum ether (pet ether) and EtOAc to give compound **11** as colorless liquid (5.3 g, 85% yield); R_f = 0.5 pet ether/EtOAc (70:30); [α]²⁵_D -17.8° (c 1, Methanol); IR (neat cm⁻¹) 3358, 2975, 2098, 1702, 1511, 1445, 1359, 1247, 1159, 1054, 859, 775, 636, 557; ¹H NMR (400 MHz, CDCl₃), δ, 5.25 (s, 1H), 4.35 (s 1H), 3.72 (s, 3H), 3.36 (t, J = 6.6 Hz, 2H), 2.16 – 1.78 (m, 2H), 1.40 (s, 9H). The peak at δ 3.9, arises from solvent impurity. ¹³C NMR (100 MHz, CDCl₃) δ 172.5 (s), 155.4 (s), 80.2 (s), 58.6 (s), 52.6 (s), 51.3 (s), 47.8 (s), 31.8 (s), 28.3 (s). HRMS (ESI-TOF) m/z calcd. for C₁₀H₁₈N₄NaO₄ [M Na]⁺ 281.1226, Obsd. 281.1223.

4-Azido-[2(S)-amino(N-ethylamino-t-butoxycarbonyl)] methyl butanoate (12)

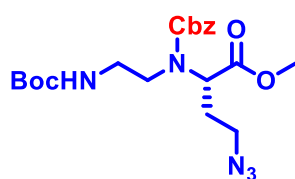


(12)

TFA (1 ml) was added to compound **11** (500 mg) which was dissolved in DCM (1 mL) at 0 °C. The resulting mixture was stirred for 1 h. The volatiles were removed under vacuum, washed crude product by toluene (5 ml) to obtain TFA salt of deprotected amine of **11** (760 mg) as crude oil. Without further purification, this was dissolved in methanol (10 mL) to which N-Boc-amino acetaldehyde (250 mg 1.50 mmol) in MeOH (2.5 ml) was added, while continuous stirring at 0 °C. DIPEA (0.75 ml, 4.26 mmol) was slowly added dropwise to the stirred solution. After stirring for 30 min, glacial acetic acid (0.2 ml) and NaCNBH₃ (130 mg, 2.13 mmol) were sequentially added and the reaction mixture was stirred for 3 h. All volatiles

were removed under vacuum and the residue was dissolved in EtOAc and extracted with saturated aq. NaHCO₃. The organic phase after drying with MgSO₄, was purified by column chromatography to obtain **12** (430 mg, 75.9%) ¹H NMR (400 MHz, DMSO-d₆) δ 3.49 – 3.36 (m, 2H), 3.35 – 3.22 (m, 3H), 3.06 – 2.86 (m, 2H), 2.55 (dt, *J* = 13.1, 6.6 Hz, 1H), 2.44 – 2.07 (m, 2H), 1.90 – 1.62 (m, 2H), 1.37 (s, 9H). ¹³C NMR (100 MHz, DMSO-d₆) δ 174.6 155.6, 115.6, 77.5, 57.95, 51.55, 47.61, 46.97, 31.62, 28.23. HRMS (ESI-TOF) *m/z* calcd. for C₁₂H₂₃N₅O₄ [M+H]⁺ 302.1828, Obsd. 302.1829

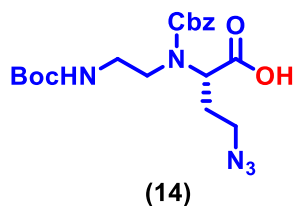
4-Azido-[2(S)-amino(N-benzyloxycarbonyl-N-ethylamino-t-butoxycarbonyl)] methyl butanoate (13)



(13)

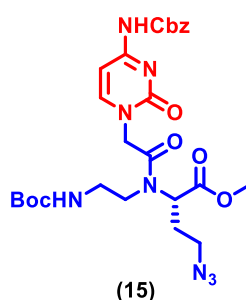
To a solution of **12** (0.65 g, 2.17 mmol) in DCM (15 ml) at 0°C was added benzyl chloroformate (0.75 g, 4.35 mmol) dropwise followed by addition of N, N-diisopropylethylamine (1.2 g, 8.7 mmol). After stirring at room temperature for 6 h, saturated aq. NH₄Cl solution (30 ml) was added and extracted with DCM (4 x 30 ml). The organic layers were dried over MgSO₄, concentrated *in vacuo* and purified with flash chromatography (Eluent: 50-60% EtOAc in hexane) to afford compound **32** (0.67 g, 70%) as yellow oil; [α]_D²⁵ -66.4° (*c* 1, MeOH); IR (neat, cm⁻¹): 3887, 3735, 3388, 2973, 2100, 1697, 1509, 1455, 1362, 1246, 1162, 1084, 1046, 1002, 864, 735, 699, 607; ¹H NMR (400 MHz, CDCl₃) δ: 7.35 – 7.3 (m, 5H), 5.21– 5.04 (m, 3H), 4.15 – 4.09 (m, 1H), 3.76 – 3.69 (m, 2H), 3.54 – 3.45 (m, 2H), 3.35 – 3.28 (m, 3H), 3.21 – 3.15 (m, 1H), 2.36 – 2.17 (m, 2H), 2.11 – 1.8 (m, 1H), 1.42 ppm (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ: 171.6, 156.0, 136.2, 128.7, 128.4, 128.3, 127.9, 79.4, 67.8, 58.9, 52.6, 48.9, 48.5, 39.6, 29.9, 28.9 ppm; HRMS (ESI-TOF) *m/z* calcd. C₂₀H₂₉N₅O₆Na [M+Na]⁺ 458.2016, obsd. 458.2018

4-Azido-[2(S)-amino(N-benzyloxycarbonyl-N-ethylamino-t-butoxycarbonyl)] butanoic acid (14)



To a stirred solution of compound **13** (0.65 g, 1.55 mmol) in THF (12mL) was added aq. NaOH (2 M, 12 ml) at 0 °C, and the reaction mixture was stirred for 30 min at 0 °C. Upon completion of the reaction, as confirmed by TLC, water (10 mL) was added, and the resulting mixture was extracted with EtOAc (2×30 ml). The aqueous layer was neutralized with saturated aq. KHSO₄ at 0 °C to pH 3.4 and then extracted with EtOAc (4×30 ml). The combined organic extracts were dried over anhydrous Na₂SO₄. The solvent was evaporated in vacuo, and crude product was purified by column chromatography (Eluent: 60–70% EtOAc in hexane) to afford monomer **14** (0.6 g, 91%) as a hygroscopic solid. $[\alpha]^{25}_D$ -42.0° (c 1, MeOH); IR (neat) 3825, 3736, 3359, 2977, 2313, 2100, 1696, 1513, 1459, 1420, 1364, 1247, 1159, 1083, 1047.10, 858, 771, 734, 698, 561, 531cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ: 7.37 – 7.29 (m, 5H), 6.87 – 6.51 (m, 1H), 5.11– 4.97 (m, 2H), 4.27 - 4.23 (dd, *J* = 9.0, 5.0 Hz, 1H), 3.49 – 3.33 (m, 3H), 3.12 - 2.95 (m, 3H), 2.19 - 1.92 (m, 2H), 1.37 ppm (s, 9H); ¹³C NMR (100 MHz, DMSO-d₆, 25°C) δ: 172.3, 155.5, 136.7, 128.3, 127.7, 126.6, 126.4, 79.2, 66.3, 62.8, 58.1, 48.1, 46.9, 28.4, 28.1 ppm, HRMS (ESI-TOF) *m/z* calcd. C₁₉H₂₈N₅O₆ [M+H]⁺ 422.2040, obsd 422.2045.

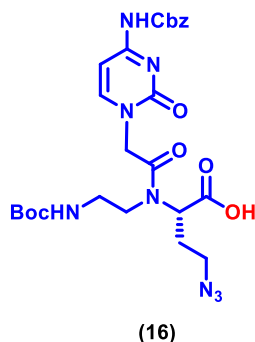
4-Azido-[2(S)-amino(N-ethylamino-t-butoxycarbonyl)-N-acetamido(2-oxopyrimidin-1(2H)-yl)-N⁴(benzyloxycarbonyl)] methyl butanoate (15)



To a stirred solution of N⁴-benzyloxycarbonyl cytidinyl-N¹-acetic acid, **22** (900 mg, 2.9 mmol) in dry DMF (25 ml) under an inert atmosphere were added DCC (680 mg, 3.3 mmol) and DhbtOH (540 mg, 3.3 mmol). The resulting mixture was stirred at room temperature for 1 h. Compound **12** (1 g, 3.3 mmol) in dry DMF (30 ml) was added to the above reaction mixture, and stirred at 50 °C for 24 h. The solvent was removed under reduced pressure, and the remaining residue was dissolved in EtOAc (300 ml) and washed with a saturated aq. NaHCO₃

(300 ml). The organic layer was washed with brine (3×150 ml) and dried over Na₂SO₄. The solvent was removed under reduced pressure. The crude product was purified by column chromatography to afford **15** (800 mg ,45.6%) Eluent: 80-90 % EtOAc in petroleum ether, m.p.75-78 °C; [α]_D²⁵ -23.2° (c 1, MeOH); IR (neat, cm⁻¹): 3254, 2972, 2101,1740, 1659, 1557, 1498, 1447, 1366, 1193, 1063, 997, 853, 790, 736, 698, 629, 573; ¹H NMR (400 MHz, CDCl₃) δ : 7.83 (s, 1H), 7.56 (s, 1H), 7.38 (d, *J* = 3.0 Hz, 5H), 7.24 (d, *J* = 7.3 Hz, 1H), 5.57 (s, 1H), 5.25 –5.17 (m, 2H), 5.03 (d, *J* = 15.4 Hz, 1H), 4.25 (dd, *J* = 14.2, 7.0 Hz, 1H), 3.96 – 3.78 (m, 2H),3.75 (s, 3H), 3.52 (dt, *J* = 12.5, 4.9 Hz, 2H), 3.38 (dt, *J* = 19.0, 5.8 Hz, 2H), 3.25 (d, *J* = 5.4 Hz,1H), 2.29 (dd, *J* = 12.6, 7.0 Hz, 2H), 1.43 (d, *J* = 8.6 Hz, 9H); ¹³C NMR (100 MHz, CDCl₃) δ : 171.2, 167.2, 162.9, 156.0, 155.6,152.2, 149.6, 134.93, 128.7, 128.3, 95.0, 80.0,68.0, 59.0, 52.8, 50.3, 49.5, 48.1, 38.93, 28.5, 28.1, 20.3; HRMS (ESI-TOF) *m/z* calcd for C₂₆H₃₅N₈O₈ [M + H]⁺ 587.2578, found 587.2581. HRMS (ESI-TOF) *m/z* Calcd for C₂₆H₃₅N₈O₈ [M+H]⁺ 587.2578, Found [M+H]⁺ 587.2581

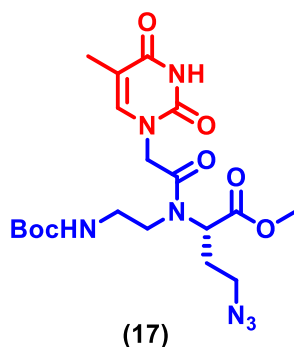
4-Azido-[2(S)-amino(N-ethylamino-t-butoxycarbonyl)-N-acetamido(2-oxopyrimidin-1(2H)-yl)-N4(benzyloxycarbonyl)] butanoic acid (16)



To a stirred solution of compound **15** (0.8 g, 1.36 mmol), in THF (15 mL) was added aq NaOH (2 M, 15 ml) at 0 °C, and the reaction mixture was stirred for 30 min at 0 °C. Upon completion of the reaction as confirmed by TLC, H₂O (50 ml) was added, and resulting mixture was extracted with EtOAc (2× 5 ml). The aqueous layer was neutralized with saturated aq. KHSO₄ at 0 °C to pH 3.4 and then extracted with EtOAc (4x35 ml). The combined organic extracts were dried over anhydrous Na₂SO₄. The solvent was evaporated in vacuo, and crude product was purified by column chromatography to afford yield compound **16** (85%), m.p = 172-175°C; [α]_D²⁵ -24.80° (c 1, MeOH); IR (neat, cm⁻¹) 3387, 2974, 2100, 1656,1561, 1502, 1452, 1369, 1206, 1066, 999, 789, 743, 696, 563 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ : 12.86 (s, 1H), 10.80 (s, 1H), 7.95 (d, *J* = 7.3Hz, 1H), 7.45 – 7.33 (m, 5H), 7.02 (d, *J* = 7.3 Hz, 1H), 6.92 (s, 1H), 5.20 (s, 2H), 4.85 – 4.72(m,2H), 4.24 (dd, *J* = 8.8, 5.6 Hz, 1H), 3.58 – 3.25

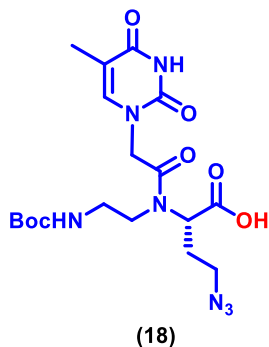
(m, 6H), 2.18 – 1.97 (m, 2H), 1.38 (d, $J = 8.7$ Hz, 9H); ^{13}C NMR (101 MHz, DMSO) δ : 171.5, 167.2, 163.2, 155.7, 155.0, 153.2, 150.9, 136.0, 128.5, 128.1, 127.9, 93.8, 78.1, 66.5, 57.6, 49.9, 47.8, 47.2, 28.2 ppm; HRMS (ESI-TOF) m/z Calcd. $\text{C}_{25}\text{H}_{33}\text{N}_8\text{O}_8$ $[\text{M} + \text{H}]^+$ 573.2421, Found: 573.2424. HRMS (ESI-TOF) m/z Calcd. $\text{C}_{25}\text{H}_{33}\text{N}_8\text{O}_8$ $[\text{M} + \text{H}]^+$ 573.2431, Found: 573.2421.

4-Azido-[2(S)-amino(N-ethylamino-*t*-butoxycarbonyl)-N-acetamido(5-methyl-2,4-dioxo-3,4 dihydropyrimidin-1(2H)-yl)] methyl butanoate (17)



To a stirred solution of thymine-1-yl acetic acid **25**, (1.0 g, 5.3 mmol) in dry DMF (50 ml) under an inert atmosphere were added DCC (1.1 g, 5.3 mmol) and DhbtOH (0.9 g, 5.3 mmol). The resulting mixture was stirred at room temperature for 1 h. Compound **12** (1.5 g, 4.8 mmol) in dry DMF (30 ml) was added to the above reaction mixture, and stirred at 50 °C for 24 h. The solvent was removed under reduced pressure, and the remaining residue was dissolved in EtOAc (300 ml) and washed with a saturated aq. NaHCO_3 (300 ml) followed by 10% aq. KHSO_4 (300 ml). The organic layer was washed with brine (3x150 ml) and dried over Na_2SO_4 . The solvent was removed under reduced pressure. The crude product was purified by column chromatography to afford compound **17** (1.45 g, 70%) as a white solid; m.p. 80-83 °C; $[\alpha]_D^{25}$ -14.0° (c 1, MeOH); IR (neat, cm^{-1}) 3732, 3379, 3185, 3057, 2977, 2102, 1667, 1509, 1457, 1366, 1247, 1163, 1095, 1006, 863, 782, 731, 640, 554; ^1H NMR (400 MHz, CDCl_3) δ : 9.13 (s, 1H), 7.04 – 6.92 (m, 1H), 5.61 (s, 1H), 4.80 (t, $J = 16.2$ Hz, 1H), 4.18 (d, $J = 16.2$ Hz, 1H), 3.93 – 3.89 (m, 1H), 3.81-3.07 (m, 4H), 3.53 (dt, $J = 12.5, 5.0$ Hz, 2H), 3.34 (ddd, $J = 14.0, 7.5, 3.8$ Hz, 2H), 3.29 – 3.17 (m, 1H), 2.31 (tt, $J = 14.1, 7.0$ Hz, 2H), 1.94 – 1.91 (m, 3H), 1.45 ppm (s, 9H); ^{13}C NMR (100 MHz, CDCl_3) δ : 171.2, 167.5, 164.2, 156.1, 151.1, 141.0, 110.9, 80.1, 59.0, 53.0, 49.2, 48.6, 48.3, 39.0, 34.0, 28.5, 28.3, 12.5 ppm; HRMS (ESI-TOF) m/z Calcd. $\text{C}_{19}\text{H}_{29}\text{N}_7\text{NaO}_7$ $[\text{M} + \text{Na}]^+$ 490.2026, Found 490.2031.

4-Azido-[2(S)-amino(N-ethylamino-t-butoxycarbonyl)-N-acetamido(5-methyl-2,4-dioxo-3,4- dihydropyrimi din-1(2H)-yl)] butanoic acid (18)



To a stirred solution of compound **17** (0.7 g, 1.55 mmol) in THF (15 mL) was added aq NaOH (2 M, 15 ml) at 0 °C, and the reaction mixture was stirred for 30 min at 0 °C. Upon completion of the reaction as confirmed by TLC, H₂O (50 ml) was added, and resulting mixture was extracted with EtOAc (2x35 ml). The aqueous layer was neutralized with saturated aq. KHSO₄ at 0 °C to pH 3-4 and then extracted with EtOAc (4x35 ml). The combined organic extracts were dried over anhydrous Na₂SO₄. The solvent was evaporated in vacuo, and crude product was purified by column chromatography to afford compound **18** as a white solid (0.63 g, 89%), m.p. 132-135°C; $[\alpha]^{25}_D$ -22.6° (c 1, MeOH); IR (neat) 3825, 3733, 3186, 3058, 2979, 2312, 2101, 1662, 1511, 1461, 1365, 1246, 1162, 1096, 1013, 964, 857, 780, 732, 637, 555 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ: 11.30 (s, 1H), 7.36 (s, 1H), 6.91 (s, 1H), 4.70 – 4.45 (m, 2H), 4.42 – 4.14 (m, 1H), 3.56 – 3.2 (m, 6H), 3.13 – 2.87 (m, 1H), 2.34 – 1.86 (m, 2H), 1.75 (d, *J* = 0.7 Hz, 3H), 1.38 ppm (s, 9H); ¹³C NMR (100 MHz, DMSO-d₆) δ: 171.6, 168.0, 167.3, 164.4, 155.7, 151.0, 142.1, 108.0, 78.1, 57.6, 47.98, 47.92, 47.80, 47.1, 28.1, 11.9 ppm; HRMS (ESI-TOF) *m/z* Calcd. C₁₈H₂₇N₇O₇ [M+Na]⁺ 476.1869, Found 476.1840.

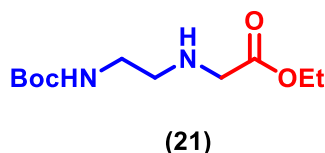
N1(Boc)-1, 2-diaminoethane (20)



To an ice-cold stirred solution of 1,2-diamonoethane **19** (2.0 g, 33.3 mmol) in DCM (300 ml) was added solution of Boc-anhydride (5 g, 35 mmol) in DCM (50 ml) solution. The

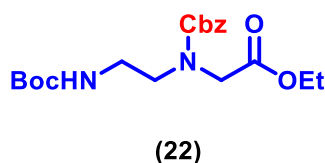
mixture was stirred for 12 h and after completion of reaction DCM removed from reaction mixture. The residue was washed with diethylether to remove the unreacted boc anhydride, solvent was evaporated and triturated with water. The insoluble diboc derivative was filtered out and the aqueous layer upon concentration gave the corresponding mono *N*-Boc derivative, **20** (3.4 g, 60%) which was used for further reaction without any purification.

Ethyl N2 (*t*-Boc-aminoethyl) glycinate (21)



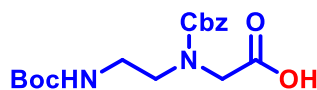
The (N1-Boc)-1,2-diaminoethane (**20**) (3.2 g, 20 mmol) was treated with ethyl bromoacetate (2.2 mL, 20 mmol) in acetonitrile (100 mL) in the presence of triethylamine (5.5 mL, 40 mmol) and the mixture was stirred at ambient temperature for 10 h. The completion of reaction was monitored by TLC. After completion of reaction solvent was removed on rota evaporator. Reaction mixture was then diluted with water (50 mL) and extracted with ethyl acetate (3x50 mL), followed by washing with brine. The collected organic layer was dried over anhydrous sodium sulphate and filtered and then concentrated to get crude product. The crude product was used further for next reaction without purification.

Ethyl [N-ethylamino(*t*-butoxycarbonyl)-N(benzyloxy)carbonyl] glycinate (22)



To a solution of **21** (0.8 g, 3.25 mmol) in DCM (20 ml) at 0 °C was added benzyl chloroformate (0.8 g, 5.0 mmol) dropwise followed by addition of N,N-diisopropylethylamine (1.7 g, 13.4 mmol). After stirring at room temperature for 5 h, saturated aq. NH₄Cl solution (10 ml) was added and extracted with DCM (4x20 ml). The organic layers were dried over MgSO₄, concentrated *in vacuo* and purified with flash chromatography in hexane to afford **22** (Yield, 1.16 g, 3.0 mmol, 92%), TLC in EtOAc 4:1, R_f = 0.26; HRMS (ESI-TOF) m/z Calcd for C₁₉H₂₈N₂O₆Na [M+Na]⁺ 403.1845, Found, 403.1844.

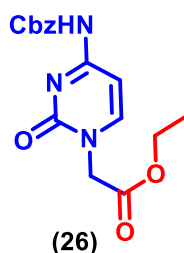
N-ethylamino(*t*-butoxycarbonyl) N(benzyloxy)carbonyl glycine (23)



(23)

Compound **23** (0.87 g, 2.47 mmol) was prepared, purified, and characterized the same way as described for compound **17**, starting from compound **22** (1.0 g, 2.63 mmol), to afford yield (94%); HRMS (ESI-TOF) m/z Calcd for $C_{17}H_{24}N_2O_6Na$ $[M+Na]^+$ 375.1532, Found 375.1532.

Ethyl (N4 –benzyloxycarbonyl) cytidin-1-yl acetate (**26**)

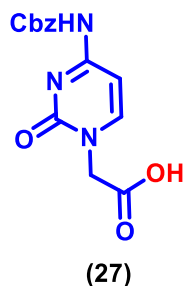


(26)

(a) To a suspension of cytosine **24** (5.0 g, 45.0 mmol) in dry DMF (100 ml) was added NaH (1.1 g, 45.0 mmol) under N_2 at 0 °C. The mix was stirred for 2 h at rt, followed by addition of ethyl bromoacetate (4.3 ml, 45.0 mmol). The mix was stirred for 48 h at rt and the solvent was evaporated in vacuo. The crude residue was triturated with water (100 ml), the precipitate was filtered and recrystallised from MeOH/ H_2O to yield compound **25** (5.3 g, 26.7 mmol 60%) as white crystals.

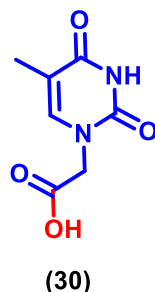
(b) Cbz-Cl (3.1 ml, 21.8 mmol) and DMAP (2.67 g, 21.8 mmol) were dissolved at -15 °C in DCM (20 ml). The mixture was stirred for 15 min and compound **25** (2.0 g, 10.15 mmol) was gradually added. After stirring for 15 min at -15 °C, it was kept for 5 h at rt and then evaporated in vacuo followed by dissolution in $CHCl_3$. The organic layer was washed with 1 M aq.HCl, water and then dried over Na_2SO_4 and concentrated under vacuum. Trituration of the crude residue in ether gave a white precipitate that was filtered to afford compound **26** (2.6 g, 7.82 mmol, yield 77%) as an amorphous powder. TLC (EtOAc/MeOH 1:1) R_f = 0.79. ^1H NMR (400 MHz, DMSO) δ 8.04 (d, J = 7.3 Hz, 1H), 7.45 – 7.33 (m, 5H), 7.04 (d, J = 7.2 Hz, 1H), 5.19 (s, 2H), 4.60 (s, 2H), 4.14 (q, J = 7.1 Hz, 2H), 1.20 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, DMSO- D_6) δ 168.0, 163.5, 155.1, 153.2, 150.5, 136.0, 128.6, 128.3, 128.0, 94.2, 66.6, 61.2, 50.7, 14.1 ppm.

N⁴(Benzyloxycarbonyl) cytidin-1-yl acetic acid (**27**)



Compound **26** (2.0 g, 6.0 mmol) was dissolved in dioxane (50 ml) and aqueous 1 M NaOH (8.8 ml) was added. The mix was stirred 5 h, at rt and then concentrated under reduced pressure. The residue was taken up in a aqueous KHSO₄ (1 M). The resultant white precipitate was isolated by filtration, washed with water and dried in a vacuum desiccator affording **27** (1.6 g, 5.24 mmol, 87%). TLC (EtOAc/MeOH 1:1) R_f = 0.10. ¹H NMR (400 MHz, DMSO-d₆) δ: 10.87 (s, 1H), 8.04 (d, 1H), 7.39 (m, 5H), 7.03 (d, 1H) 5.19 (s, 2H), 4.52 (s, 2H) ¹³C NMR (101 MHz, DMSO-d₆) δ: 169.4, 163.3, 155.1, 153.21, 150.5, 135.9, 128.5, 128.2, 128.0, 94.1, 66.6, 50.6, 39.5. HRMS (ESI-TOF) m/z Calcd for C₁₄H₁₄N₃O₅ [M+H]⁺ 304.0933, Found 304.0934.

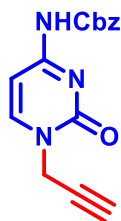
Synthesis of thymine-N1-yl acetic acid (30)



To a mixture of thymine **28** (5.0 g, 39.7 mmol) and K₂CO₃ (5.5 g, 40 mmol) in dry DMF (120 ml) was added ethyl bromoacetate (37.5 ml, 0.39 mol) and the mixture was stirred vigorously overnight under N₂. The mixture was filtered and evaporated to dryness, in vacuo. The solid residue was cooled to 0°C, treated with water (37 ml) and aq. HCl (4 N, 1.6 ml), and stirred for 30 min. The precipitate was collected by filtration and washed with water (3x20 ml). The precipitate was treated with water (40 ml) and aq. NaOH (2 M, 20 ml) and boiled for 10 min. The mixture was cooled to 0 °C, treated with aq. HCl (4 M, 13 ml), and stirred for 30 min. The compound **30** was collected by filtration, washed with water (3x20 ml), and dried to afford (4.5 g, 24.5 mmol, 62%); ¹H NMR (400 MHz, DMSO-d₆) δ: 11.34 (s, 1H), 7.48 (s, 1H), 4.36 (s, 1H), 2.50 (s, 1H), 1.75(s,1H.). ¹³C NMR (101 MHz, DMSO-d₆) δ 169.7, 164.5, 151.0, 141.9,

108.4, 48.5, 39.5, 11.9. HRMS (ESI-TOF) m/z Calcd. for $C_7H_9N_2O_4$ $[M+H]^+$ 185.0562, Found, 185.0566.

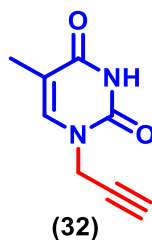
N⁴-(benzyloxycarbonyl)-N1-(propyne-2-yl) Cytosine (31)



(31)

To a solution of N⁴-Cbz-cytosine **25** (5g, 20.4 mmol) and NaH (0.98g, 40.8 mmol) in dry acetonitrile (25 ml) at 0° C was added propargyl bromide (80% in toluene, 2 mL 26.5 mmol) and the resulting mixture was stirred at rt. for 18 h. The combined organic layer was washed with water and brine solution. The organic layer was separated, dried over Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure. The crude mass thus obtained, was purified by column chromatography [Eluent: 20-50% of EtOAc in pet ether] to afford compound **31** (3.5 g, 60.6%) ¹H NMR (400 MHz, DMSO) δ 10.82 (s, 1H), 8.15 (d, 1H), 7.37 (m, 5H) 7.06 (d, 1H) 5.18 (s, 2H), 4.63 (s, 2H), 3.45 (s, 1H) ¹³C NMR (101 MHz, DMSO-d₆) δ 163.1, 148.8, 135.9, 128.5, 128.2, 128.0, 94.7, 78.3, 76.4, 66.6, 39.5. HRMS (ESI-TOF) m/z Calcd. for $C_{15}H_{14}N_3O_3$ $[M+H]^+$ 284.1035, Found, 284.1039

(N¹-propargyl) Thymine (32)

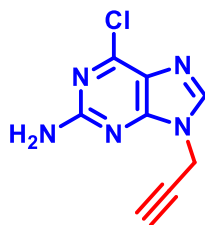


(32)

To a solution of thymine **25** (5 g, 39.65 mmol) in dry DMF (80 ml) was added anhydrous K₂CO₃ (8.2 g, 59.5 mmol) and mixture stirred under N₂ for 15 min. followed by addition of propargyl bromide (80% in toluene, 4.2 g, 35.7 mmol) was added. The resulting mixture was stirred at rt for 12 h. After checking TLC for completion of reaction, DMF was removed by vacuum. The combined organic layer was washed with water and brine solution. The organic layer was separated, dried over Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure. The crude mass obtained was purified by column chromatography [Eluent: 20- 50% of EtOAc in pet ether] to afford compound **32**. ¹H NMR (400 MHz, DMSO-

d₆) δ : 11.38 (s, 1H), 7.56 (s, 1H), 4.46 (s, 2H), 3.39 (s, 1H), 1.76 (s, 3H) ¹³C NMR (101 MHz, DMSO-d₆) δ : 164.2, 150.4, 140.2, 109.4, 78.7, 75.7, 39.5, 36.4, 12.0. HRMS (ESI-TOF) m/z Calcd for C₈H₉N₂O₂ [M+H]⁺ 165.0664, Found, 165.0668

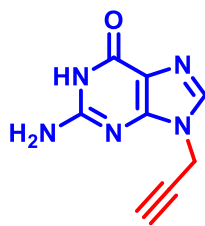
(N9-propargyl) 6-amino-9-chloro-purine (33)



(33)

2-Amino-6-chloropurine (2 g, 11.7 mmol), K₂CO₃ (1.8 g, 12.8 mmol) were stirred under N₂, in DMF (40 mL). Then propargyl bromide (1.5 g, 12.8 mmol) was added dropwise, and the resulting mixture was stirred for 48 h. After then DMF was evaporated at 60 °C under high vacuum and the residue was purified by column chromatography eluting with methanol/chloroform to afford yellowish-white solid **50** (2.4 g, 11.5 mmol, 65%) m.p. 160-165°C; ¹H NMR (400 MHz, DMSO-d₆, 25°C) δ : 8.17 (s, 1H), 7.02 (s, 2H), 4.92 (d, *J* = 2.5 Hz, 2H), 3.48 ppm (t, *J* = 2.5Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆, 25°C) δ : 159.9, 153.6, 149.5, 142.4, 123.1, 77.9, 76.1, 32.4 ppm; [Eluent: 20–70% of EtOAc in petroleum ether] to afford compound **33** HRMS (ESI-TOF) m/z Calcd for C₈H₈ClN₅ [M+H]⁺ 208.0390, Found, 208.0396.

N9-(propargyl) Guanine (34)



(34)

Compound **33** (1.0 g, 4.8 mmol) was dissolved in 3:1 mixture of TFA-H₂O (10 ml) and then stirred for 48 h at ambient temperature. The reaction mixture was evaporated and washed well with diethyl ether to afford compound **34** as brownish white solid, (0.75 g, 3.85 mmol, 80%), m.p. 167-170 °C; ¹H NMR (400 MHz, DMSO-d₆, 25 °C) δ : 11.45 (s, 1H), 8.69 (s, 1H), 7.16 (bs, 2H), 4.94 (d, *J* = 2.5 Hz, 2H), 3.60 ppm (t, *J* = 2.5 Hz, 1H); ¹³C NMR (100 MHz,

DMSO-d₆, 25°C) δ : 155.1, 154.5, 149.9, 136.7, 110.9, 77.1, 33.4 ppm; HRMS (ESI+), m/z Calcd. for (M+H)⁺ C₈H₉N₅O: 190.0729, Found, 190.0735.

2.6 Summary

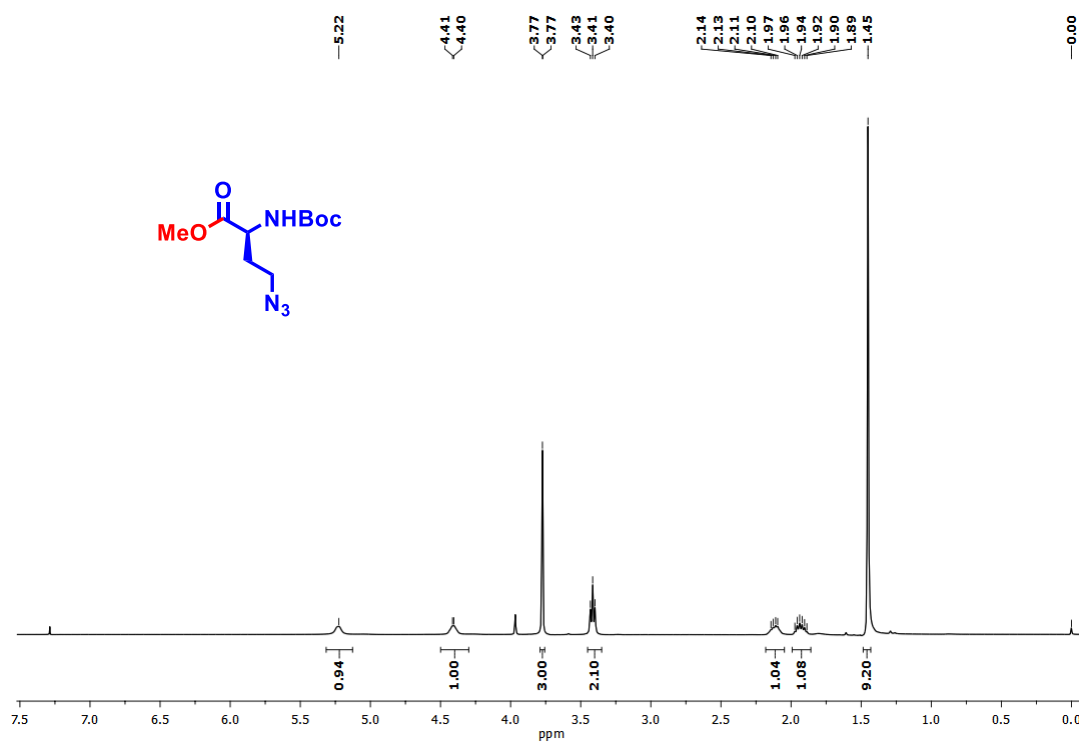
This section describes the synthesis and characterization of rationally designed C α -substituted *bm*-PNA monomers, synthesis of propargyl derivative linked nucleobases and acetic acid derivatives of nucleobases. All the intermediates have been characterized by ¹H and ¹³C NMR spectroscopy and mass spectral characterization. These monomers and propargyl were derivatives used for solid phase synthesis of the *bimodal*-PNA oligomers described in the next section.

2.7 Appendix I: Index

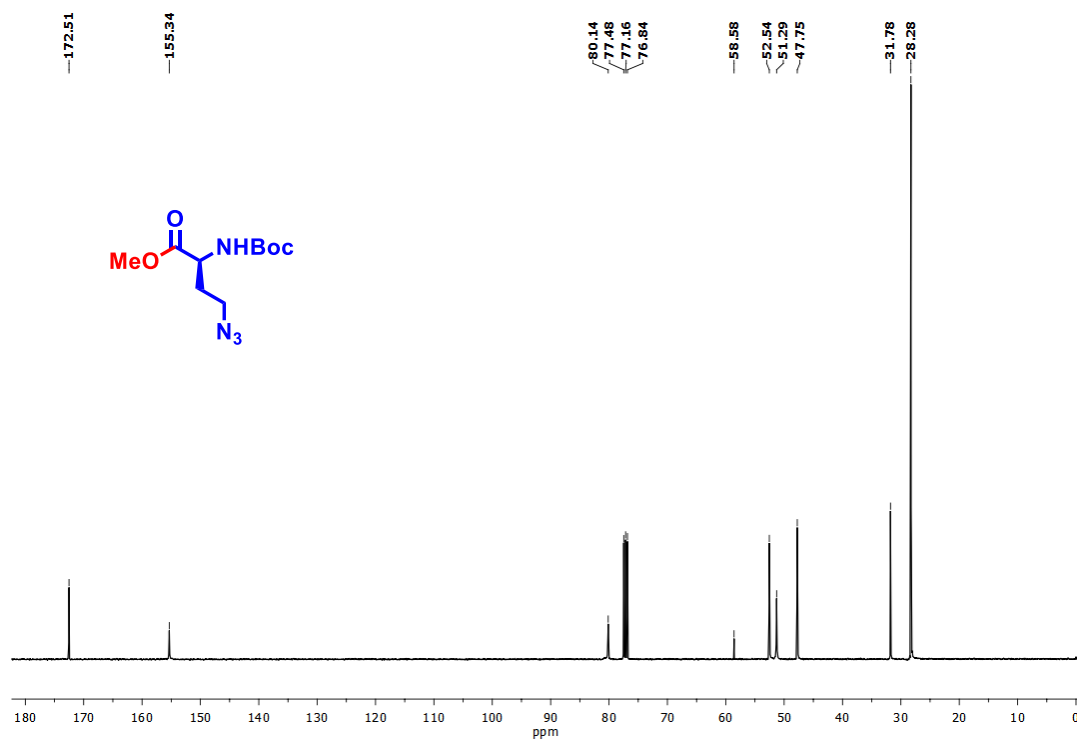
Entry	Table of contents	Page No
01	Introduction	55
02	Retrosynthesis	58-59
03	Scheme of synthesis	60
04	Experimental procedure	64
05	¹ H and ¹³ C NMR Spectrum	79-91
06	HRMS Spectrum of compounds	92-100
07	References	101

NMR Spectra (^1H and ^{13}C) of Compounds

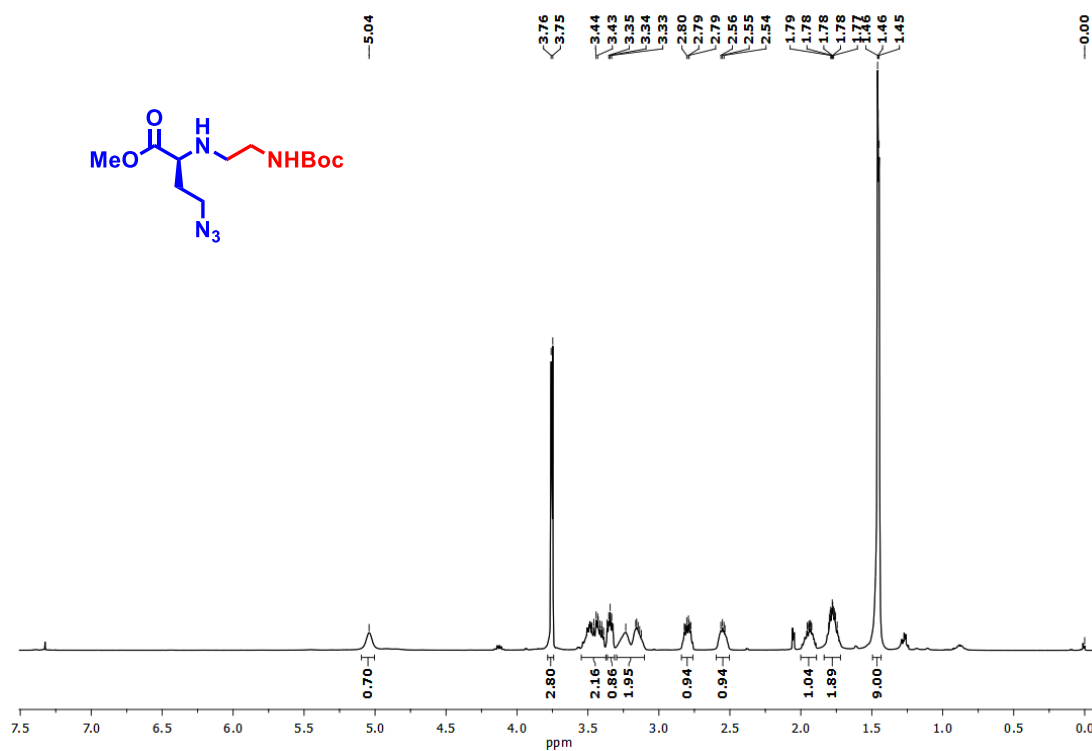
^1H spectrum of compound 11



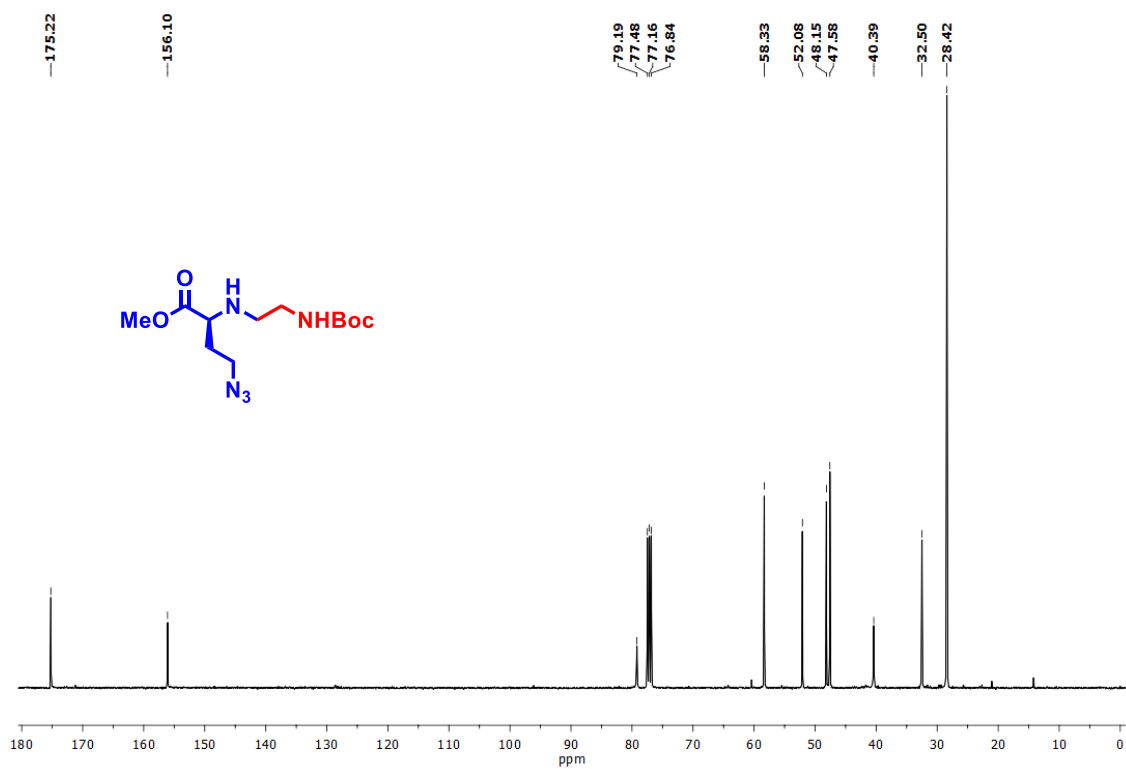
^{13}C spectrum of compound 11



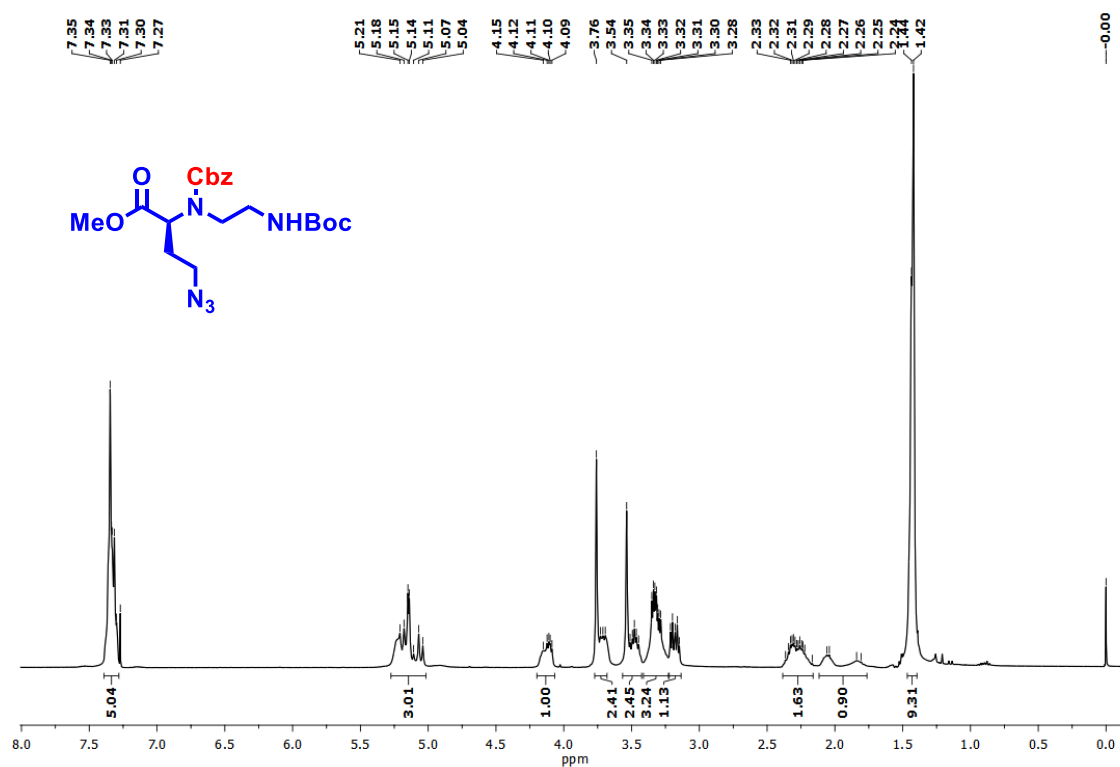
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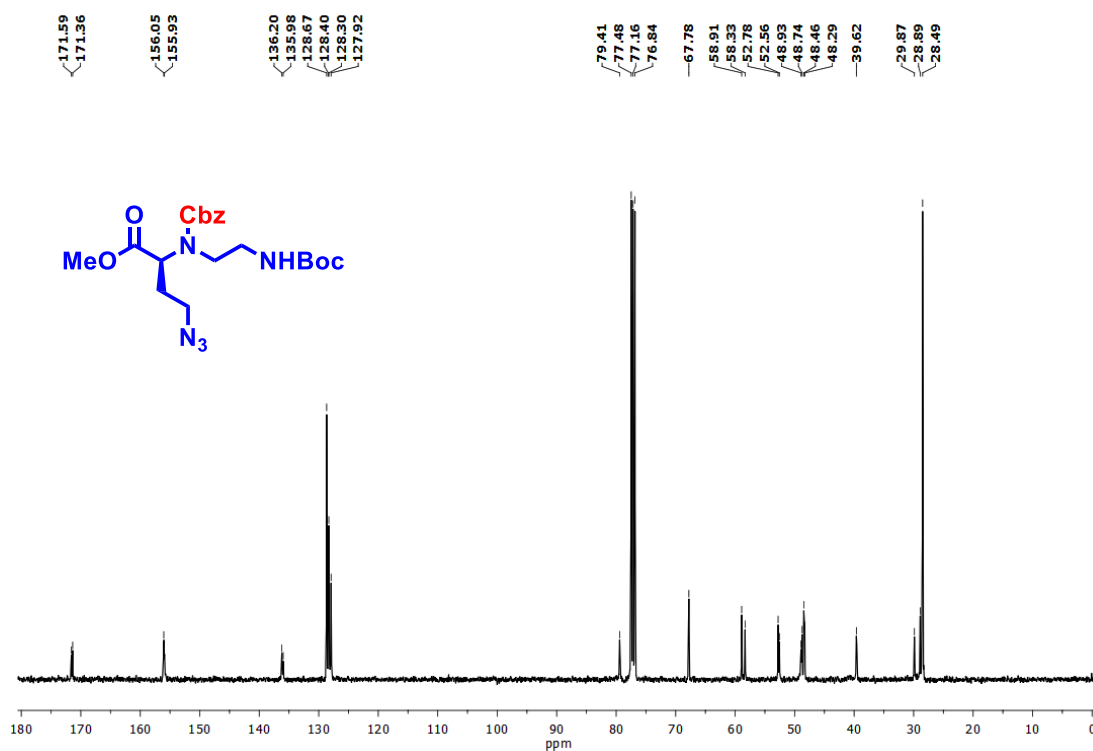
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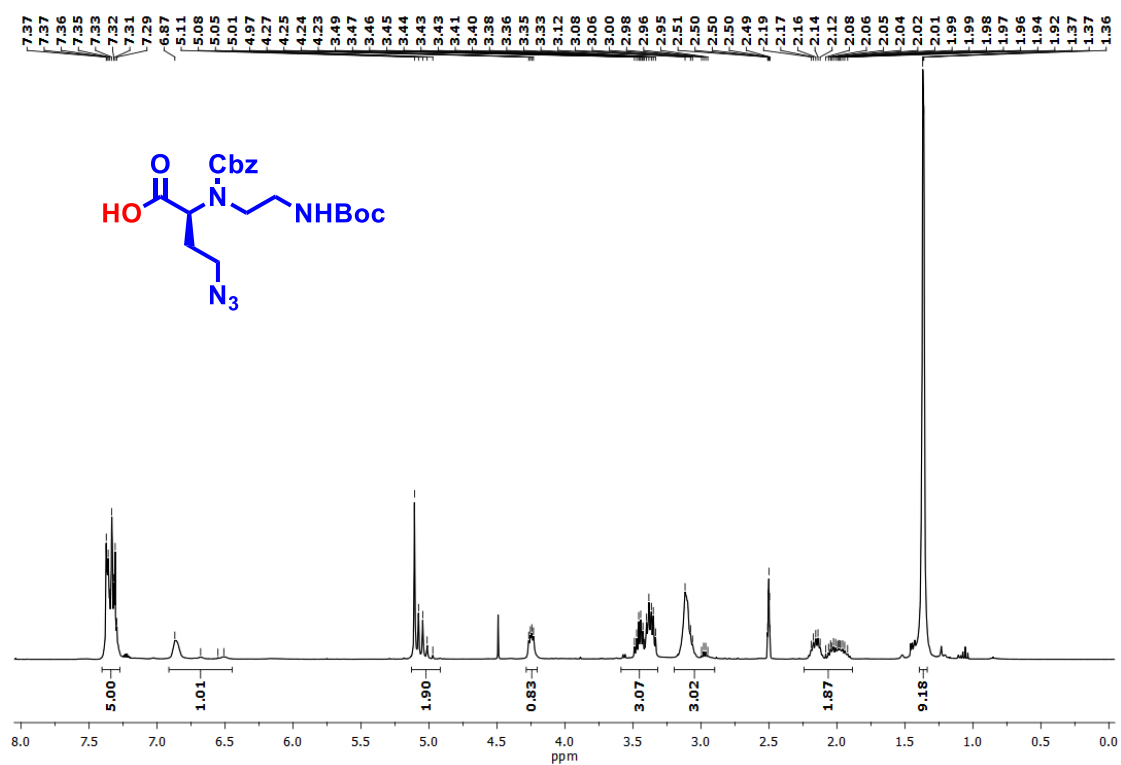
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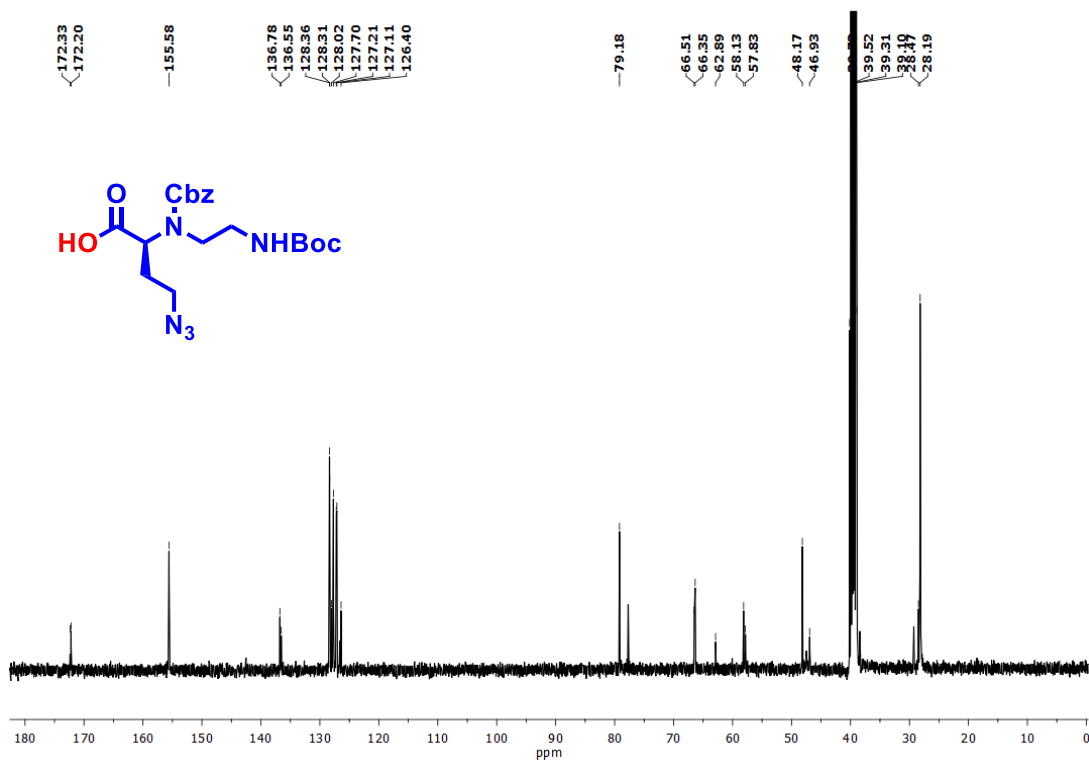
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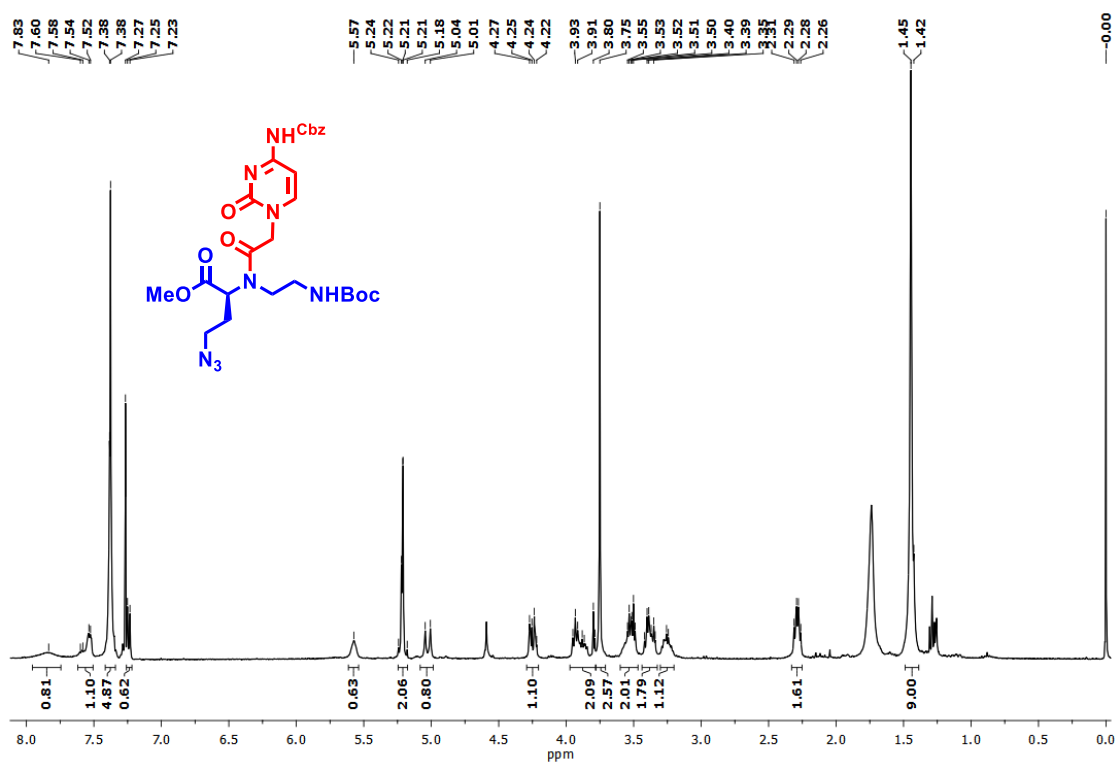
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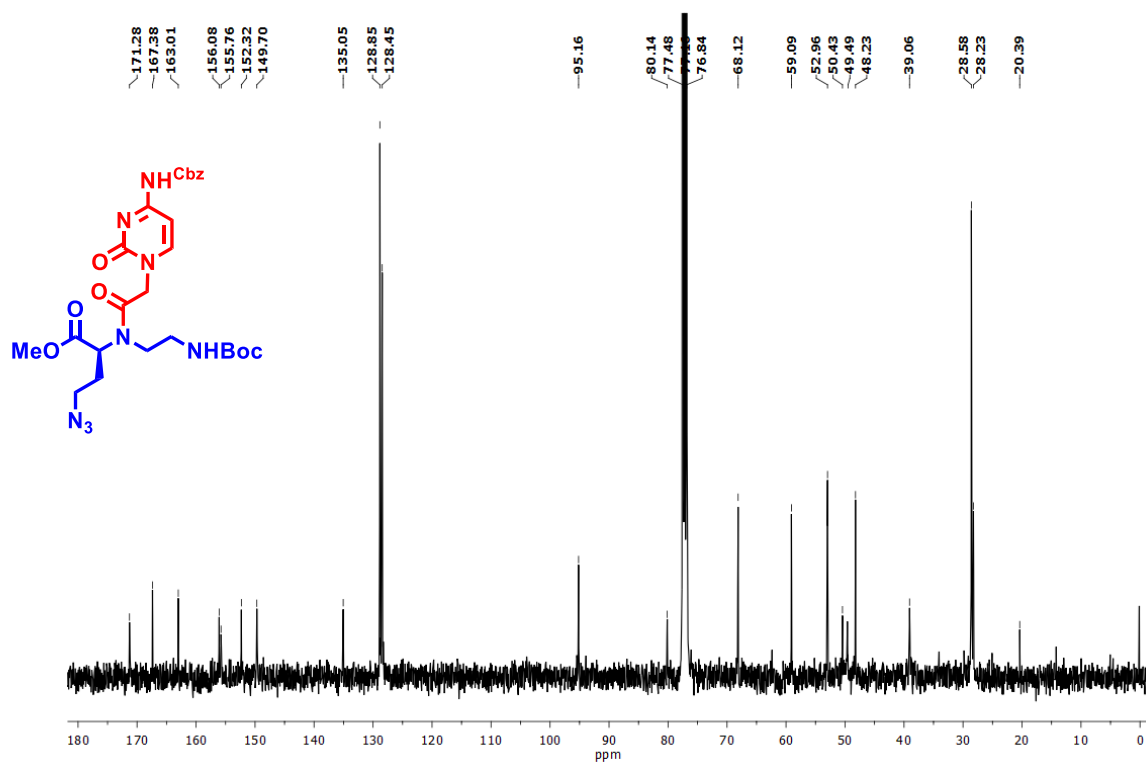
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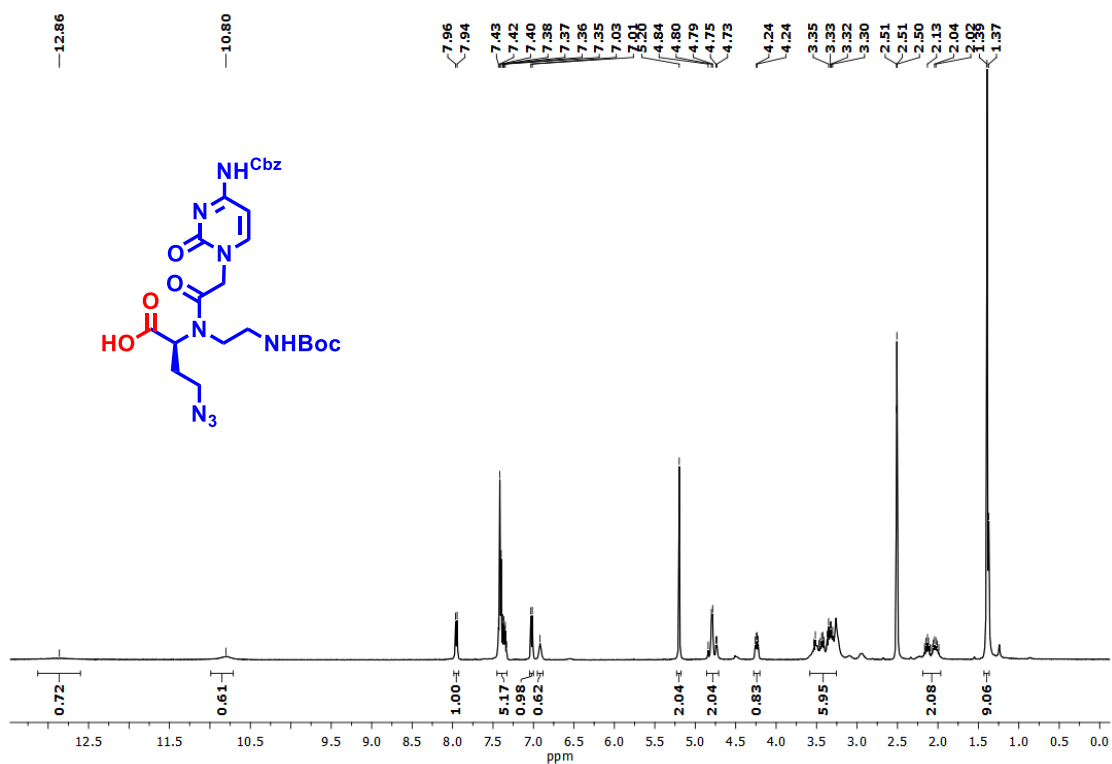
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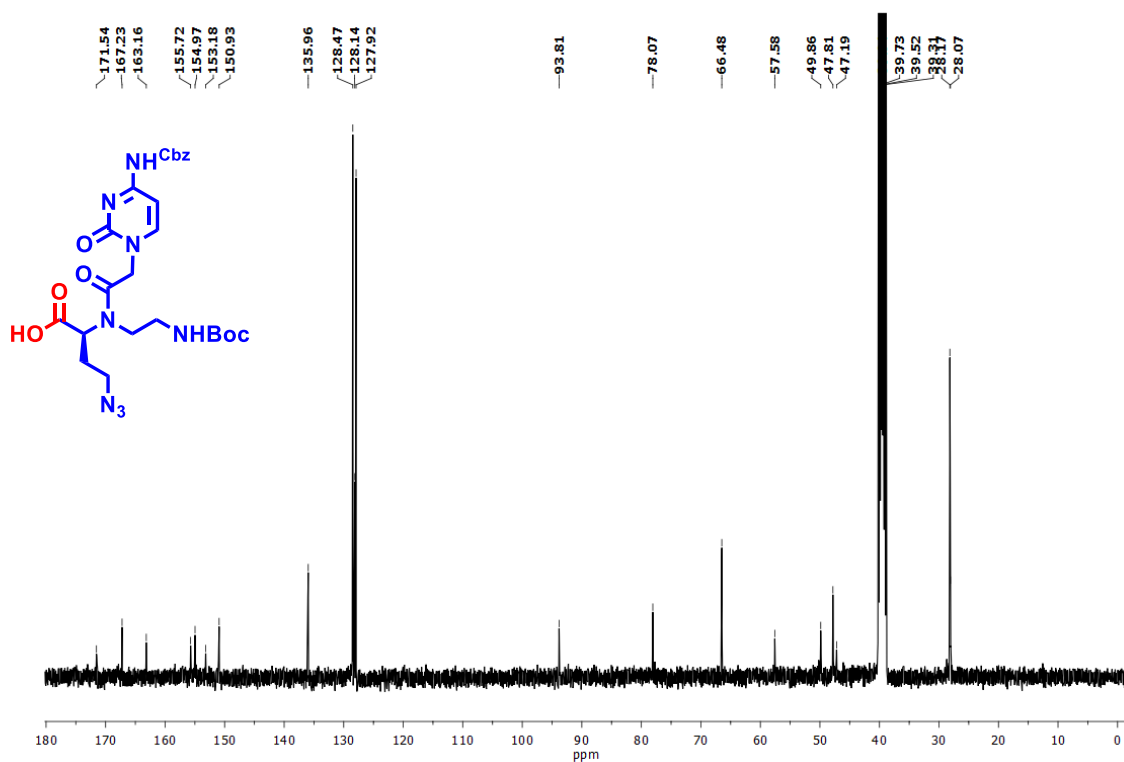
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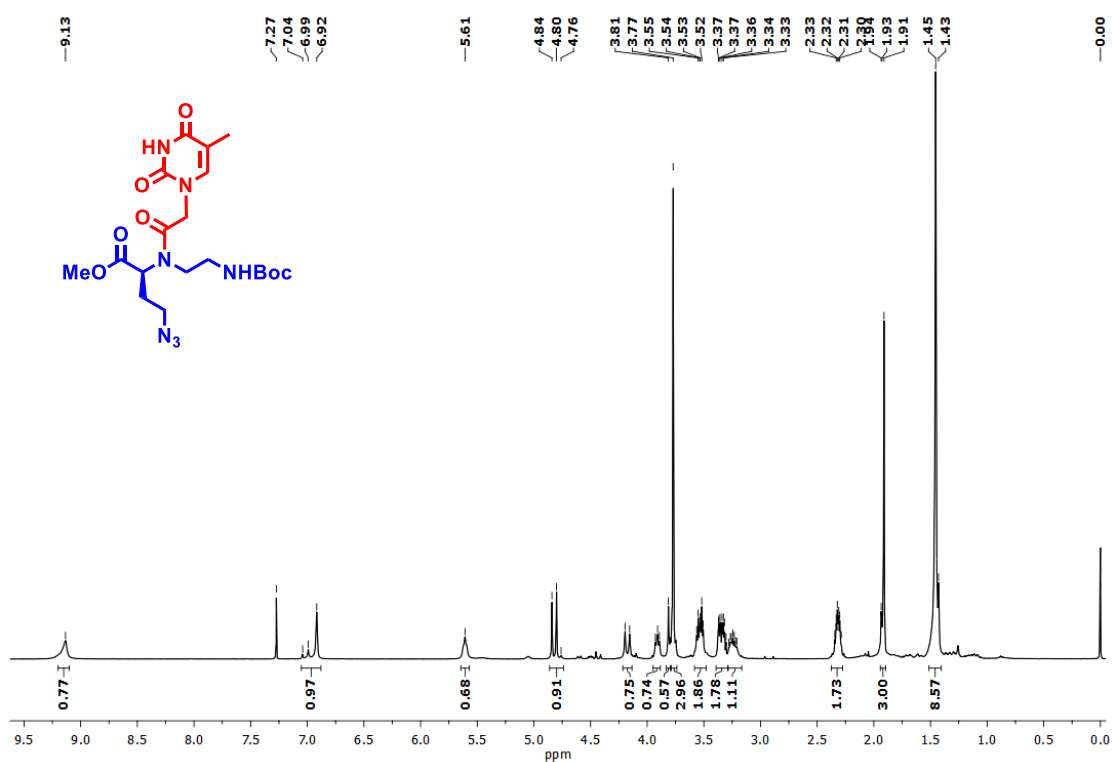
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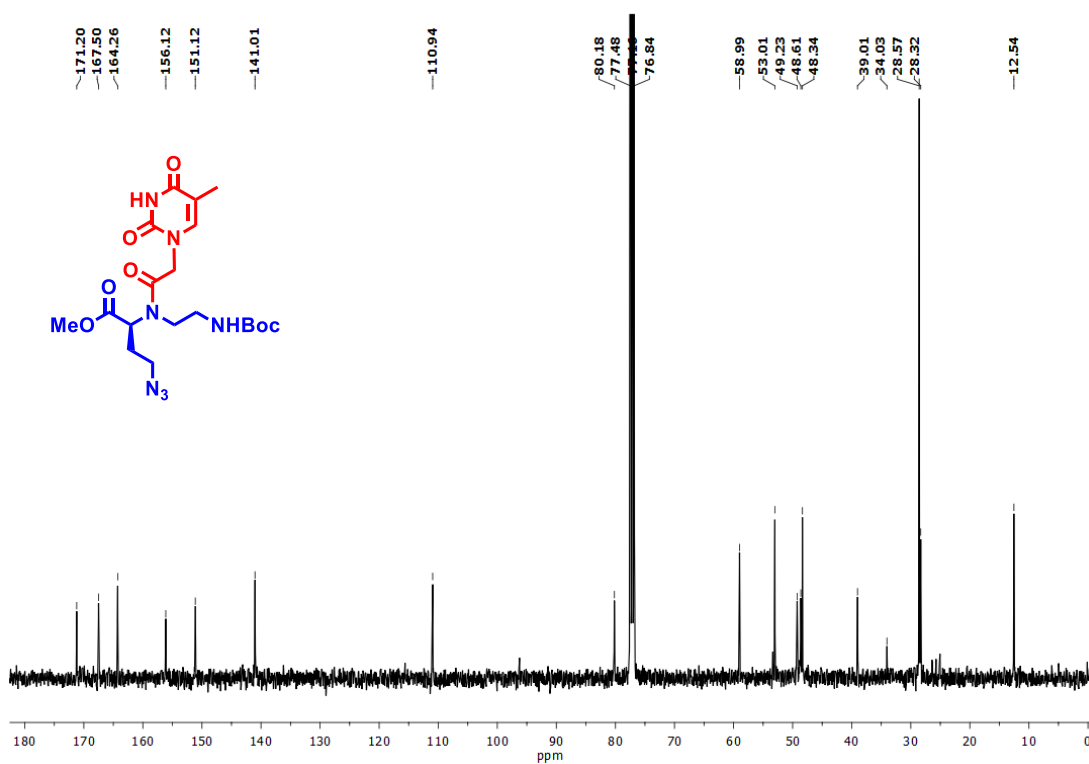
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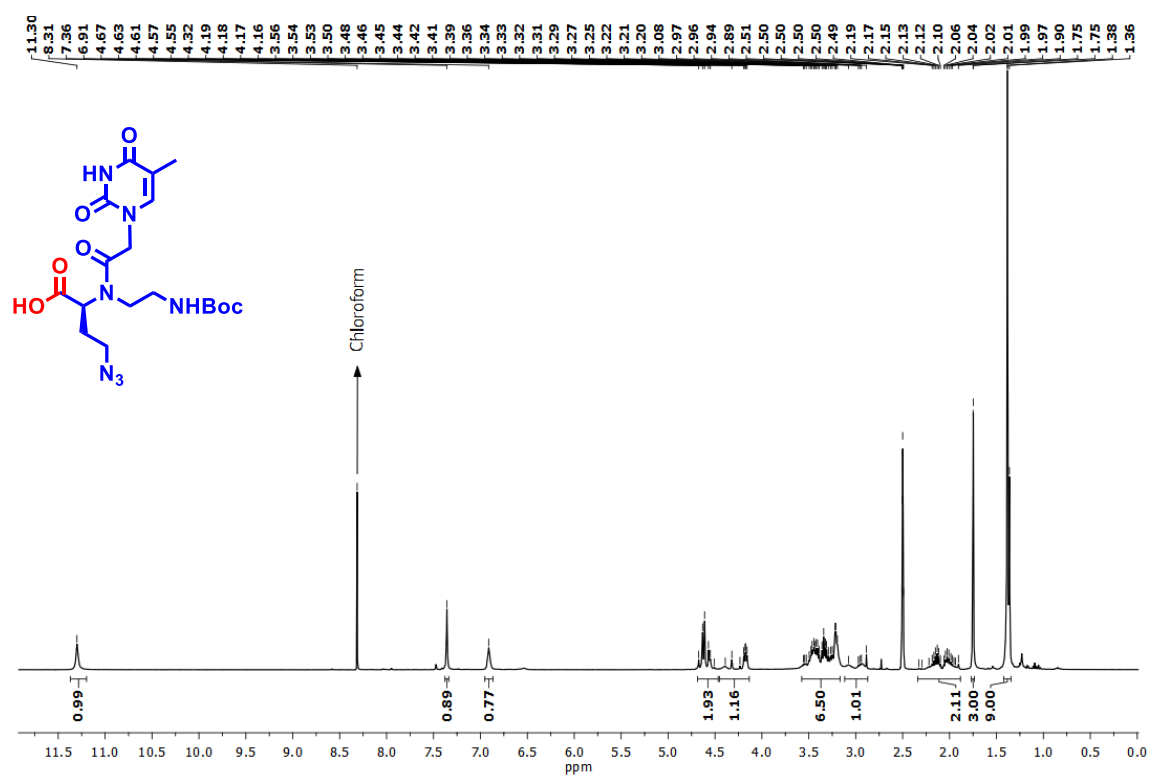
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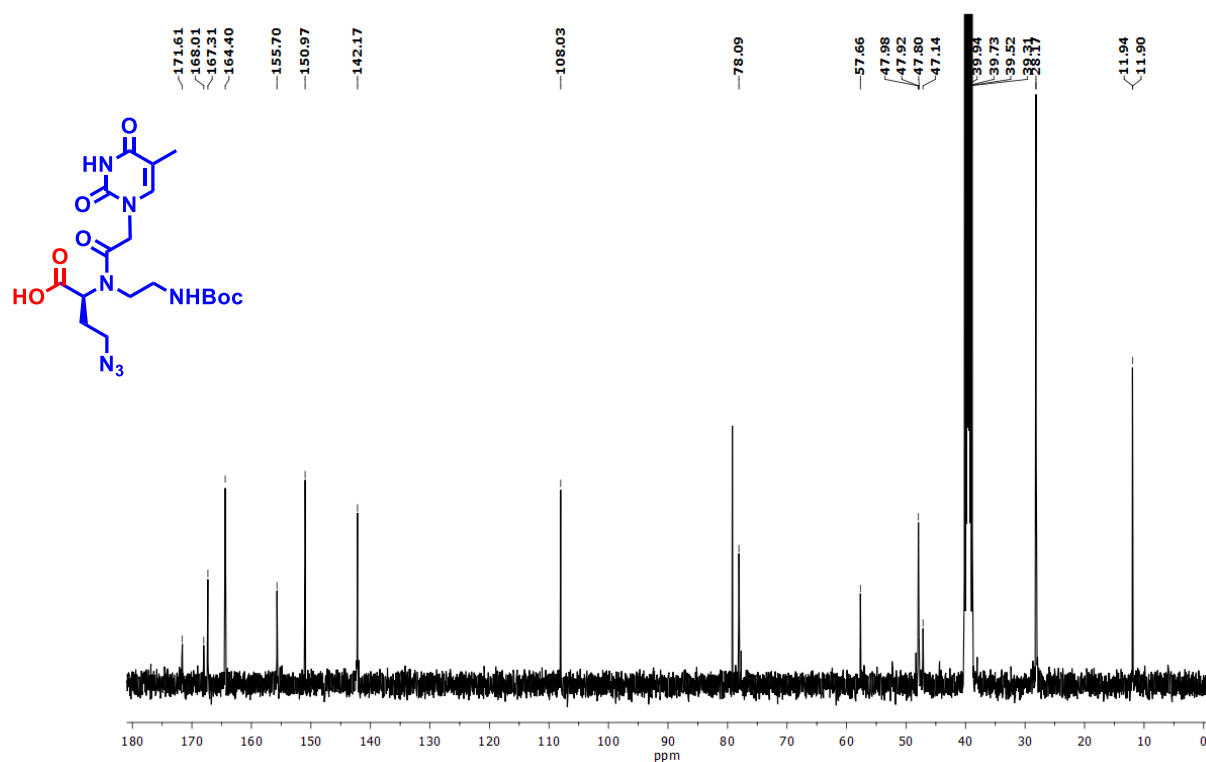
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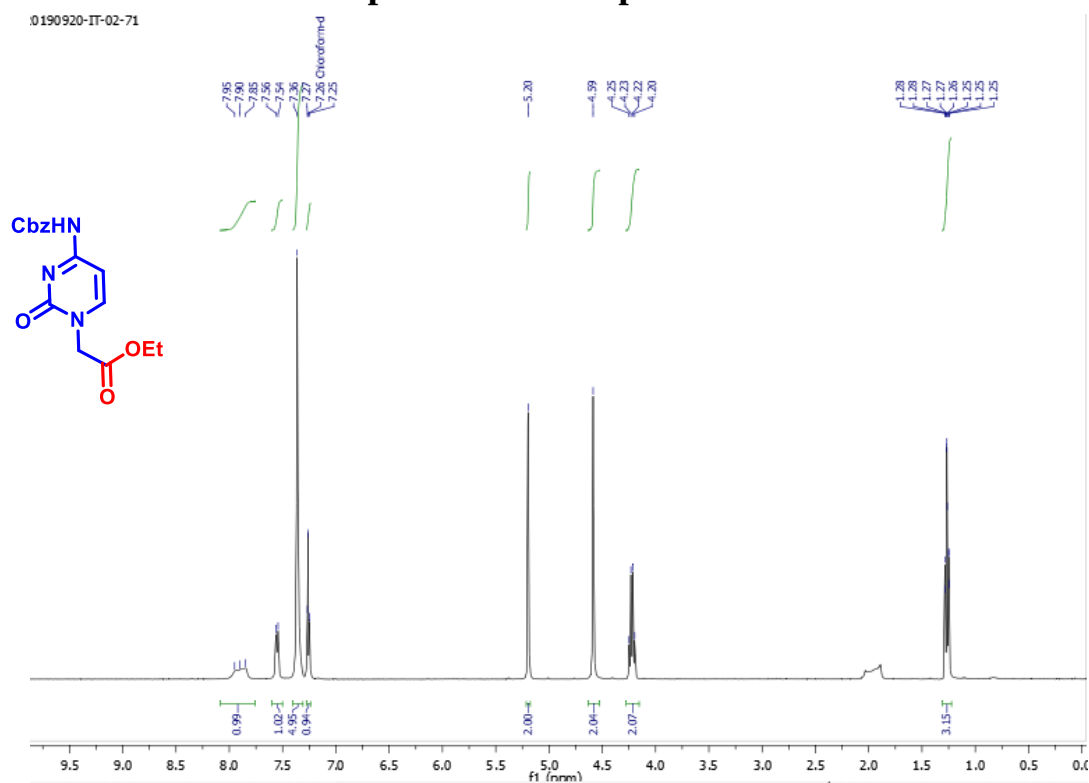
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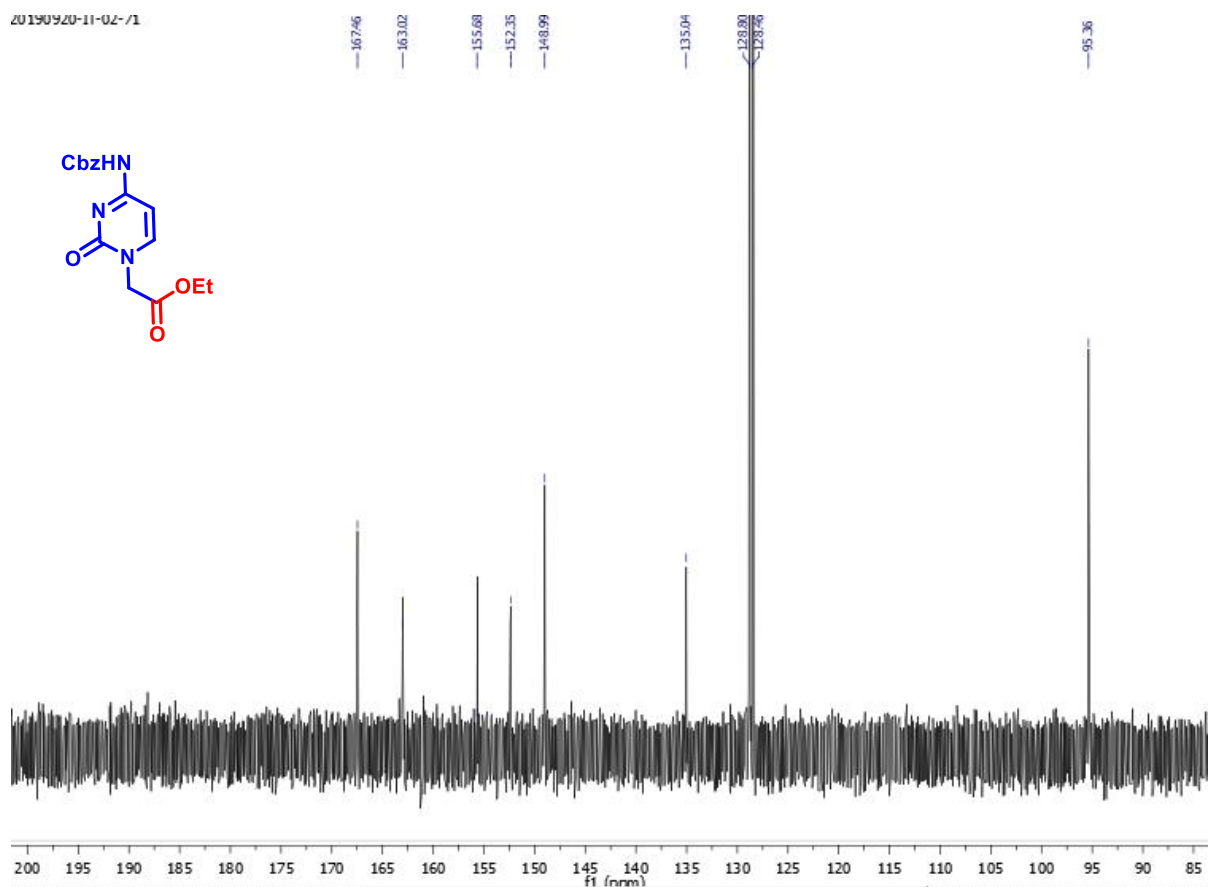
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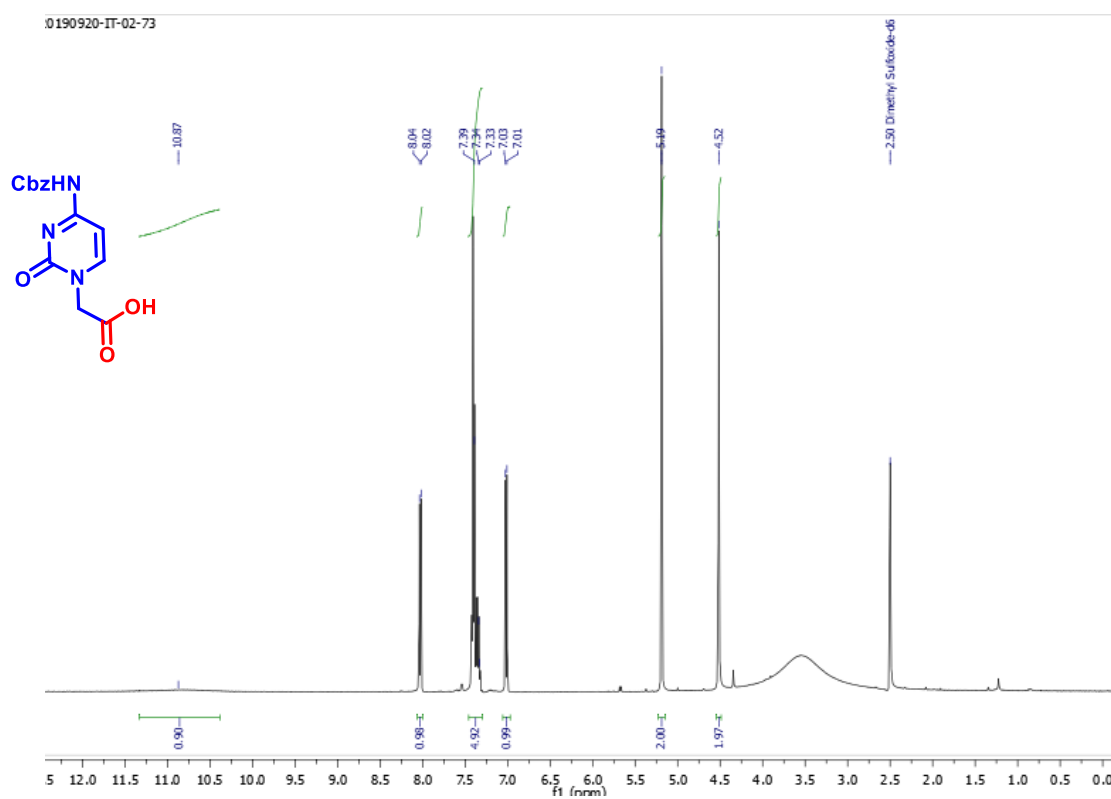
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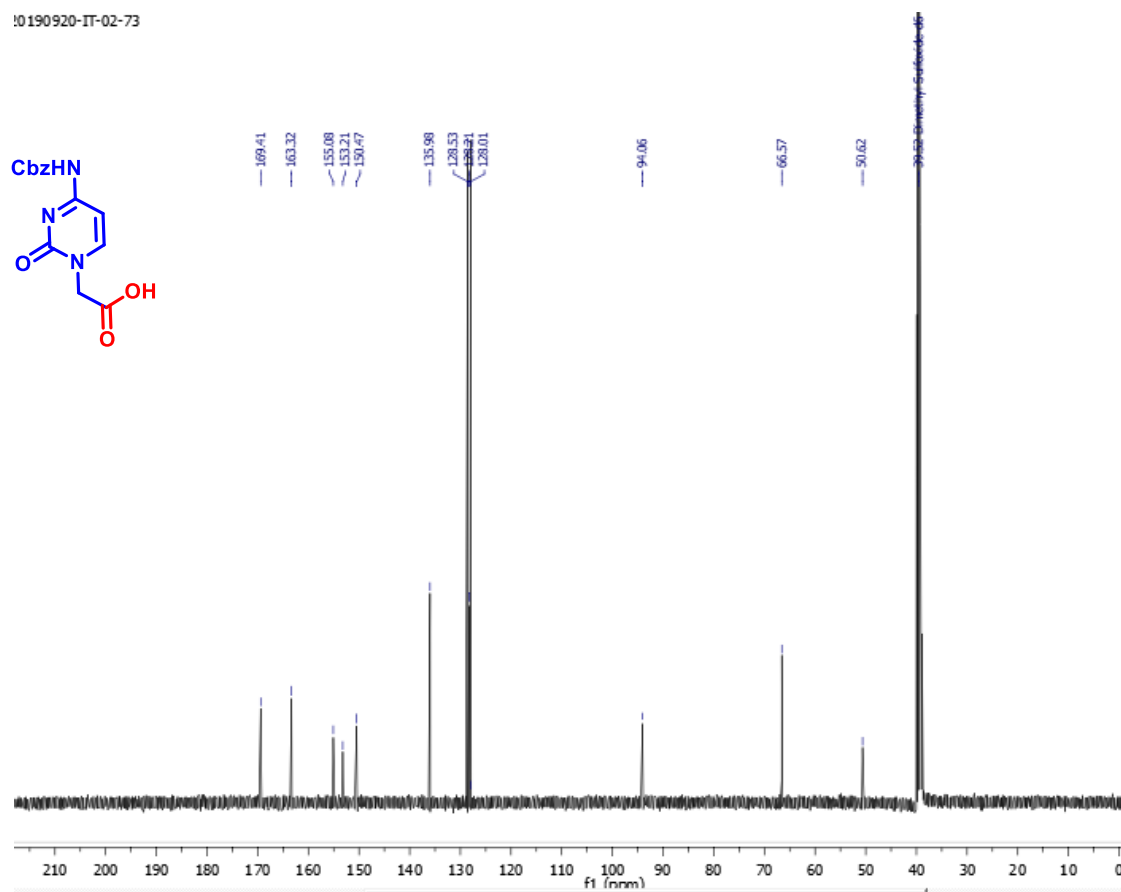
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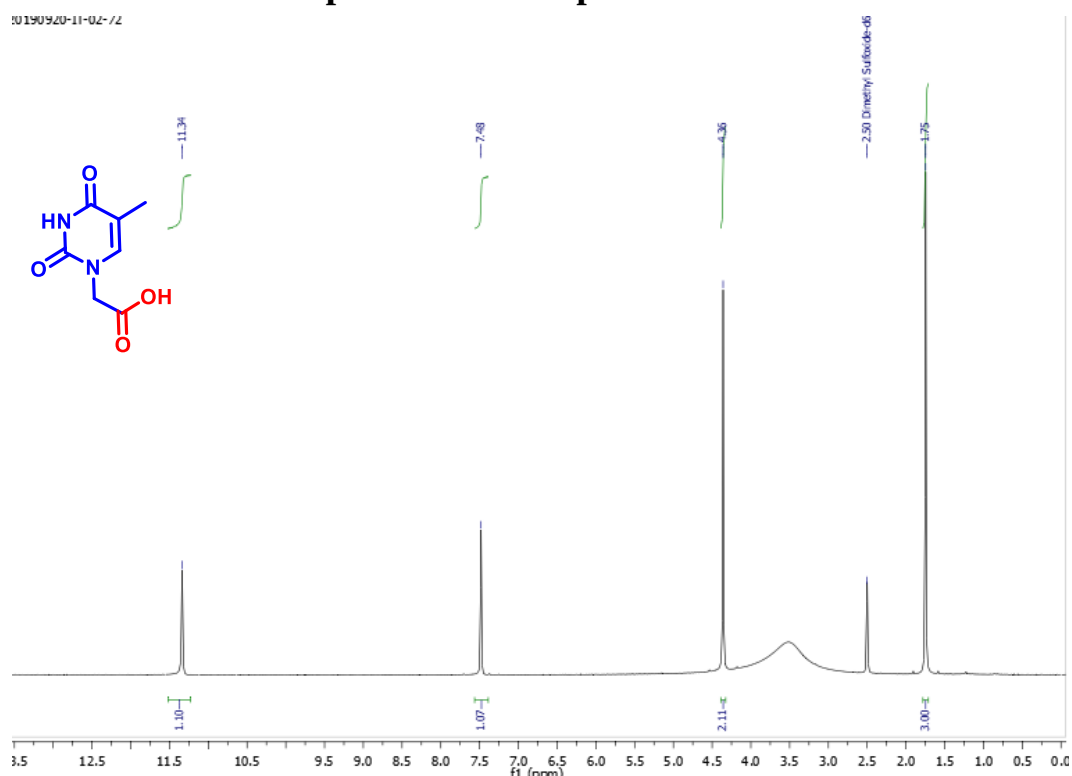
^1H spectrum of compound 27



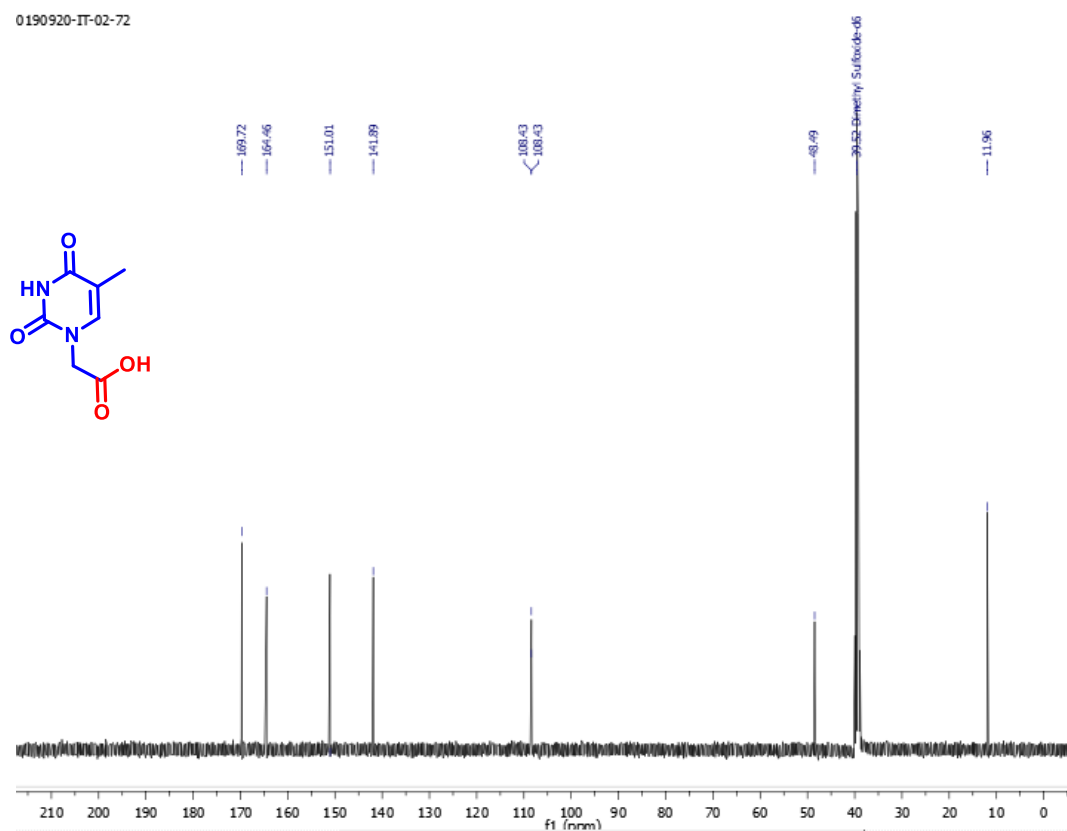
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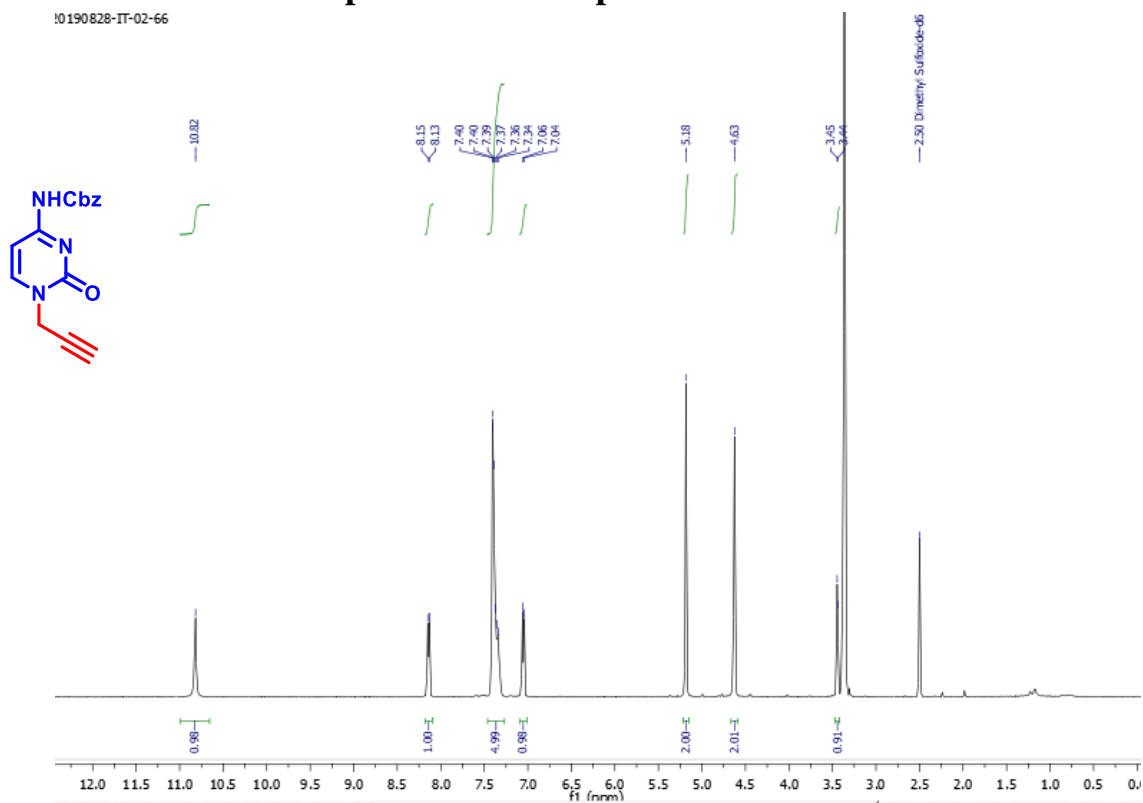
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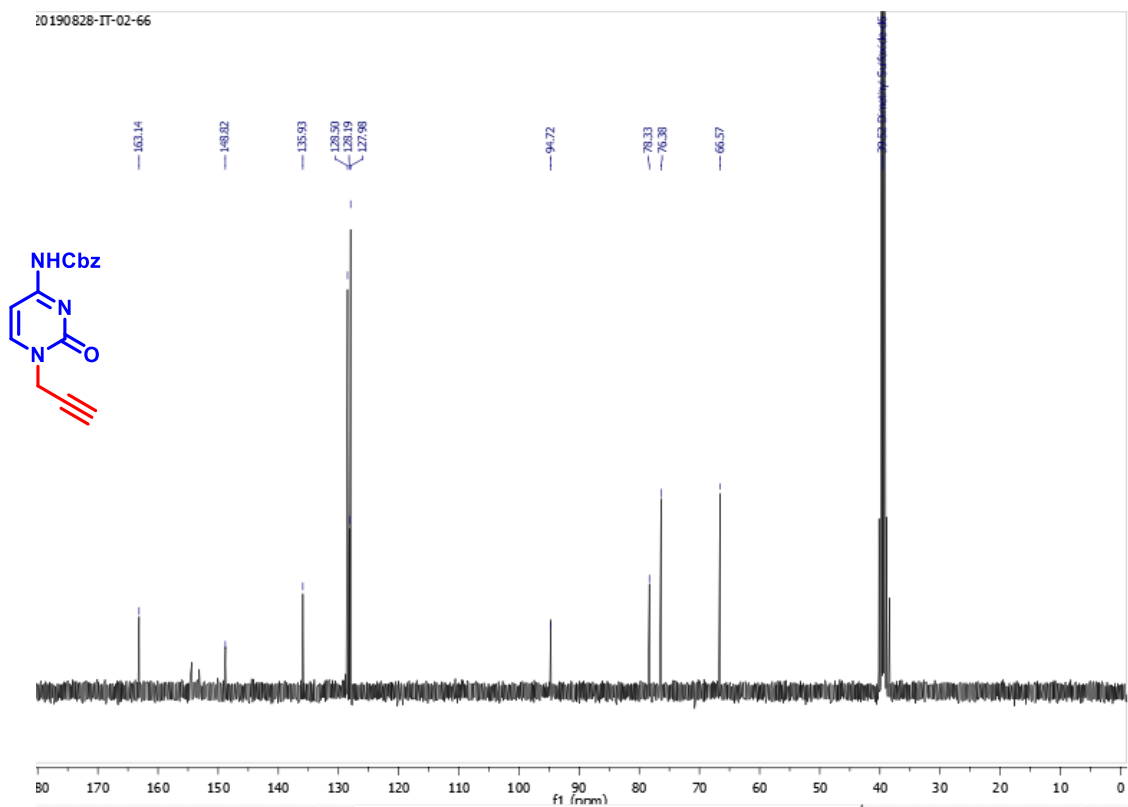
¹³C spectrum of compound 30



¹H spectrum of compound 31

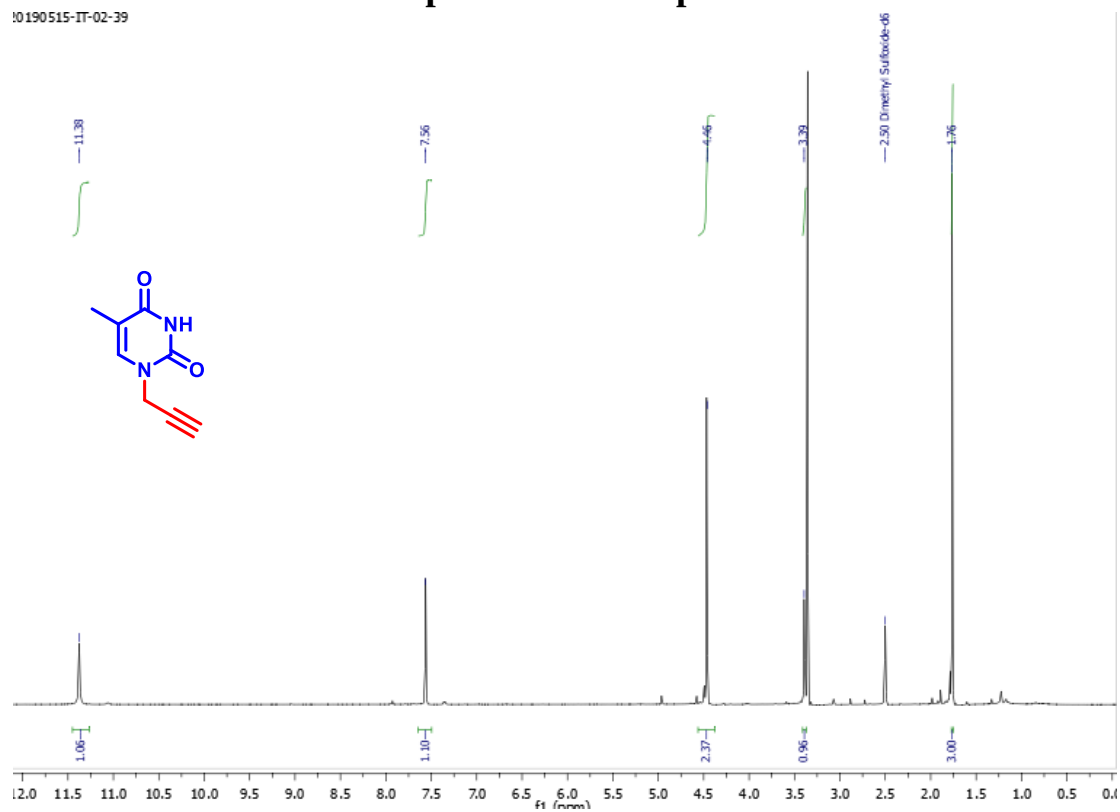


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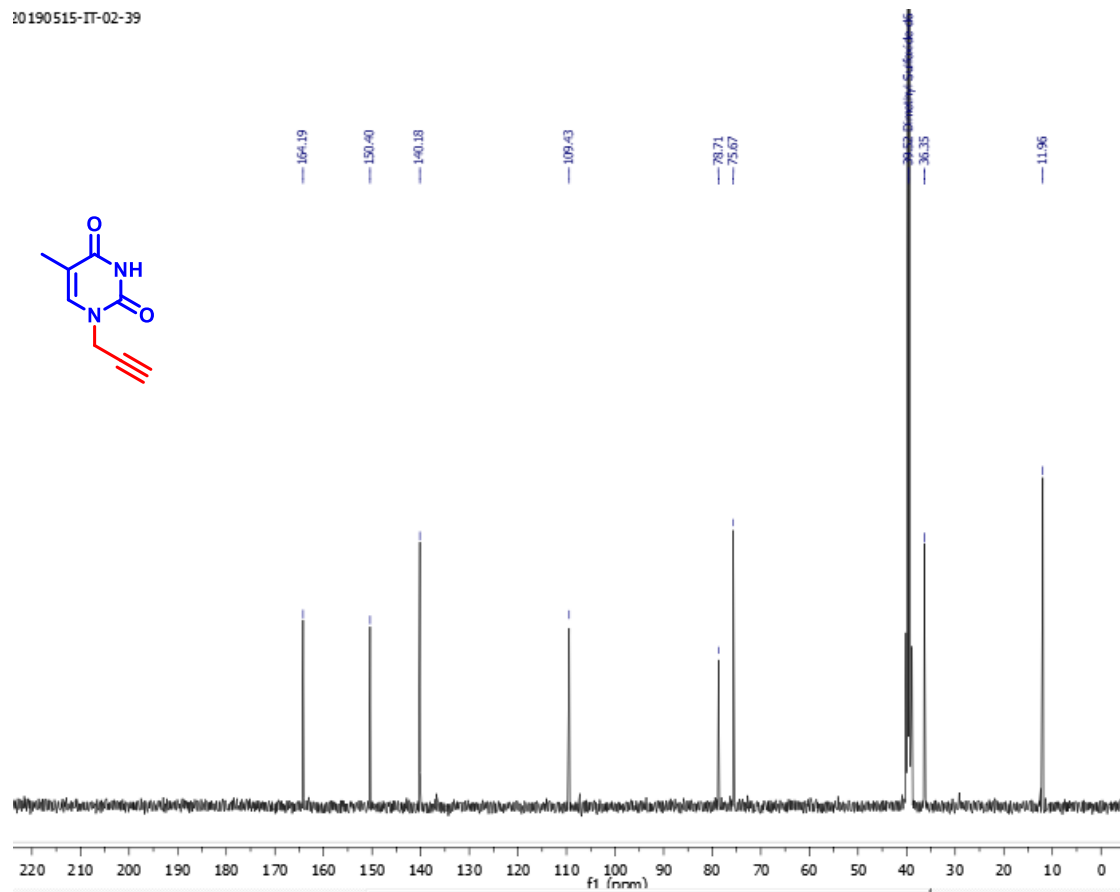
¹H spectrum of compound 32

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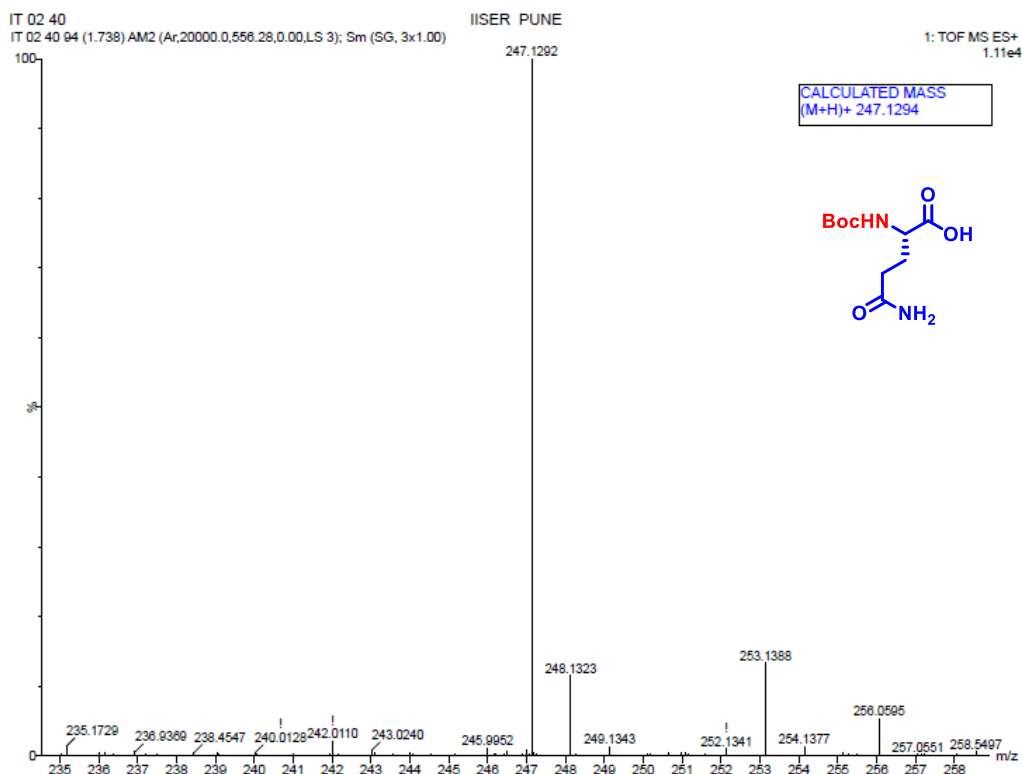
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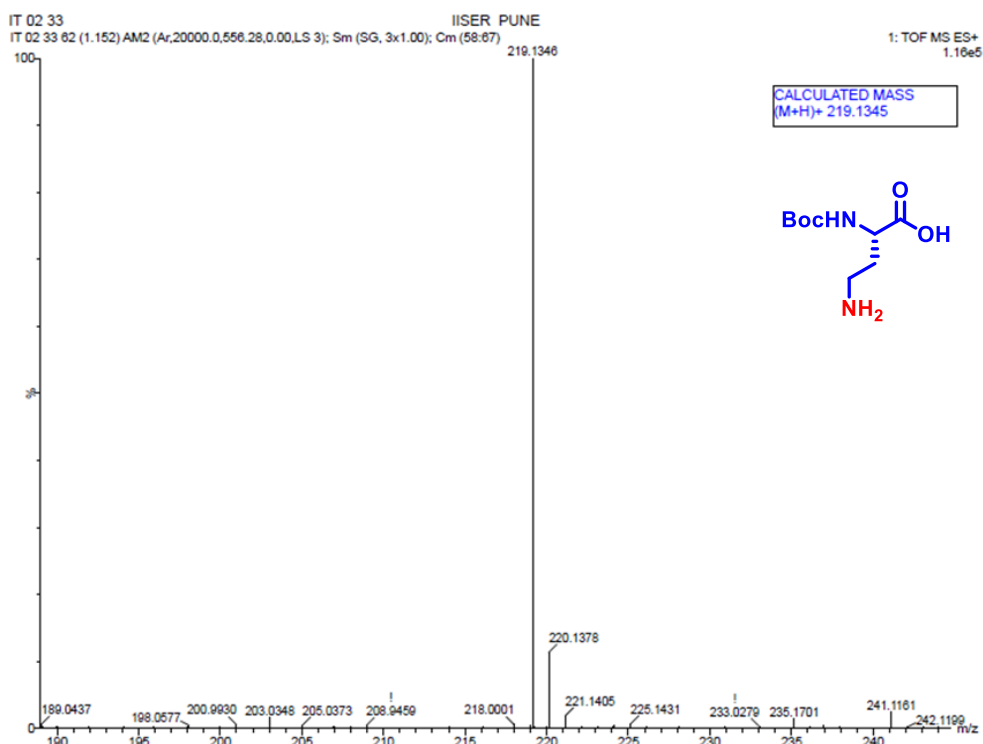


HRMS Spectra

HRMS Spectra of Compound 8



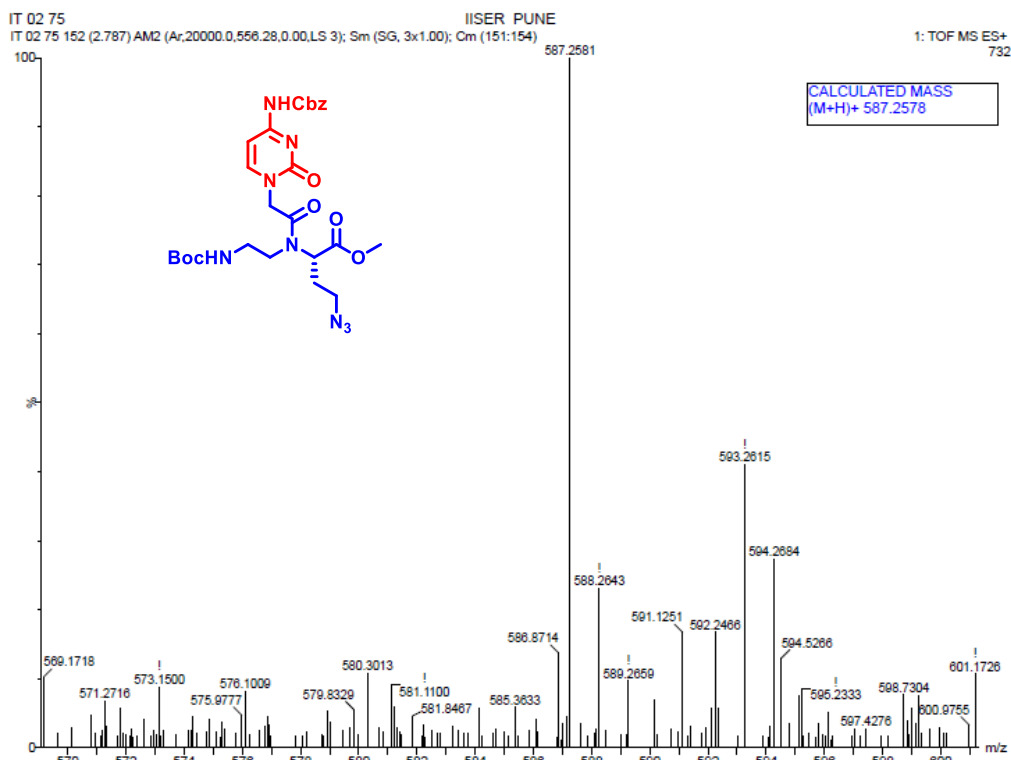
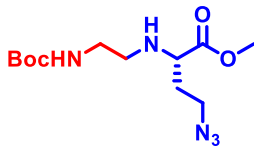
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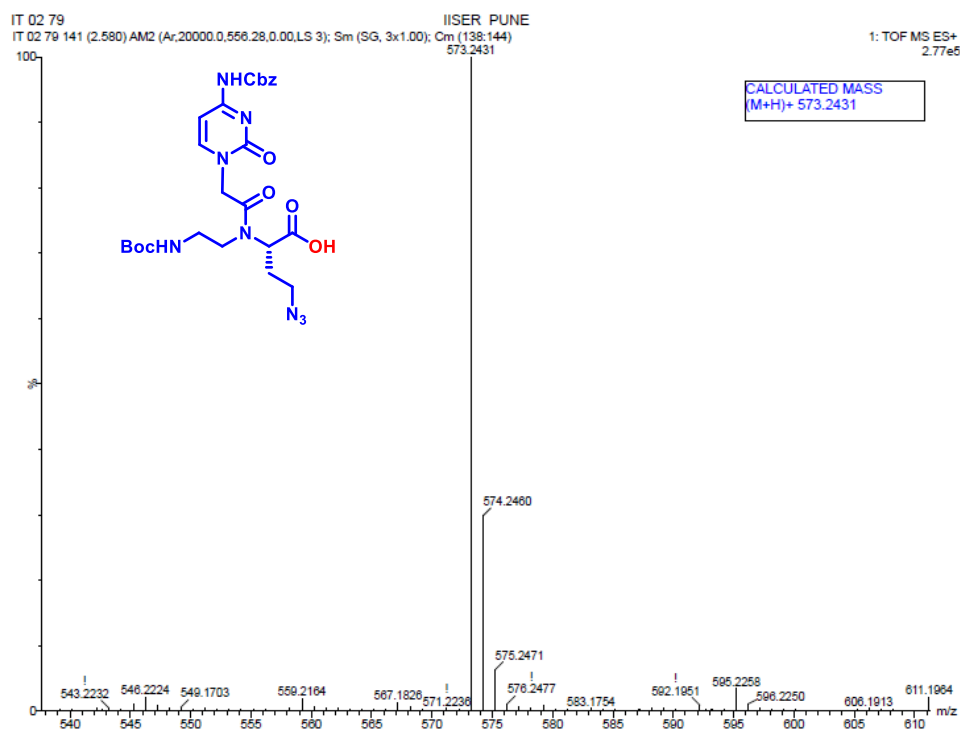


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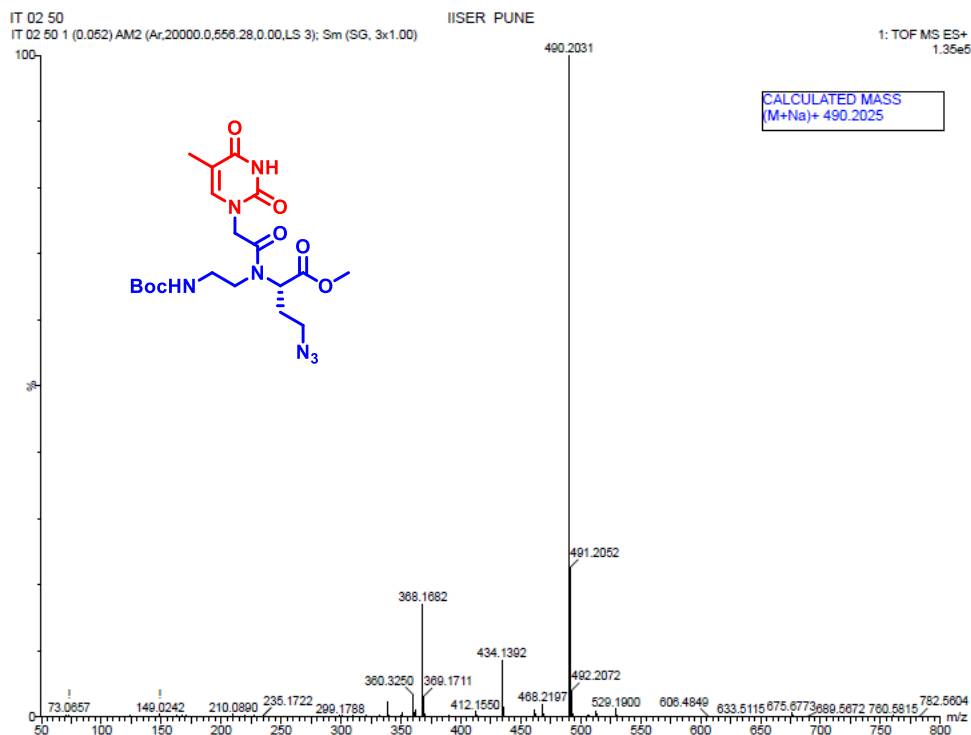




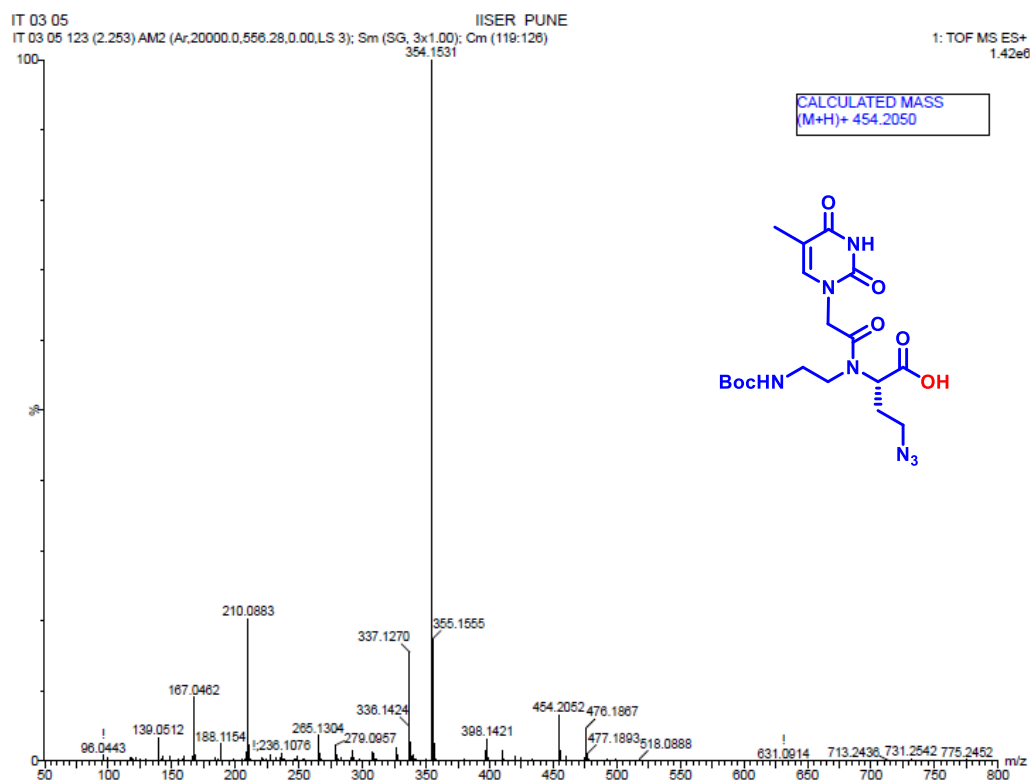
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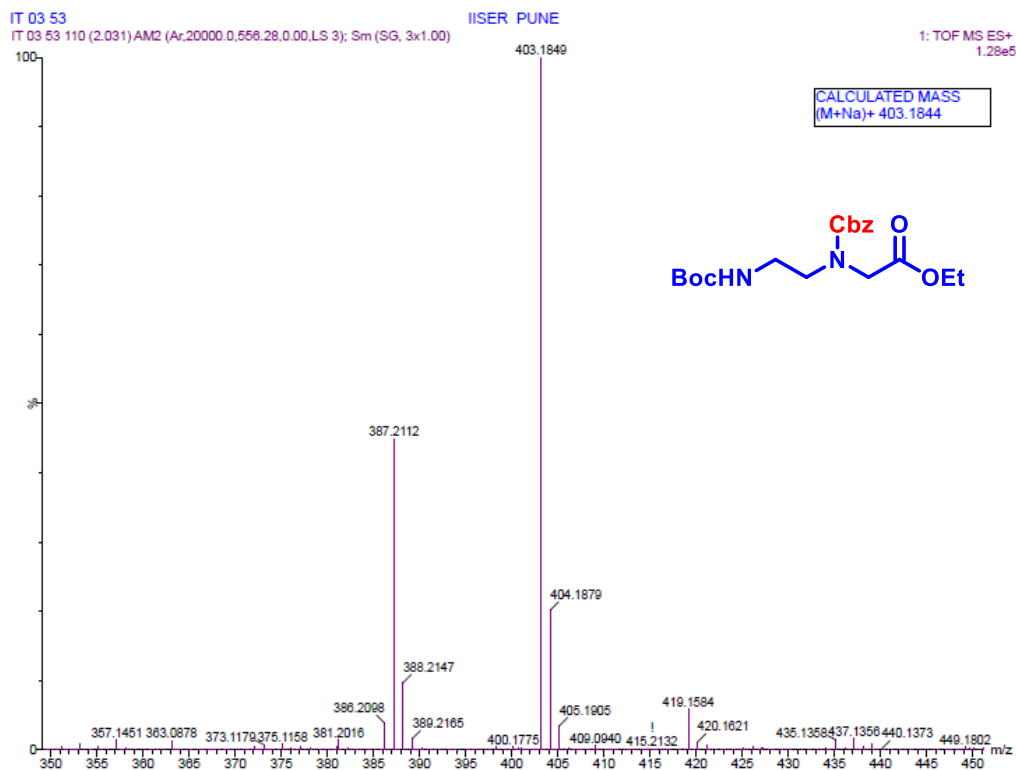
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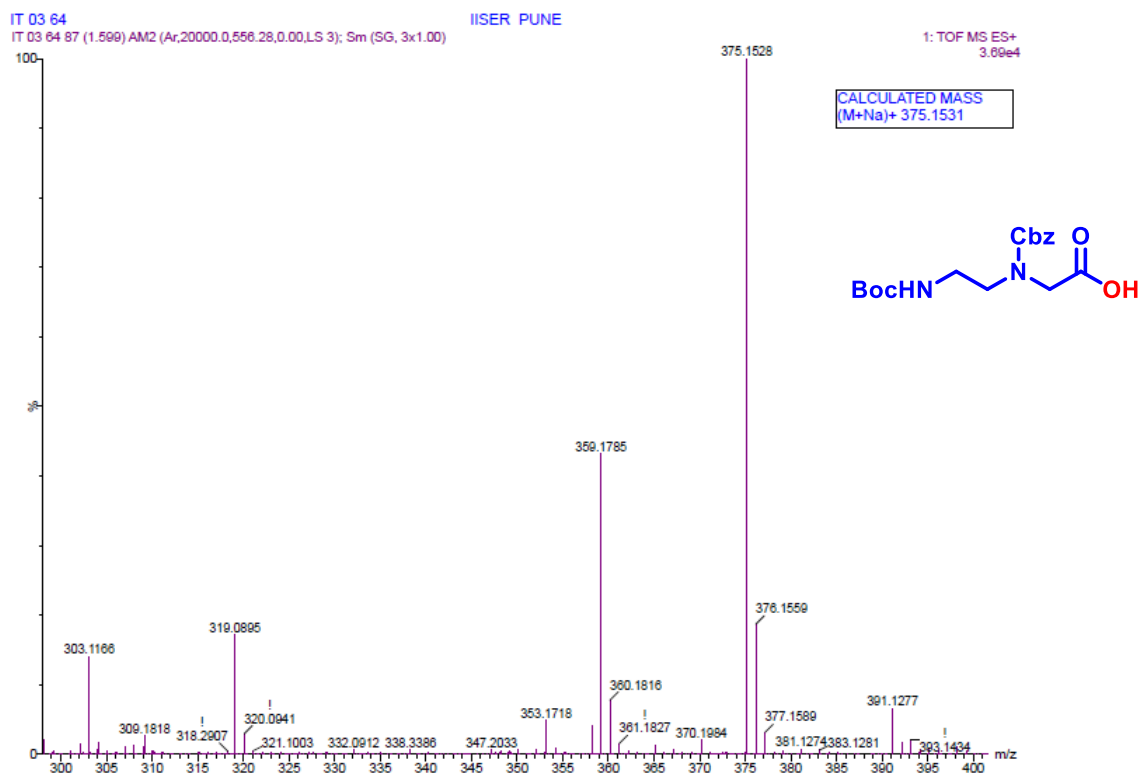
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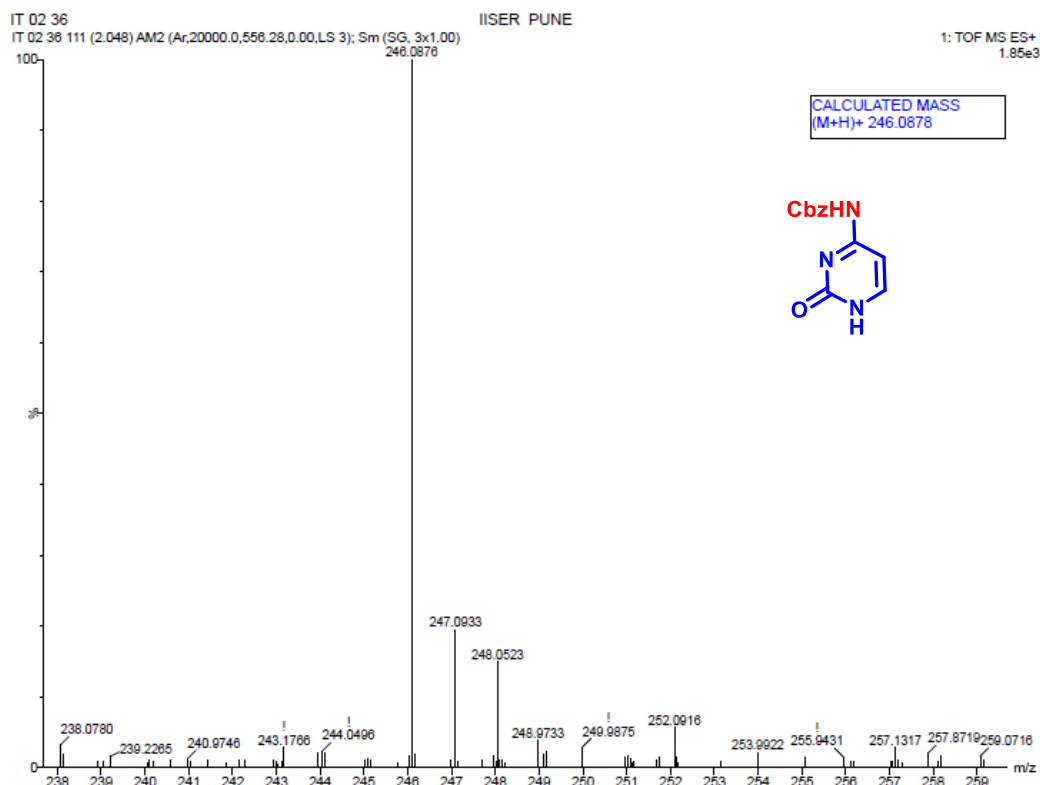
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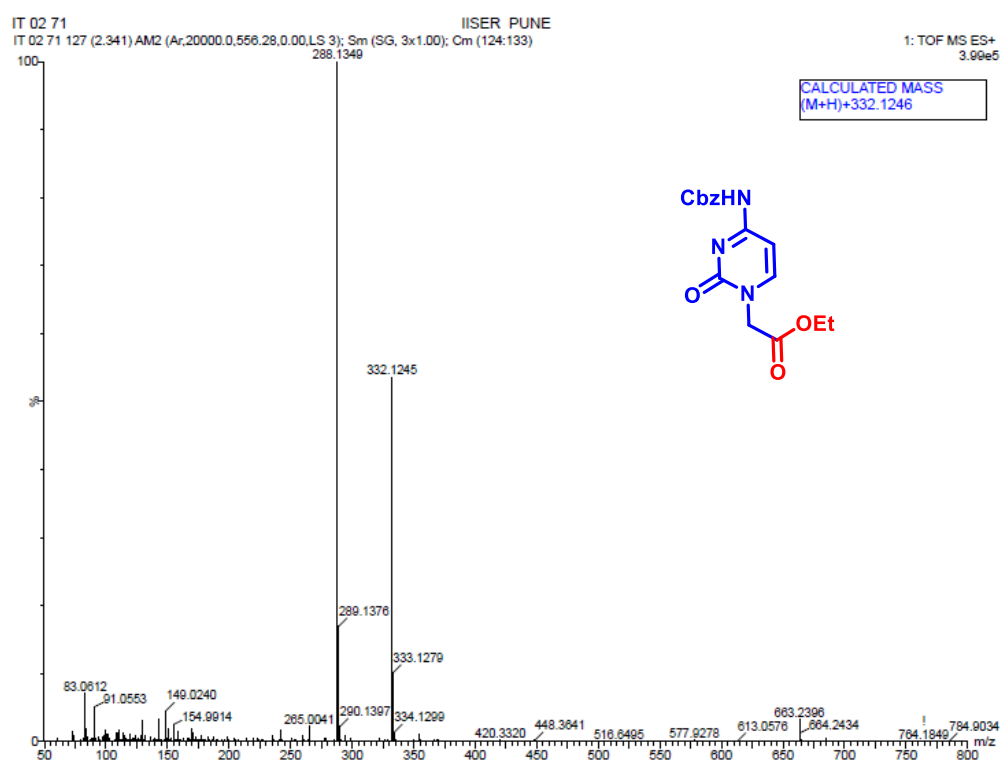
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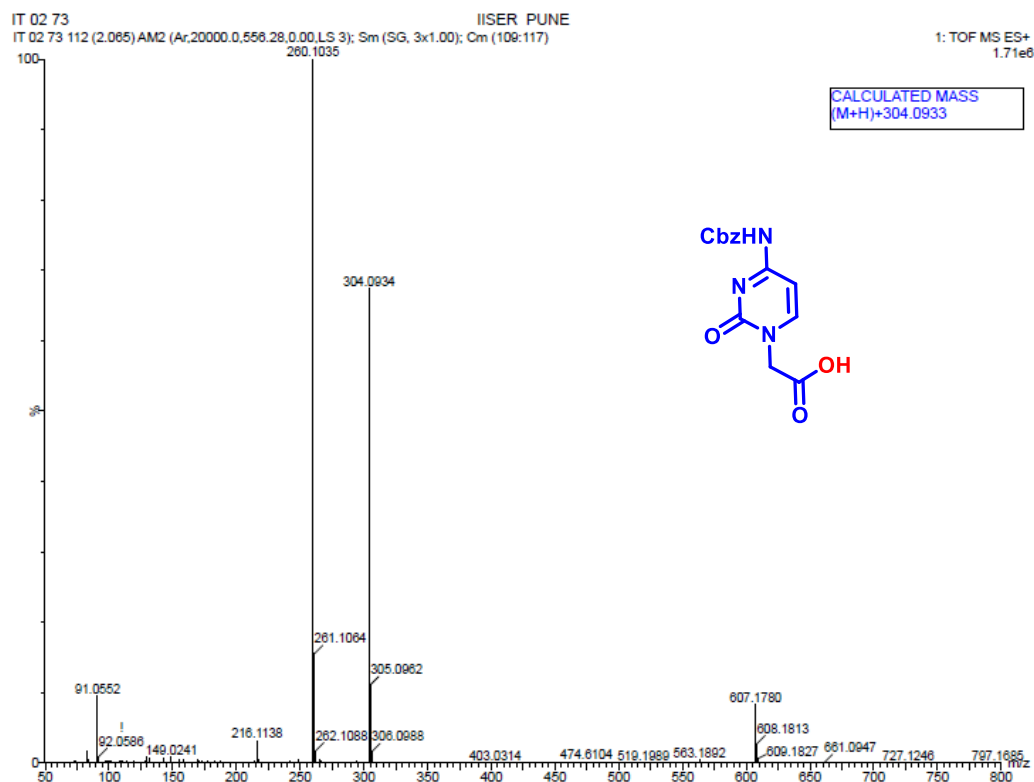
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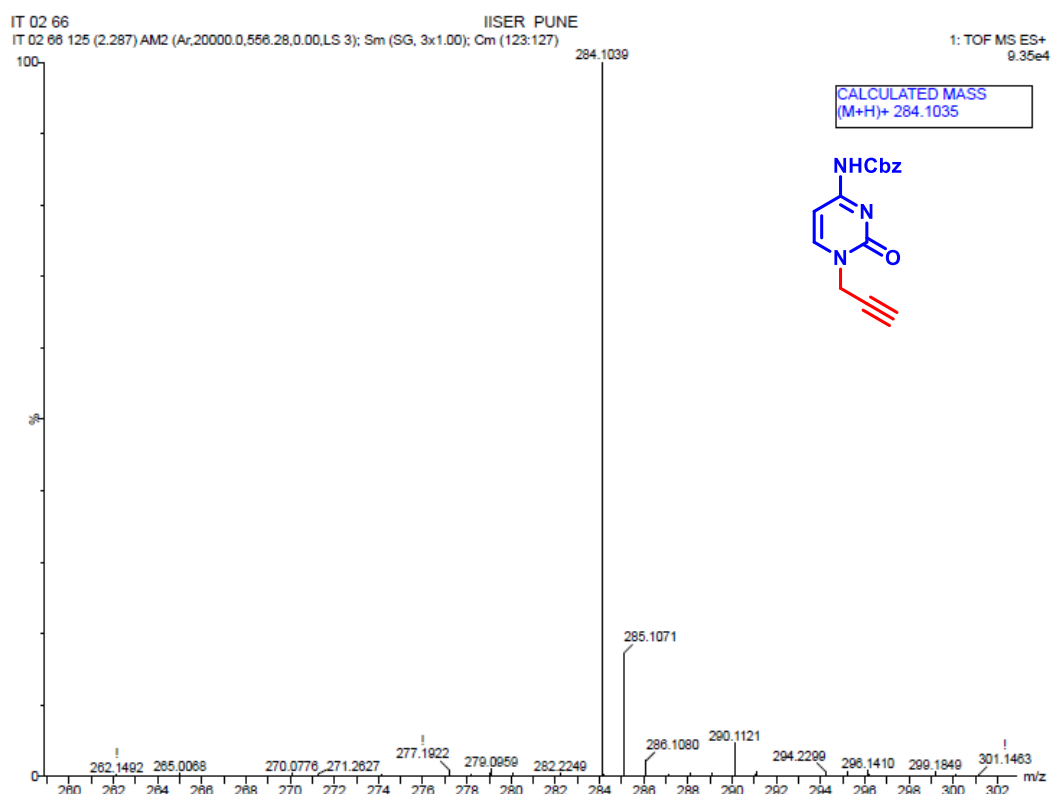
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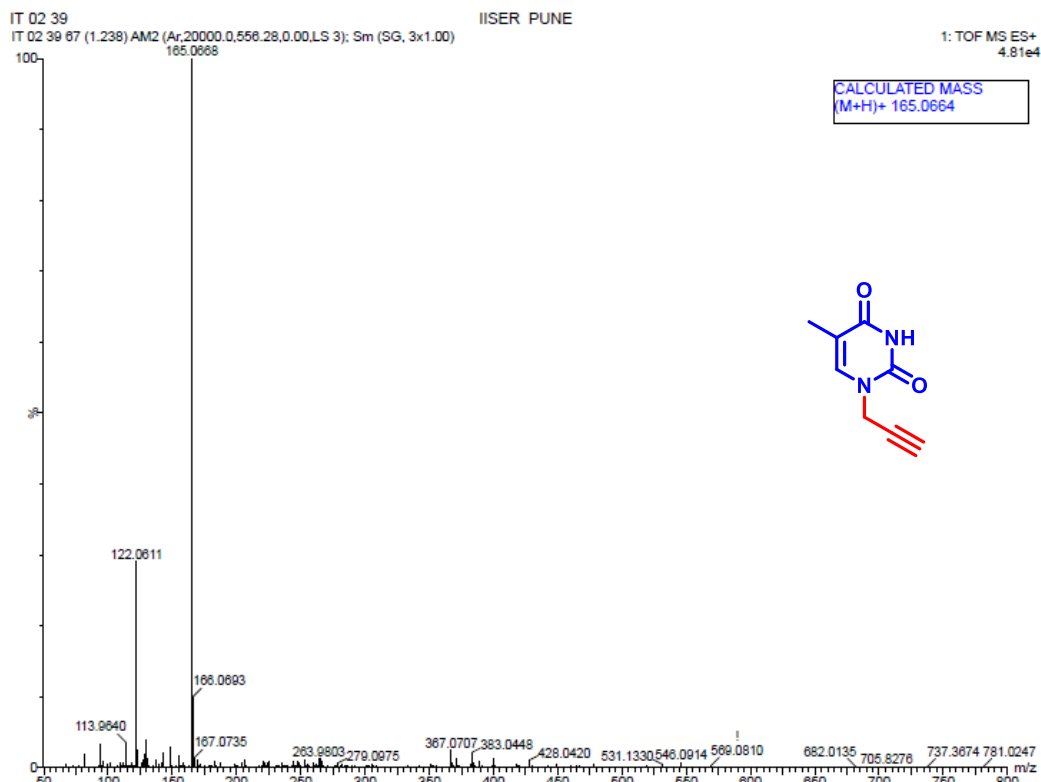
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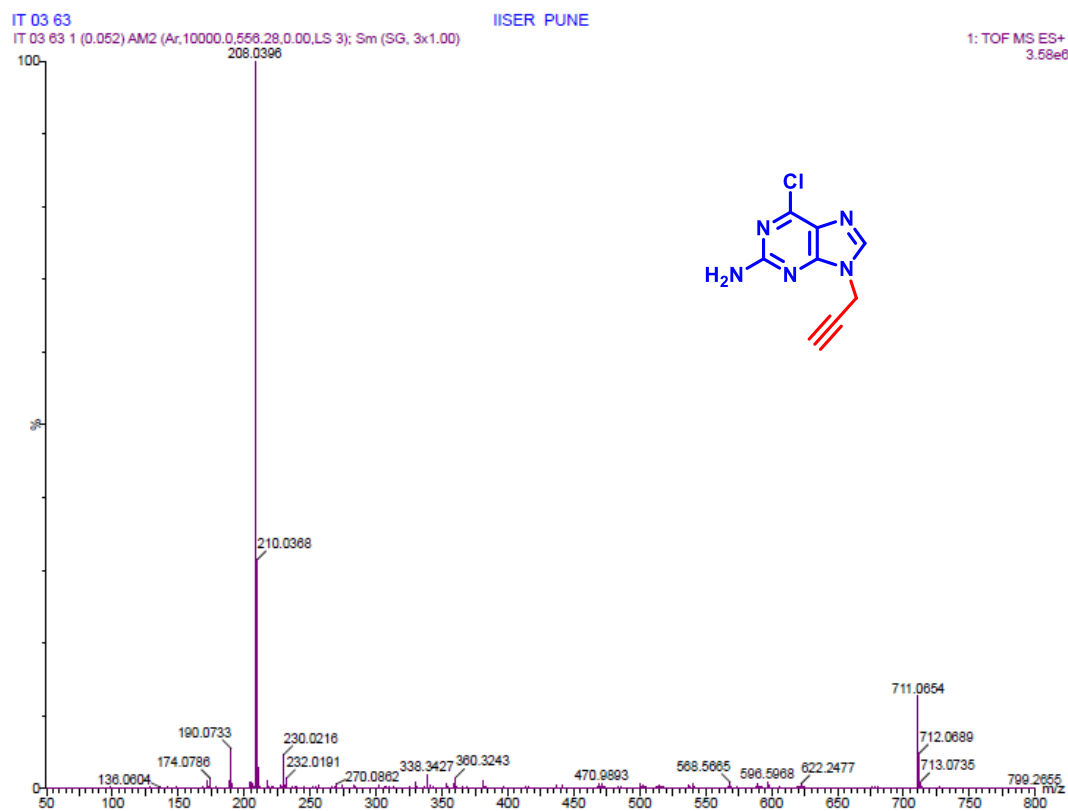
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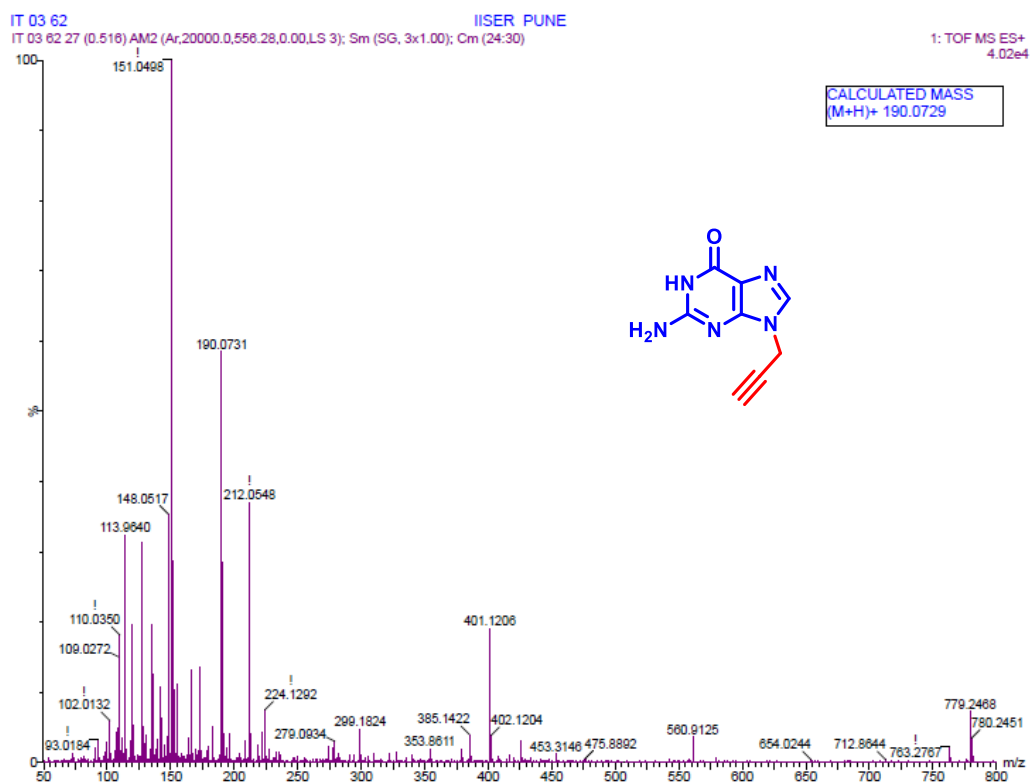
HRMS Spectra of Compound 32



HRMS Spectra of Compound 33



HRMS Spectra of Compound 34



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Chapter 3

Solid phase synthesis, Purification and Characterization of *bimodal* PNA Oligomers

3.1 Introduction: Solid phase peptide synthesis

Since Emil Fischer synthesized the first peptide in solution phase in 1901, the laborious and time-consuming method has been revolutionised by introduction of solid phase synthesis by Merrifield¹⁻⁵ in 1963. The method involves coupling of the desired N-protected amino acid to solid resin bound amino acid (free NH₂) employing suitable coupling reagent. After the coupling reaction, the excess amino acid and reagents are washed out, and the deprotection/coupling reactions are repeated in that order until the desired peptide is achieved. The need to purify the intermediates at every step as in solution phase is avoided. The resin bound peptide and the side chain protecting groups are cleaved in one final step. Merrifield's technique has undergone a series of modifications and improvements to overcome the physicochemical incompatibility of the growing peptide chain on the solid support. This has led to design of new types of solid supports to suit both Boc and Fmoc chemistries. Some resins which are frequently employed in solid phase peptide synthesis are shown (Figure 3.1).⁶ The main advantage is the amenability of Merrifield technique of solid phase synthesis to automation, resulting in development of several brands of automated peptide synthesizers, including microwave reactor for difficult couplings.

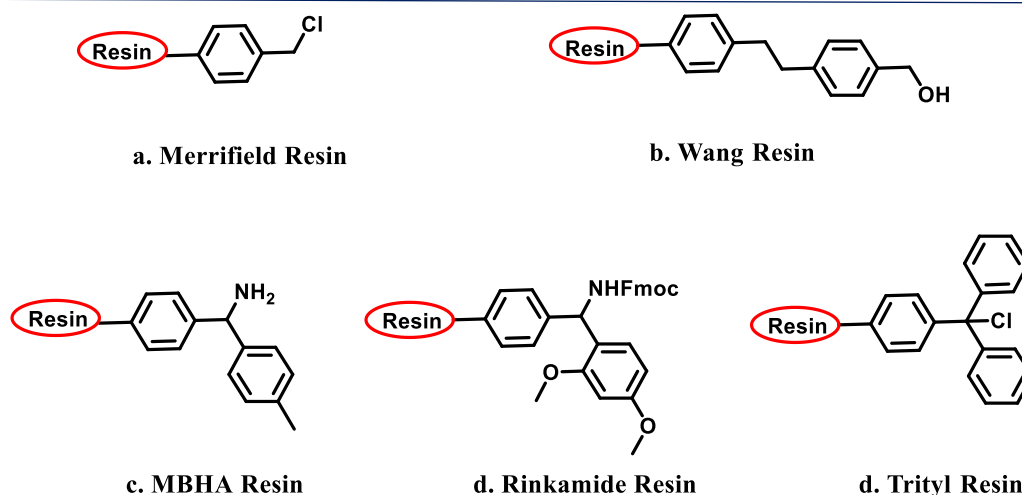
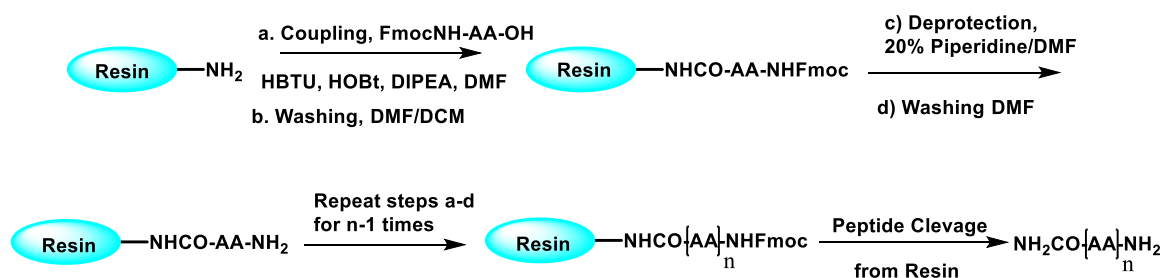


Figure 3.1 Resins commonly used for solid phase peptide synthesis

There are two standard chemical strategies for solid phase peptide synthesis depending on the type of temporary protecting group used in every cycle: (i) Fmoc strategy uses base labile protecting group and (ii) Boc strategy uses acid labile protecting group (Figure 3.2). Solid-phase peptide synthesis proceeds from C-terminus to N-terminus direction. The monomeric amino acid protected on α -amino group either as NHFmoc or NHBoc with free carboxylic acid is coupled on the solid support carrying free amino group. In the first protocol, fluorenylmethyloxycarbonyl (Fmoc) group used for N-protection is stable to acidic conditions and can be cleaved efficiently at the end of every cycle with the base piperidine and the side chain amino groups are protected as acid labile groups (*t*-Boc, Cbz). The final peptide and side chain protecting groups are then cleaved with acid (50% TFA). The second protocol uses *t*-butoxycarbonyl (*t*-Boc) group for N-protection removed by acid conditions such as 50% TFA in DCM at the end of each cycle. The reactive side chains are protected with groups that are stable for normal acid (Cbz, Bhoc etc) and finally removed under strong acidic conditions using HF in dimethylsulfide or TFMSA in TFA (Figure 3.2)⁷.

A) Fmoc-chemistry:



B) Boc-chemistry:

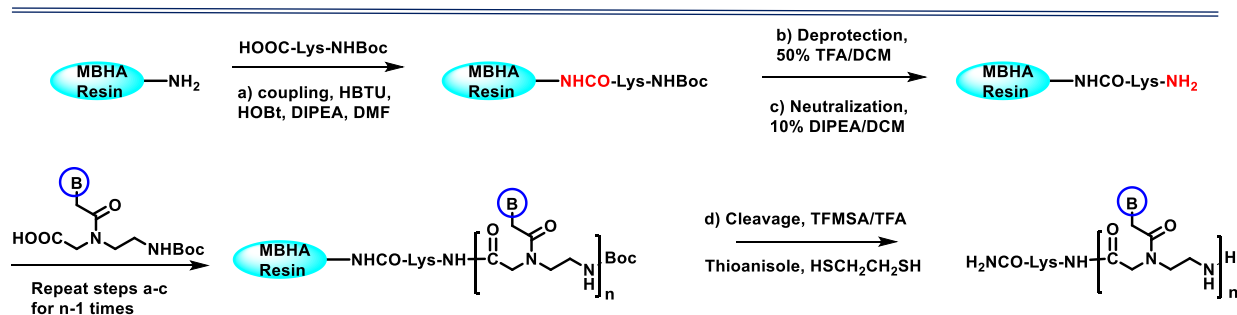


Figure 3.2 General protocols for solid phase peptide synthesis: (A) Fmoc-chemistry and (B) Boc-chemistry

3.2 Objectives of the present work

The triazole linker was chosen for the ease of synthesis by click chemistry. The side chain amide linker is structurally less complex, but needs careful orthogonal protection strategy to avoid side products during synthesis the main backbone amide chain. Since the nucleobase on α -sidechain is far removed from the backbone via flexible chain, the nature of linker is unlikely to have any significant effects on hybridization properties. Triazoles are bio isosteres of amides and it is stable to chemical transformations.

The specific objectives of this chapter are

- Incorporation of α -(*S-eaz*) *aeg* (T/C) monomers and *aeg* PNA (A/T/C/G) monomers by solid phase into PNA to yield oligomers with α -ethylazido side chains synthesis
- Click reaction of α -azide with base propynes on solid phase to yield *bm*- PNA oligomers
- Cleavage of oligomers from the solid support, purification by RP-HPLC and characterization by MALDI-TOF spectrometry

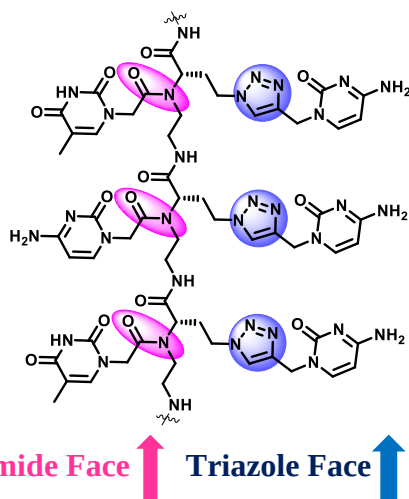


Figure 3.3 Representation of *bimodal* PNA oligomer

3.3 Results and discussion

This section describes the solid phase synthesis, purification and characterization of *bimodal* PNA oligomers incorporating modified ($C\alpha$ -ethylazide/ N^4 Cbz) monomers as well as unmodified *aeg* PNA monomers into PNA sequences at desired positions. This is followed by click reaction to obtain *bm*-PNA oligomers, deprotection, purification by HPLC and characterization of *bm*-PNA oligomers by mass spectral data.

3.3.1 Rationale for choice of sequences

The *bimodal* PNA oligomers has two (amide and triazole) faces, which can recognise two different DNAs or RNAs simultaneously through Watson- hydrogen bonding to both faces to form double duplex.^{8, 9} Homo-oligomeric *bimodal* PNA can form higher order structures such as triplexes and triplex of double duplexes.^{10, 11} The *bimodal* PNA oligomers having poly C_n (Figure 2.3) on $C\alpha$ side chains have potential to form *i*-Motif structure ($C:H^+:C$) at acidic pH.^{12, 13} Such *i*-Motifs from triazole face of *bm*-PNA (figure 3.4) possess free nucleobases on amide face to which complementary DNA strand can bind to create tetra duplex of tetraplexes.

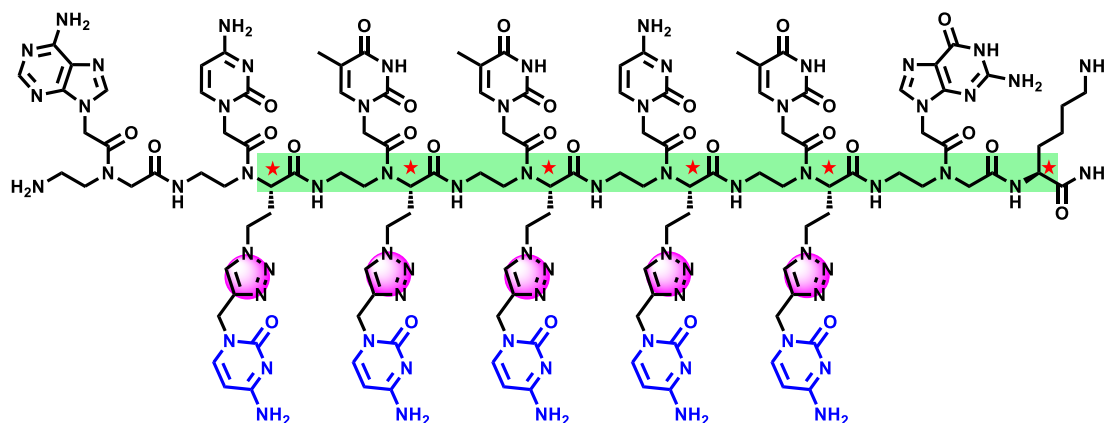


Figure 3.4 Target *bm*-PNA oligomer *bm*-PNA 1

C bases can self-pair through mediation of metal ions at neutral pH,^{14, 15} similar to H^+ at acidic pH. Hence *bm*-PNA duplexes with metal mediated base pairs ($C:M^+:C$) presents a promising strategy to generate self-assembling supramolecules with metal-based functionality. Specifically, Ag^+ has specificity for insertion into C:C mis-pairs, while Hg^{++} prefers insertion into T:T mis-pairs and stabilise them.¹⁶ Thus *bimodal* PNAs having poly C, on triazole or t-amide faces can form duplex from either side in the presence of silver. If the other face has mixed

sequence, such designed *bimodal* PNAs can form both metal mediated duplexes from one face and WC duplex from the other face resulting in triple duplexes.

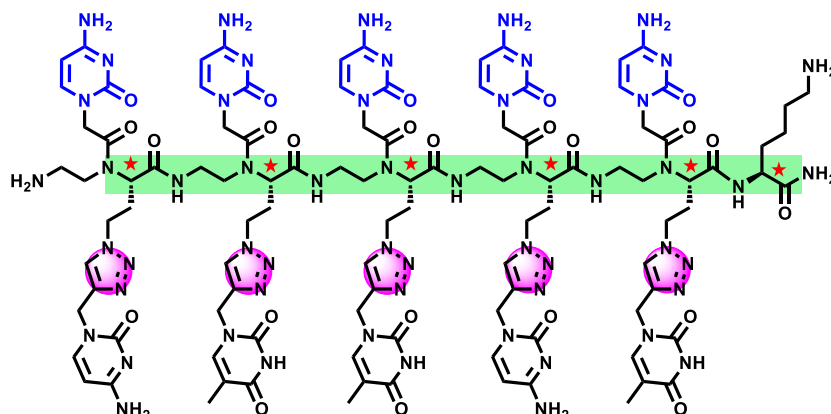


Figure 3.5 Target *bm*-PNA oligomer *bm*-PNA 2

The *bm*-PNA having poly Guanine on triazole face (Figure 3.6) can form G4-tetraplex structure^{17, 18} from that face in presence of Na^+ / K^+ under neutral pH and the resulting tetraplex having mixed base sequence on the second face can lead to formation of duplex resulting in tetra-duplex of triplex in presence at neutral pH.

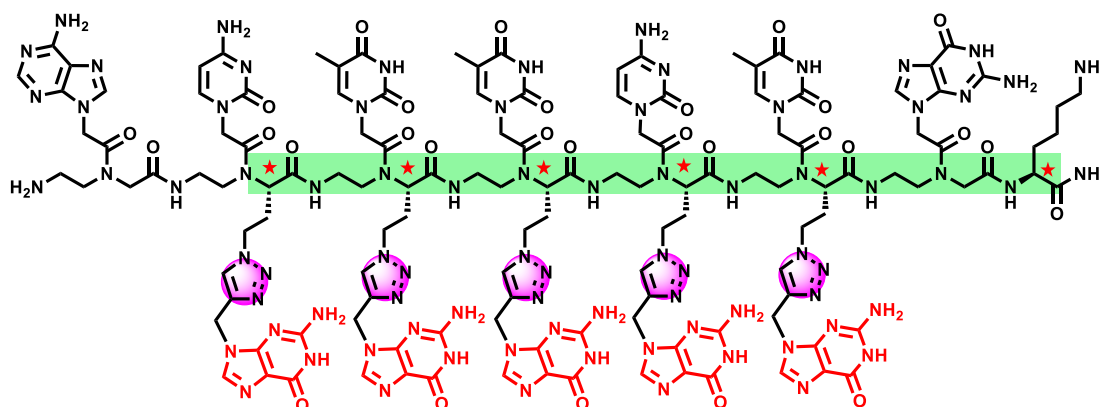


Figure 3.6 Target *bm*-PNA oligomer *bm*-PNA 3

Thus, depending on the modifications on either of the two faces (amide and triazole), various kinds of *bimodal* PNA oligomers were synthesized as shown in (Figure 3.7).

- (i) Amide face poly- C_n bimodal PNA oligomer (*bm*-PNA 2) for *i*-motif formation.
- (ii) Triazole face poly- C_n *bimodal* PNA oligomer (*bm*-PNA 1) for *i*-motif formation
- (iii) Triazole face poly-G bimodal PNA oligomer (*bm*-PNA 3) for quadruplex study
- (iv) Only triazole face poly- C_n : (*iso*-PNA1) and poly-Gn (*iso*-PNA 2)
- (v) Poly-T_n bimodal PNA oligomer *bm*-PNA 4 for metal assisted base pairing.

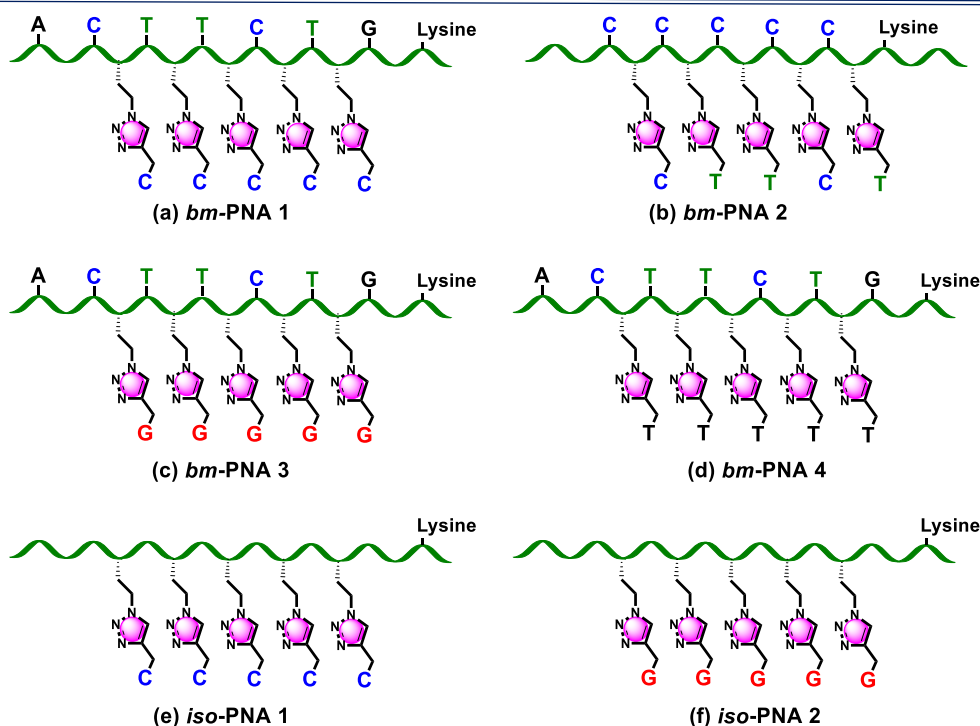


Figure 3.7 Different types of *bimodal* PNA oligomers.

3.3.2 Solid phase Synthesis of PNA oligomers

A most important factor in the synthesis of chiral PNAs from C α -substituted PNA monomers used in C α bimodal PNA is the possibility of epimerization at C α , during solid phase synthesis leading to racemic products. In C α -substituted N-acylated monomers, the C α -H becomes acidic. The coupling agent HATU is known to cause racemization and should be avoided during solid phase synthesis.¹⁹ By careful choice of the coupling reagents, the racemization can be reduced to very low levels. In particular, the best results were obtained using a DIC/HOBt combination, without preactivation in the presence of a base. Hence all couplings were done using HBTU-HOBt as HOBt is known to suppress racemization,¹⁹ and also avoiding pre-activation.

The synthesis of PNA oligomers was carried out using solid phase synthesis protocol using the Boc strategy on MBHA (4-methyl-benzhydryl amine) resin.²⁰ The modified PNA monomers were incorporated at specific positions in *aeg*-PNA sequences. PNA oligomer synthesis was carried out from the C-terminus to N terminus end using modified monomers with protected amino and carboxylic acid functions maintaining the orthogonality. The monomers were coupled by *in situ* activation with HBTU/HOBt. The synthesis of all oligomers was initiated by first coupling the orthogonally protected NHBoc/NHCbz -L-lysine (S) as the

C-terminal spacer amino acid, linked to MBHA resin through an amide bond. The amine loading on the resin was reduced from 0.6 mmol/g to 0.22 mmol/g by partial acylation of amine groups using the calculated amount of acetic anhydride.¹ This was done to increase the efficiency of coupling by lessening steric crowding with lower loading on the resin.

The deprotection of the NHBoc protecting group to NH₂ and the completion of coupling reactions were monitored by Kaiser's test^{21, 22} which is the most widely used qualitative test for the presence or absence of free amino group (deprotection/coupling). The *t*-Boc deprotection leads to a positive Kaiser's test in which the resin beads show blue color (Rheumann's purple). After completion of the coupling reaction, the resin beads will become colourless leading to a negative Kaiser's test. Using the standard solid phase synthesis protocol (Figure 3.8), the PNA oligomers of the desired length incorporating PNA monomers were synthesized.

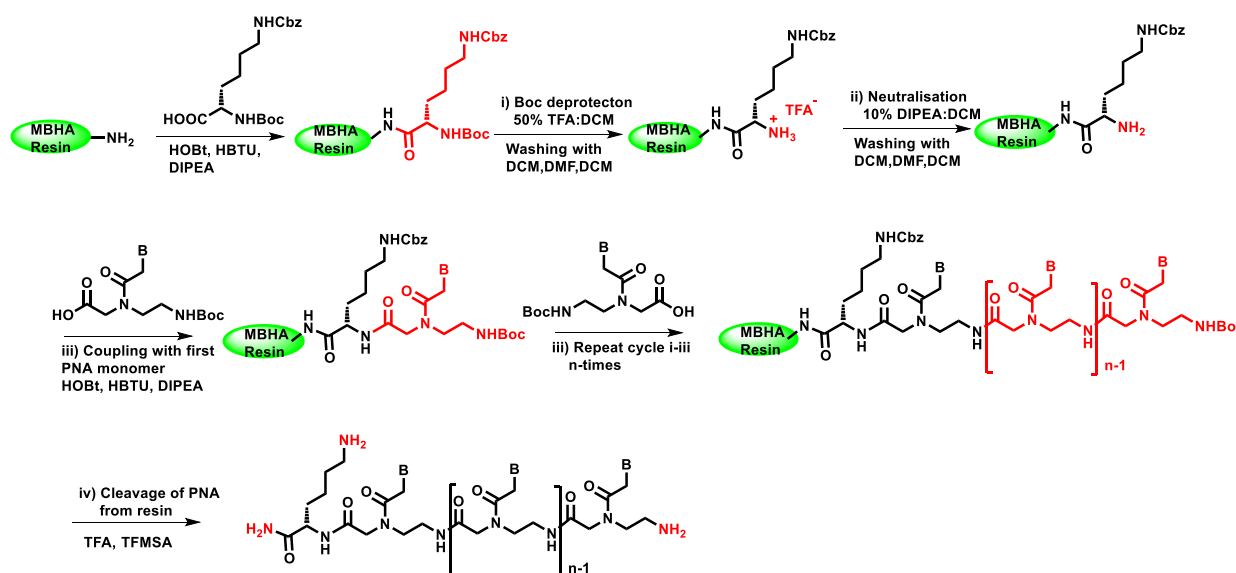


Figure 3.8 Solid phase PNA synthesis protocol by *Boc* strategy; B = Nucleobase such as Adenine(A), Guanine (G), Cytosine (C) and Thymine (T).

3.3.3 Synthesis of bimodal PNA having polycytosine on triazole face by click chemistry on solid phase (*bm*-PNA 1)

The strategies for the synthesis of various kinds of *bimodal* PNA oligomers has been described in this section. PNA monomers were incorporated into 7-mer PNA sequence by solid phase synthesis on L-lysine derivatized MBHA functionalized resin having 0.22 mmol/g loading value. Deprotection, neutralization followed by coupling of nucleobases (Figure 3.9) and repetition of the same steps gave the oligomer *bm*-PNA having five ethyl azide sidechains. In this PNA, both C and N termini used unmodified *aeg*-PNA monomers to avoid the steric

hindrance in synthesis and DNA hybridization reactions. In the subsequent step, a global click reaction of ethyl azido group on solid phase with N1-propynyl cytidine (**4**) gave solid phase bound MBHA- *bm*-PNA **1** which was cleaved from the resin by reaction with TFA/TFMSA to yield *bimodal* PNA oligomer ***bm*-PNA 1** (Figure 3.10).

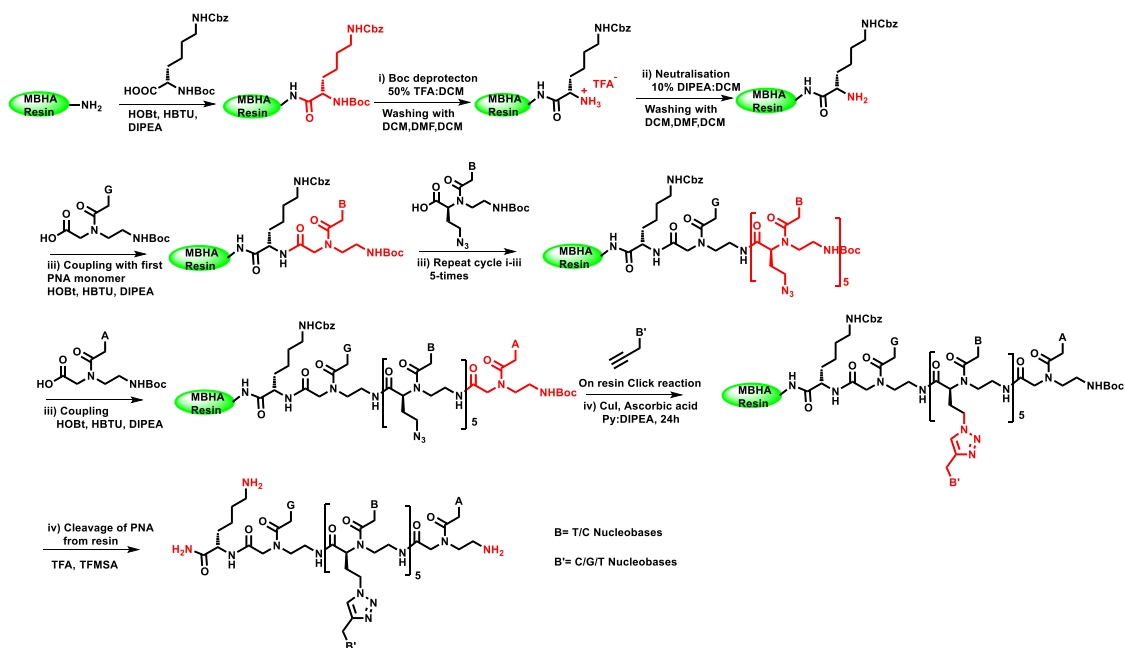


Figure 3.9 Solid phase synthesis protocol of *bimodal* PNA by *Boc* strategy.

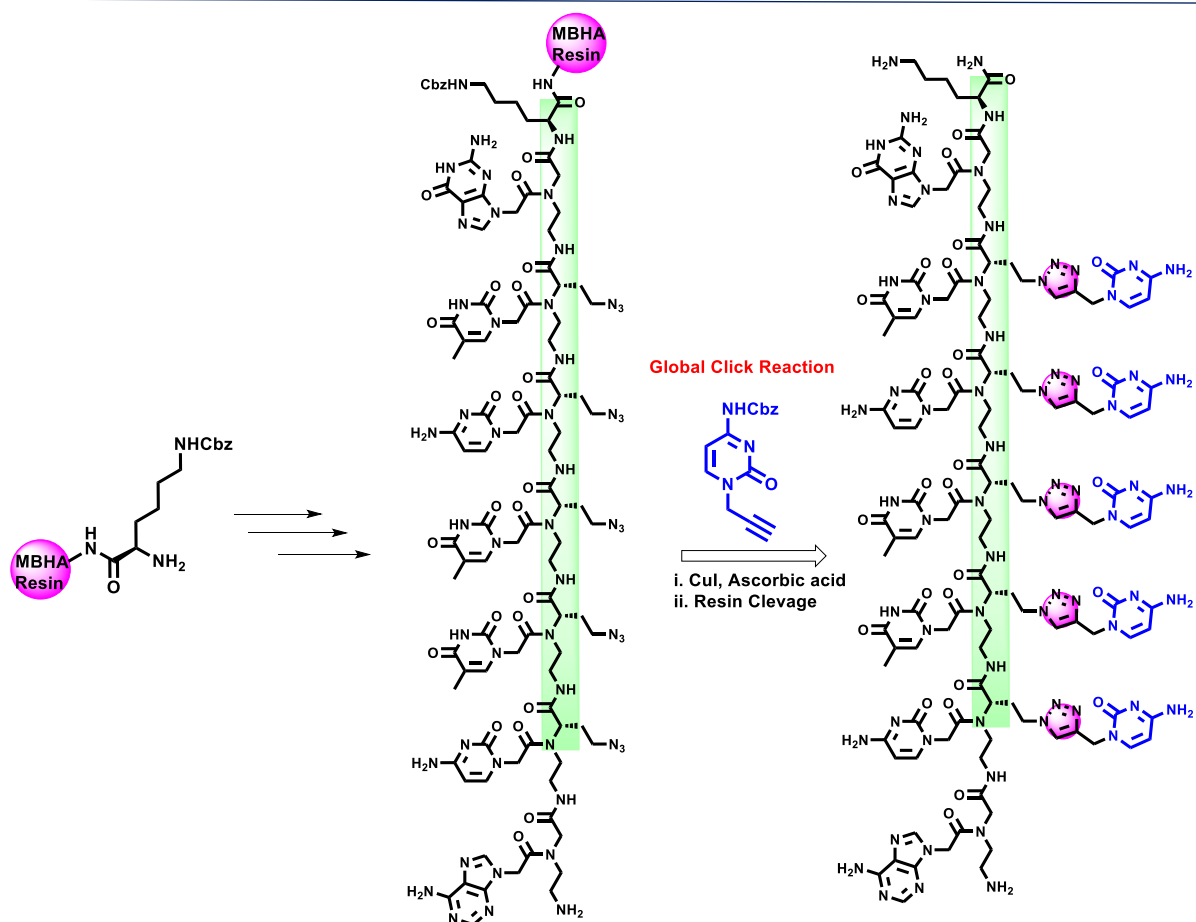


Figure 3.10 Solid phase synthesis of *bm*-PNA 1

3.3.4 Synthesis of bimodal PNA having all-cytosine on amide face by click chemistry on solid phase (*bm*-PNA 2)

The *bimodal* PNAs that possess mixed pyrimidine bases on the triazole faces and poly C₅ on the amide face were synthesized by the same solid phase protocol as above, using MBHA resin and (C^{N⁴Cbz}N1-acetamido) aminoethyl (C α S-ethylazido) *aeg*-PNA monomer (**1**). As compared to strategies shown in (Figures 3.9 and 3.10), click reactions were done with appropriate propyne bases (T/C) at desired positions in PNA sequence after each coupling step of monomers (Figure 3.11) to assemble the triazole face mixed pyrimidine sequence. One step, click reaction was avoided, as it would lead to homo pyrimidine sequence on triazole side rather than mixed sequence. This sequence of reactions leads to mixed sequences on the triazole face and homo polycytosine sequence on amide face to yield resin bound MBHA-*bm*-PNA 2 (Figure 3.12). The PNA oligomer was deprotected, cleaved from the resin, purified by HPLC and characterized by mass spectral data. To ensure successful and efficient coupling at all steps, a small amount of the resin was cleaved at intermediate stages of synthesis at difficult steps of coupling and the intermediate products were characterized by mass spectral data.

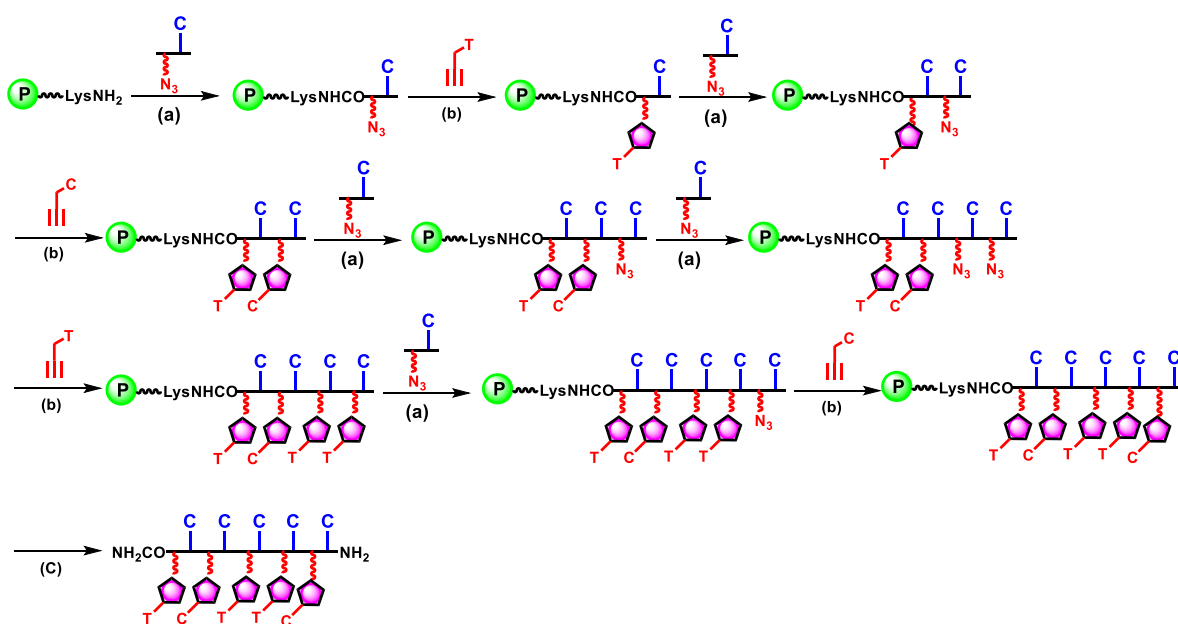


Figure 3.11 Solid phase synthesis protocol for *amide face poly C* (*bm*-PNA 2, P-MBHA resin), (a) Coupling reaction, (b) Click reaction (c) Resin cleavage

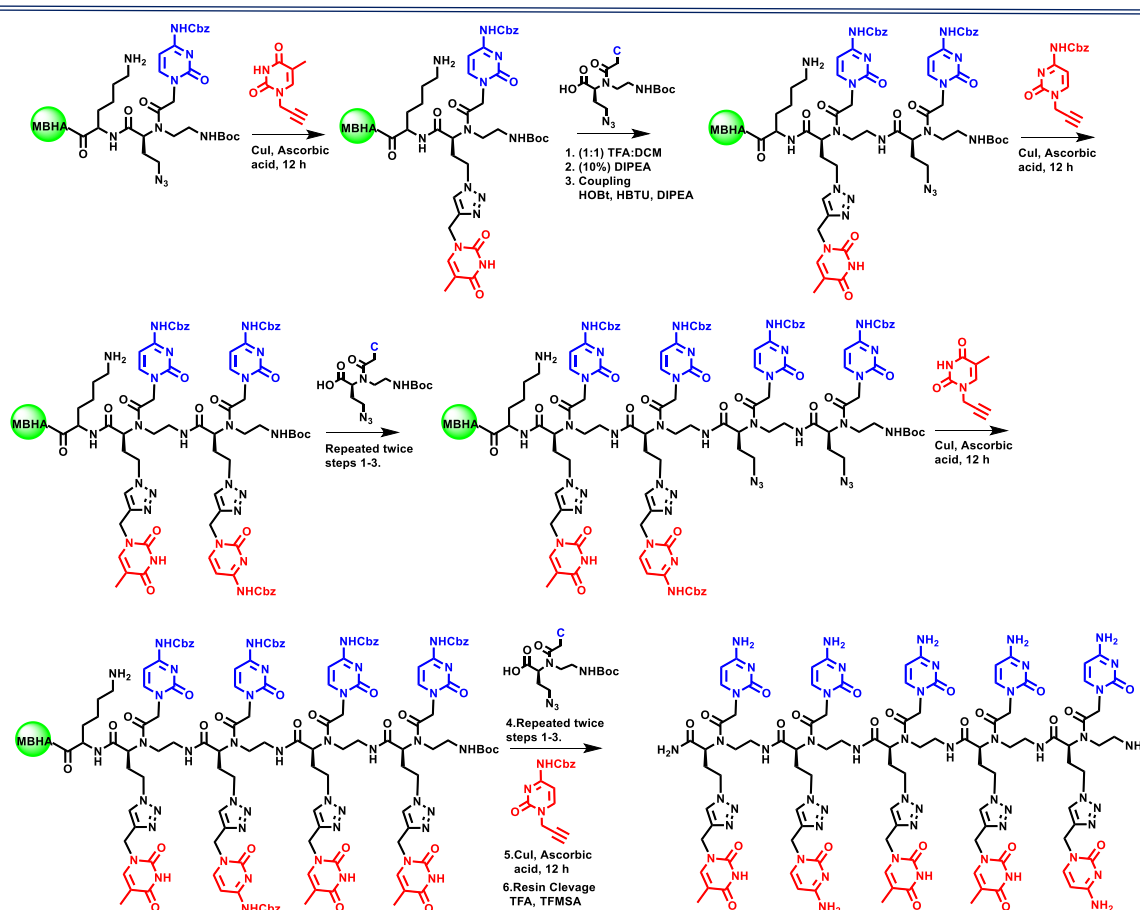


Figure 3.12 Solid phase synthesis of *bm*-PNA 2

3.3.5 Synthesis of PNA analogue with nucleobase on only on triazole face (*iso*-PNA)

For testing the hybridization efficiency of bases exclusively from the triazole face, synthesis of aminoethyl glycine backbone oligomers having only C α substituted triazole face bases (*iso*-PNA-*tz*-B5), but without any bases on t-amide face were synthesized (Figure 3.13). This involved the use of [NBoc,N'Cbz (C α S-eaz)] aeg monomer (3) and global click reaction on resin with N1-propenyl-C (4) or N9-propenyl- G (6) to yield the corresponding *iso*-PNA 1 or *iso*-PNA 2 having free secondary NH groups on the *aeg* backbone. After solid phase assembly, these were purified and characterized by mass spectral data in the same way as described for other bimodal-PNA oligomers.

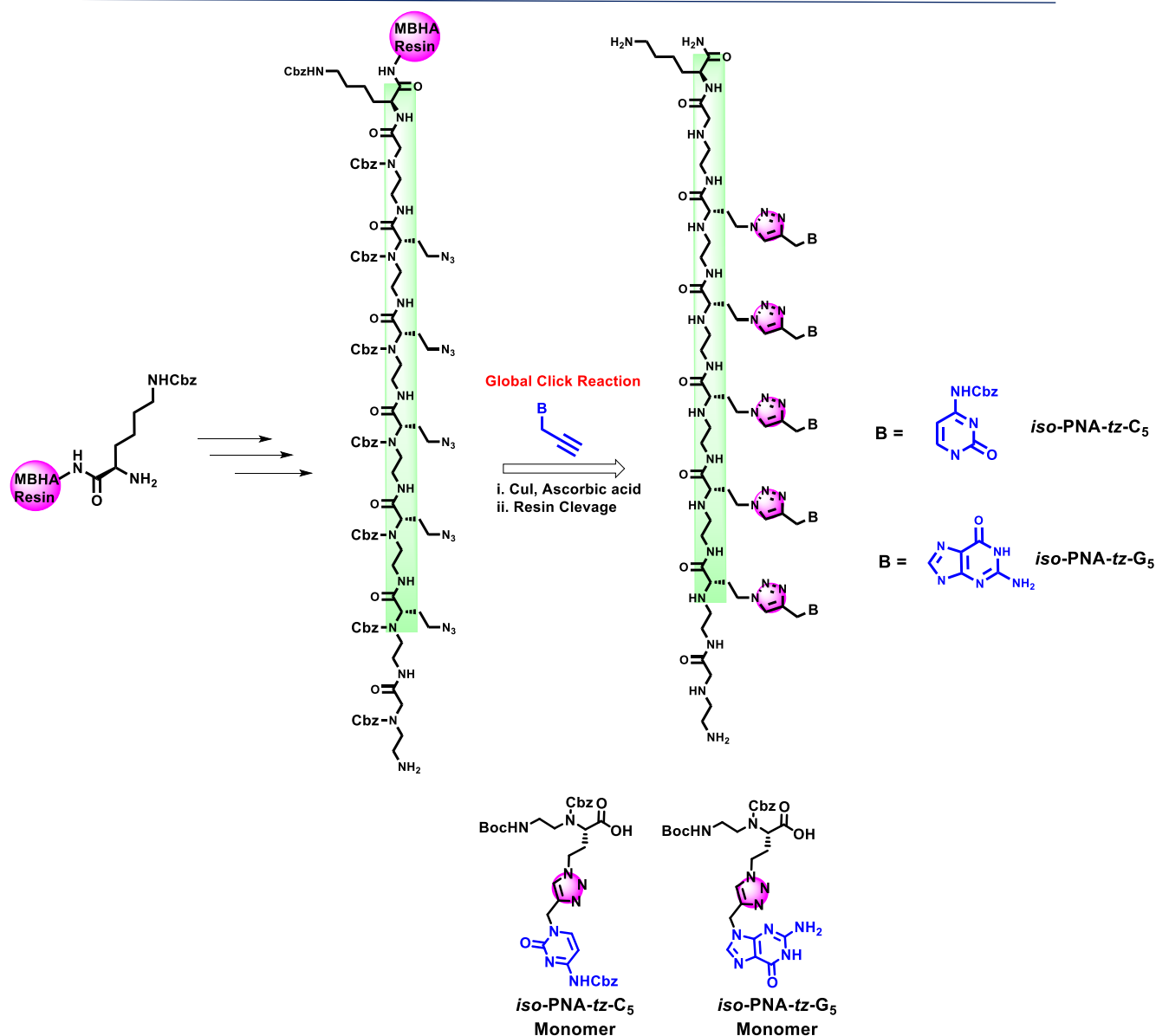


Figure 3.13 Solid phase synthesis of *iso*-PNA 1 and *iso*-PNA 2

3.3.6 Cleavage of PNA oligomers from the solid support

The resin bound bimodal and *iso*-PNA oligomers were cleaved from the solid support (L-lysine derivatized MBHA resin) using trifluoroethane sulphonic acid (TFMSA) in the presence of trifluoroacetic acid (TFA). This yielded PNA oligomers having L-lysine amides at their C-terminus.⁶ Under this cleavage condition, side chain protecting group was also removed accompanied by deprotection of nucleobases. After the cleavage reaction was over, the PNA oligomers obtained in solution were precipitated by addition of cold diethyl ether and the PNA oligomers were dissolved in de-ionized water.

3.3.7 Purification and characterization of the PNA oligomers

After the cleavage from the solid support, all PNA oligomers were purified by reverse phase high performance liquid chromatography (RP-HPLC) on semi-preparative C18 column using a gradient system of acetonitrile-water. The purity of PNA oligomers was checked by re-injecting the sample on the same C18 semi-preparative column. All HPLC chromatograms are shown in Appendix I. Any racemizations at coupling stage should lead to formation of diastomeric products should be seen as mixture on RP HPLC. However, the single peak observed in HPLC in all PNA oligomers suggested that under the coupling conditions employed with inclusion of HOBt, no significant racemizations occurred. MALDI-TOF mass spectrometry was used to confirm the integrity of the synthesized PNA oligomers. In literature, various matrices like sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 2,5-dihydroxybenzoic acid (DHB), α -cyano-4 hydroxycinnamic acid (CHCA) etc. have been reported to record the MALDI-TOF spectra. Among these, DHB and CHCA were used here as a matrix to record MALDI-TOF spectra for all the synthesized PNAs. The calculated as well as observed molecular weights for all the PNAs with their molecular formulae, the MALDI-TOF data for confirmation of mixed purine-pyrimidine PNA oligomers are shown in Table 3.2.

It should be pointed out that in *iso*-PNAs free NH groups are present in the backbone which may be protonated at physiological pH and thus *iso*-PNAs are polycationic and since they are purified with TFA in the mobile phase, they are present as salts with trifluoroacetate as counter anions. However, this did not cause any problems during purification. In a related study⁹ these were protected as acetates and these derivatives behave similarly as the free NH groups, without any differences. The amide face mixed base sequence chosen randomly in each bimodal PNA sequences

TABLE 3.1 The sequences of *bimodal*-PNA oligomers

Entry	Sequence Code	PNA Sequences
01	<i>bm</i> -PNA 1	

02	<i>bm</i> -PNA 2	
03	<i>bm</i> -PNA 3	
04	<i>bm</i> -PNA 4	
05	<i>iso</i> -PNA 1	
06	<i>iso</i> -PNA 2	
07	<i>aeg</i> -PNA-C ₅	
08	<i>aeg</i> -PNA-G ₅	
09	<i>aeg</i> -M-PNA	

Table 3.2 MALDI-TOF spectral analysis of the synthesized *bm* PNA/*iso* PNA/*aeg*-PNA Oligomers

Sr.No.	Sequence code	Mol. Formula	Calcd. Mass	Obsd. Mass	RT(min)
01	<i>bm</i> -PNA 1	[M]C ₁₂₆ H ₁₅₉ N ₆₉ O ₂₉	[M+H] 3105.12	[M+H] 3105.49	13.2

02	<i>bm</i> -PNA 2	[M]C ₁₀₄ H ₁₃₃ N ₅₅ O ₂₄	[M-3H] 2533.06	[M-3H] 2533.29	13.4
03	<i>bm</i> -PNA 3	[M]C ₁₃₁ H ₁₅₉ N ₇₉ O ₂₉	[M+K] 3343.33	[M+K] 3343.91	13.6
04	<i>bm</i> -PNA 4	[M]C ₁₃₁ H ₁₆₄ N ₆₄ O ₃₄	[M+2H] 3181.18	[M+2H] 3180.49	14.9
05	<i>iso</i> -PNA 1	[M]C ₇₉ H ₁₂₁ N ₄₇ O ₁₃	[M-5H] 1930.98	[M-5H] 1931.26	13
06	<i>iso</i> -PNA 2	[M]C ₈₄ H ₁₂₁ N ₅₇ O ₁₃	[M+K] 2176.37	[M+K] 2176.48	13
07	<i>aeg</i> -PNA-C ₅	[M]C ₅₆ H ₈₀ N ₂₈ O ₁₆	[M+H] 1401.43	[M+H] 1402.10	11.3
08	<i>aeg</i> -PNA-G ₅	[M]C ₆₁ H ₈₀ N ₃₈ O ₁₆	[M+2H] 1603.57	[M+2H] 1603.21	11.3
09	<i>aeg</i> -M-PNA	[M]C ₈₁ H ₁₀₉ N ₃₉ O ₂₄	[M+Na] 2036.00	[M+Na] 2036.27	12.3

3.4 Summary

The *aeg*-PNA, *iso*-PNA and *bimodal* PNA oligomers were designed to incorporate *homo* and *mix base* sequences on either face. The monomers (**1-6**) incorporated by solid phase synthesis on MBHA resin, using HOBt, HBTU and DIPEA as coupling reagent by standard solid phase synthesis protocols. The synthesised *aeg* PNA, *iso*-PNA and *bimodal* PNA oligomers on solid phase were cleaved from the solid support using standard procedures. The *bimodal* PNA, *aeg*-PNA and *iso*-PNA oligomers after cleavage were purified by RP-HPLC and characterized by MALDI-TOF spectrometry. Chapter 4 deals with the investigation of biophysical properties of PNA oligomers.

3.5 Experimental Methods

General procedures

3.5.1 Solid phase synthesis protocol

MBHA resin (4-methyl-benzhydryl amine resin) was chosen as solid support as it is stable to acid conditions. 200 mg resin having loading value 0.6 kept for swelling in DCM in the solid phase flask for overnight. After washing with DCM-DMF-DCM each for 3 times, resin neutralised with 10% DIPEA in DCM. In the first step, added (Boc/Cl-Cbz) Lysine monomer (1 eq) which was preactivated with HBTU/HOBt (1 eq) in dry DMF and kept it for 6-8 h. After Lysine coupling extra free amines on resin capped with calculated amount of acetic anhydride (overnight) in pyridine.

Solid phase synthesis started on 50 mg resin by the deprotection of the N-t-Boc protecting group of lysine with 50% TFA in DCM and neutralisation in 10% DIPEA in DCM. Similar protocol followed for each PNA monomers with coupling reagents (3 eq each) of desired oligomer sequence. For each coupling reaction 3-4 h required for successful coupling with nitrogen purging. All coupling reaction steps were monitored by Kaiser's test. Small amounts of resin beads taken in a test tube and heated with Kaiser's solution if resulting in a blue colour it indicates the presence of free amine (incomplete coupling of monomer) in that case recoupling needed. Finally, PNA oligomers cleaved from resin, and deprotected from other orthogonal protecting group.

3.5.2 Solid phase synthesis method for *bm*-PNA 2

The synthesis of *bm*-PNA 2 that possess mixed pyrimidine bases on triazole face and poly C bases on amide face started from lysine coupled MBHA resin (50 mg). In the first step, t-Boc group on lysine deprotected with 50% TFA in DCM, after washing with DCM-DMF-DCM (X3) resin neutralised with 10% DIPEA in DCM. Before coupling of first PNA monomer resin washed with DCM-DMF. To get mixed base sequence on triazole face sequential coupling and click reaction were done. First coupling was done with t-Boc protected C α -S-(*eaz*)-*aeg*-C (3eq), which was preactivated with HBTU/HOBt/DIPEA (3eq) in dry DMF. After 3h monitored by Kaiser's test which was negative. After successful coupling reaction resin washed with DMF-DCM-DMF.

Free azide group on C α position of monomer is available for click reaction. Then resin subjected for click reaction condition with N1-propargyl thymine. For thymine installation on azide group, propargyl thymine (6 Eq) premixed with CuI (18 eq), Ascorbic acid (5 Eq) in dry DMF:Pyridine (1:1). The reaction was done for 5 min in microwave at 65 °C and 25 W followed by keeping at 24 h at room temperature. Excess reagents were removed by filtration and the resin was washed with DMF, DCM, MeOH and saturated EDTA. Before coupling of next monomer resin dried on highvac and kept for swelling (in DCM) minimum 4h. This protocol

followed for each coupling and click reaction to get resin bound *bm*-C₅-PNA-*tz*-TCT₂C oligomer. After final click reaction washed excess DMF, DCM, MeOH and saturated EDTA then resin dried and subjected for cleavage condition.

3.5.3 Synthesis of *bimodal* PNA oligomers on solid support by ‘click’ reaction

The resin-bound PNA oligomers with C α -ethylazido side chain (10 mg, 0.20 mmol/g) in DMF:Pyridine (1:1) were reacted with corresponding nucleobase alkyne (9.0 mg, 6 eq) in the presence of CuI (12 mg, 18 equiv), ascorbic acid (3.0 mg, 5 eq), and DIPEA (15 μ L, 24 eq). The reaction was done for 5 min in microwave at 65 °C and 25 W followed by keeping at 24 h at room temperature. Excess reagents were removed by filtration and the resin was washed with DMF, DCM, MeOH and saturated EDTA.

3.5.4 Cleavage of the *bimodal* PNA oligomers from solid support

The MBHA resin (10 mg) after assembly of PNA oligomers were stirred with thioanisole (20 μ L) and 1,2-ethanedithiol (8 μ L) in an ice bath for 10 min. TFA (200 μ L) was added and cooled in an ice bath. TFMSA (16 μ L) was added slowly with stirring and the reaction mixture was stirred for 1.5 to 2 h at room temperature. The resin was removed by filtration under reduced pressure and washed twice with TFA and the filtrate was evaporated on a rotary evaporator at ambient temperature. The filtrate was transferred to Eppendorf tube and the peptide was precipitated with cold dry ether. The peptide was isolated by centrifugation and the precipitate was dissolved in water. Then, the peptides were filtered and purified by HPLC.

3.5.5 Purification of the *bimodal* PNA oligomers by RP-HPLC

The purification of PNAs was carried out on Dionex ICS 3000 HPLC system with semipreparative BEH130 C18 (10 $\times$ 250 mm) column using solvents water: acetonitrile with composition A: 0.1% TFA in CH₃CN: H₂O (5:95) and B= 0.1% TFA in CH₃CN: H₂O (1:1). The gradient for elution was 100% A to 100% B in 20 min, with flow rate of 2 mL/min. The HPLC elution's were monitored at 220 nm and 254 nm wavelength

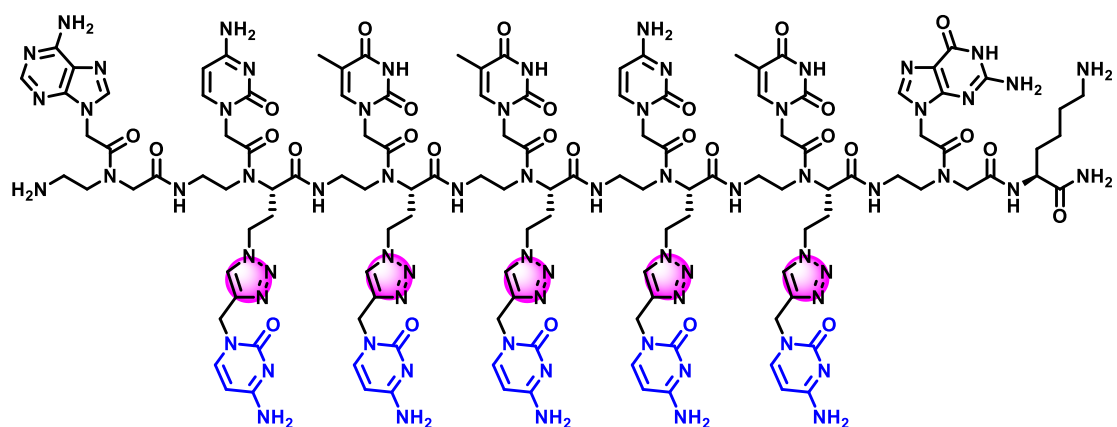
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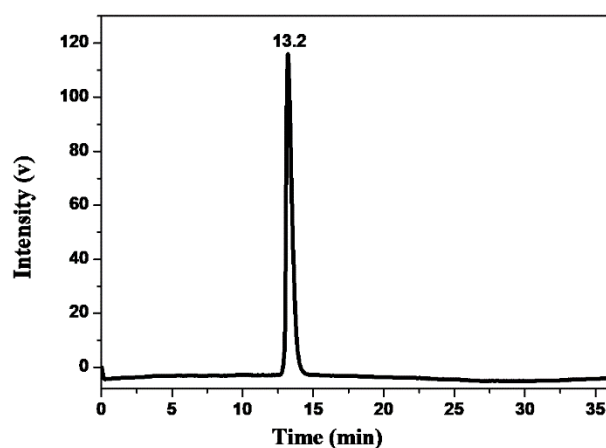
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3.7 RP-HPLC and MALDI-TOF of PNA Oligomers

3.7.1 *bm*-PNA 1



RP-HPLC



MALDI-TOF

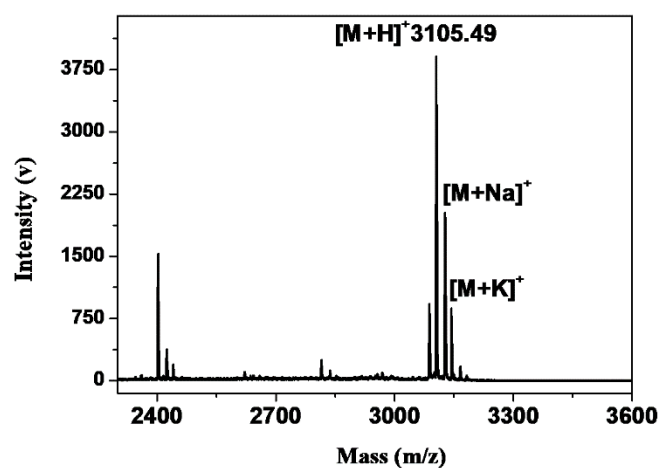
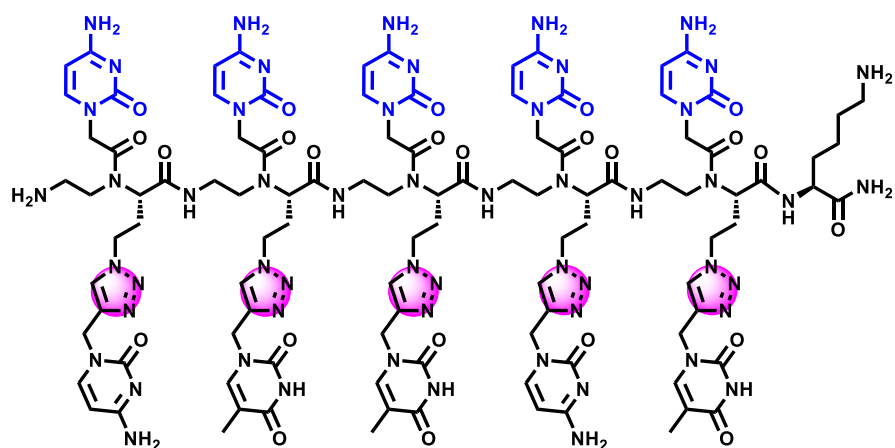
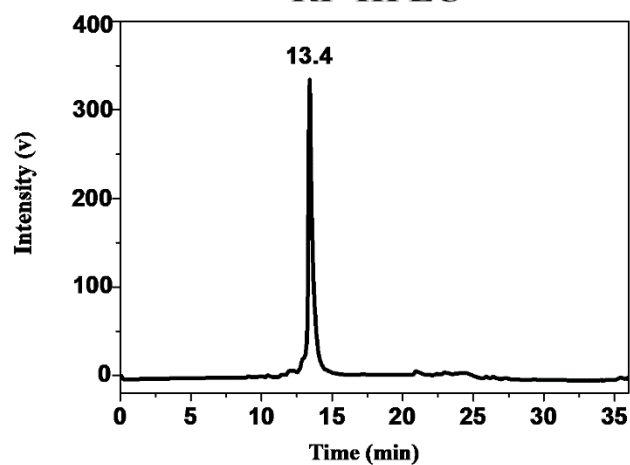


Figure 3.14 RP-HPLC and MALDI-TOF of *bm*-PNA 1
Additional peak at (m/z) 2400 corresponds to $[n-2 + K]$

3.7.2 *bm*-PNA 2



RP-HPLC



MALDI-TOF

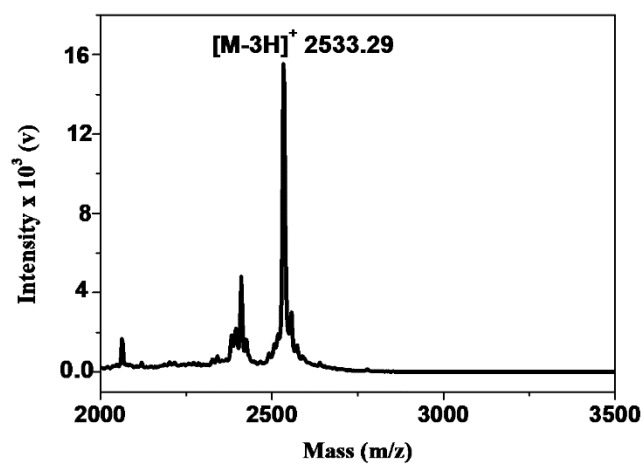
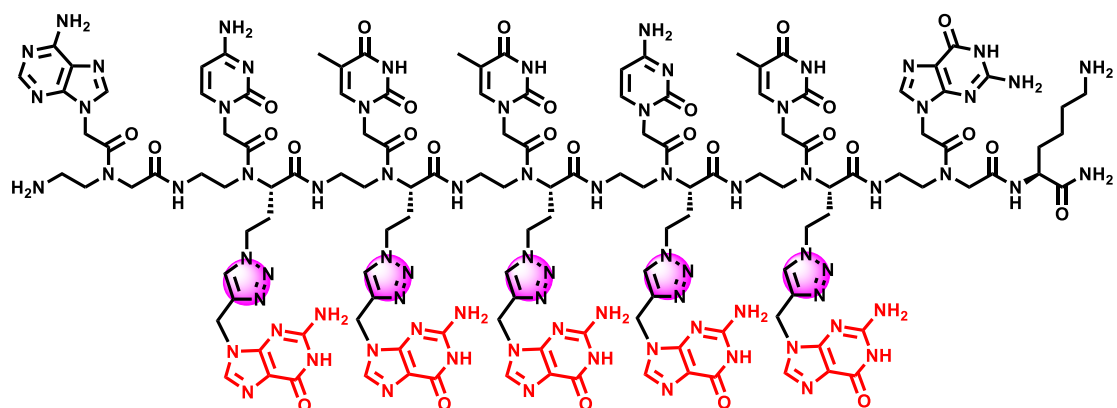


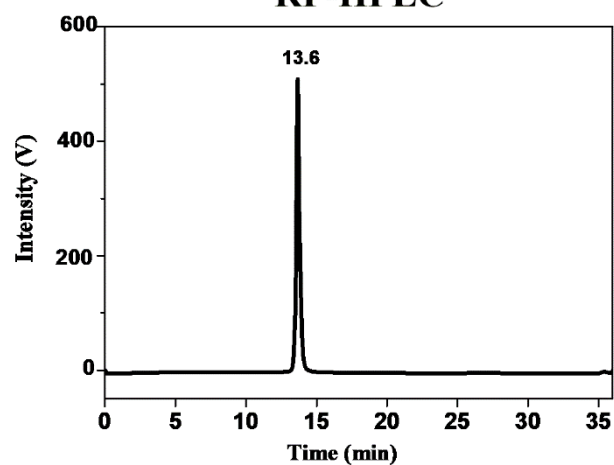
Figure 3.15 RP-HPLC and MALDI-TOF of *bm*-PNA 2

Additional peak at (m/z) 2410 represents **bm**-PNA 2 with no lysine on C-terminus

3.7.3 *bm*-PNA 3



RP-HPLC



MALDI-TOF

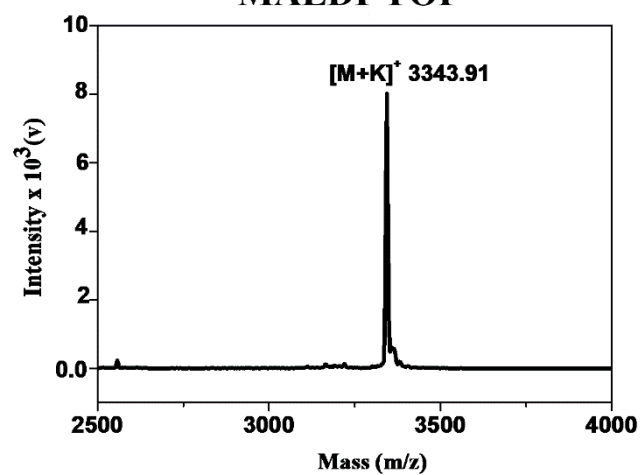
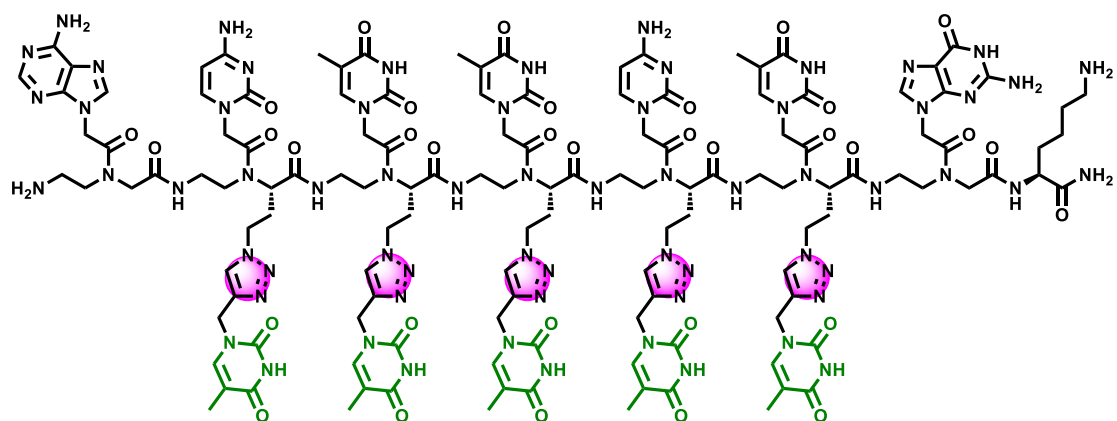
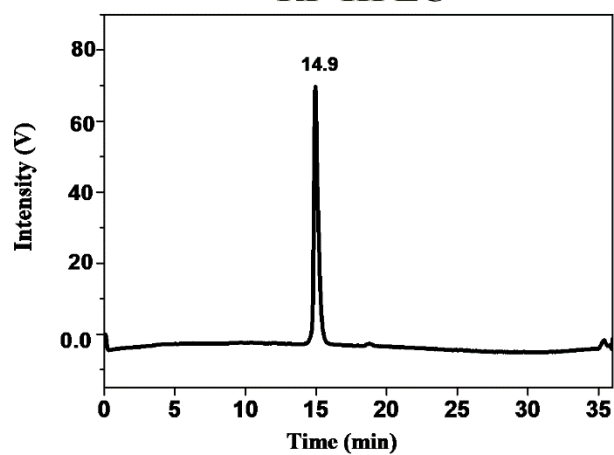


Figure 3.16 RP-HPLC and MALDI-TOF of *bm*-PNA 3

3.7.4 *bm*-PNA 4



RP-HPLC



MALDI-TOF

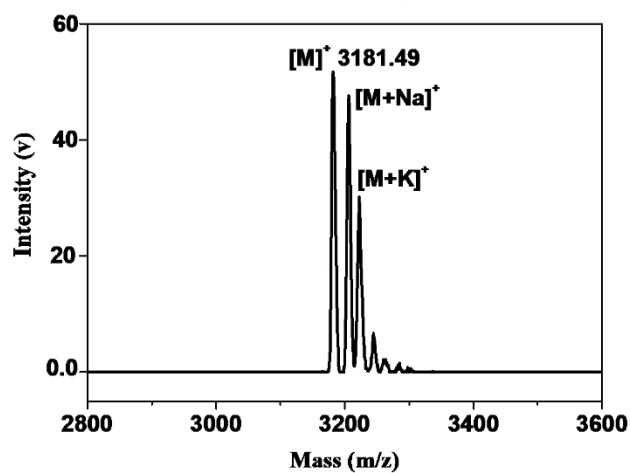
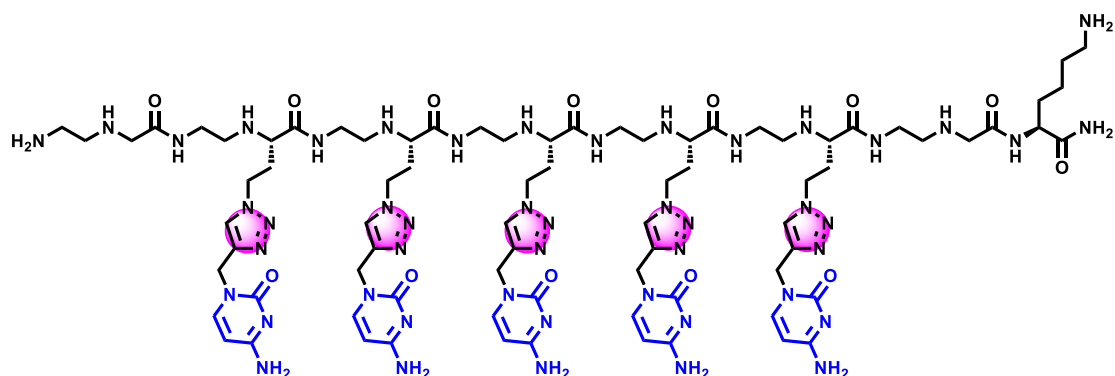
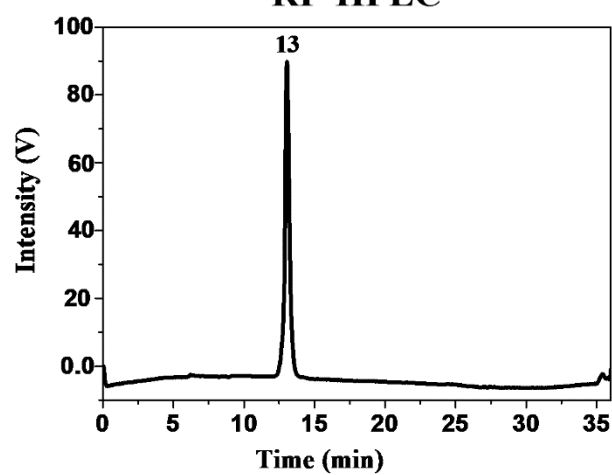


Figure 3.17 RP-HPLC and MALDI-TOF of *bm*-PNA 4

3.7.5 *iso*-PNA 1



RP-HPLC



MALDI-TOF

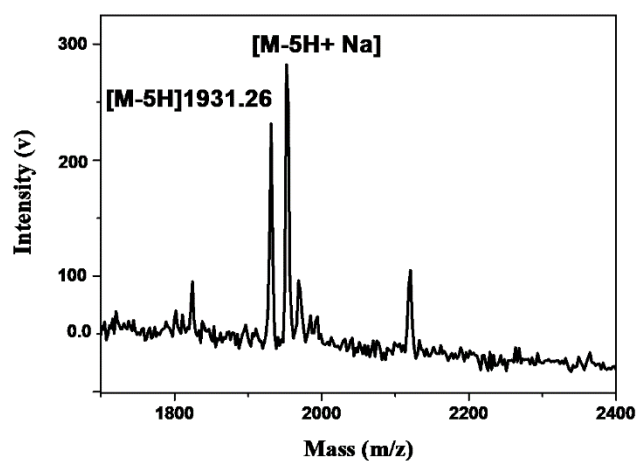


Figure 3.18 RP-HPLC and MALDI-TOF of *iso*-PNA 1

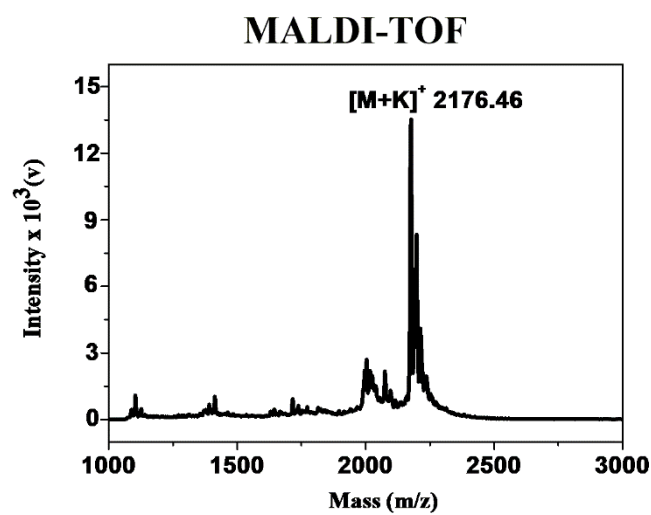
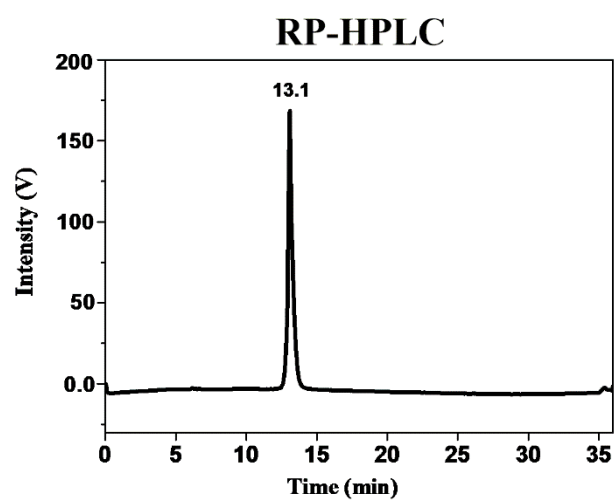
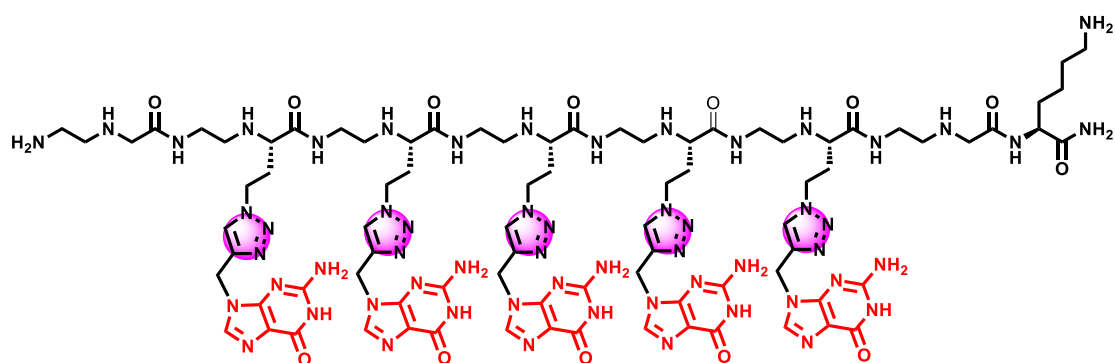


Figure 3.19 RP-HPLC and MALDI-TOF of *iso*-PNA **2**

3.7.7 *aeg*-PNA-C₅

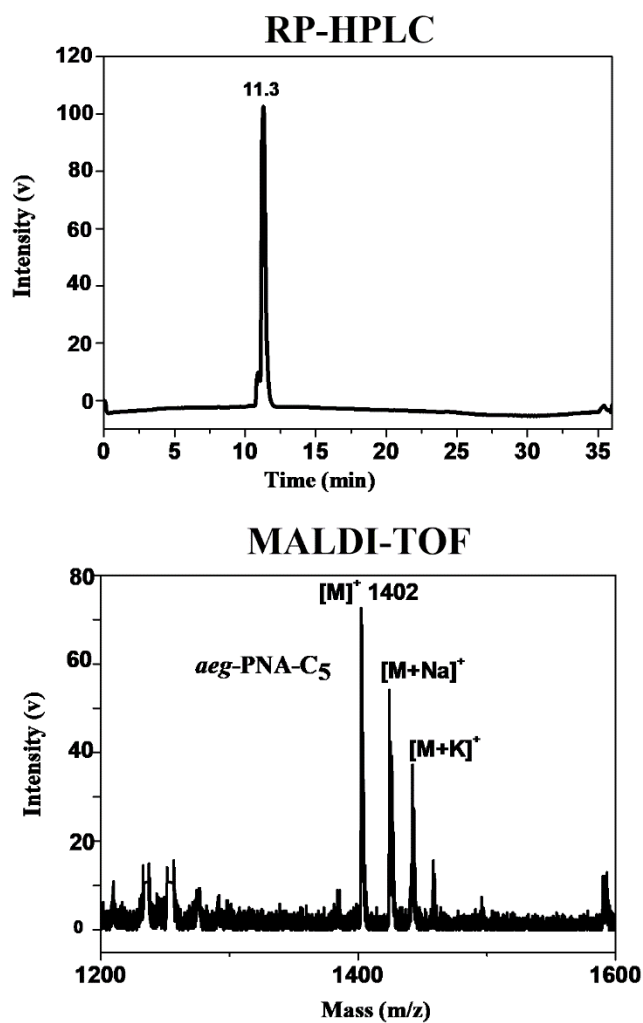
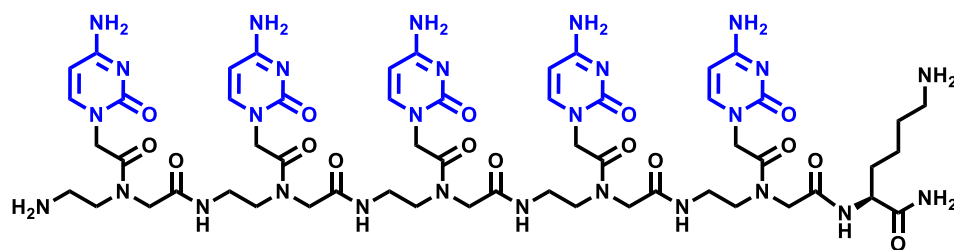


Figure 3.20 RP-HPLC and MALDI-TOF of *aeg*-PNA-C₅

3.7.8 *aeg*-PNA-G₅

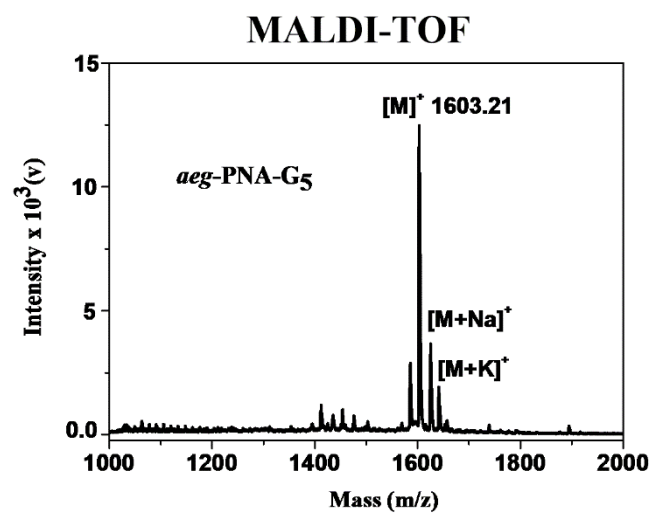
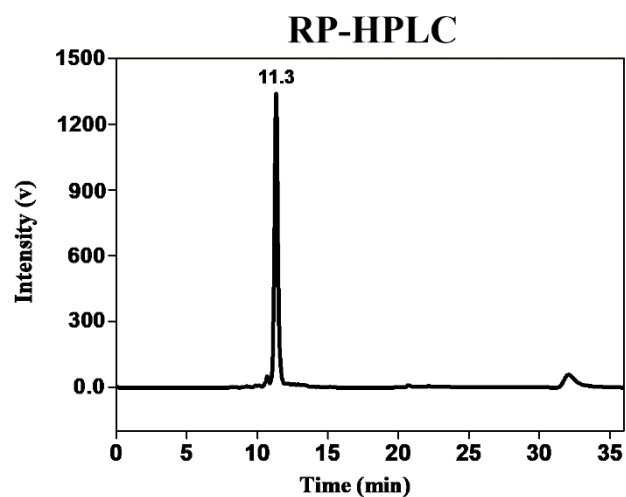
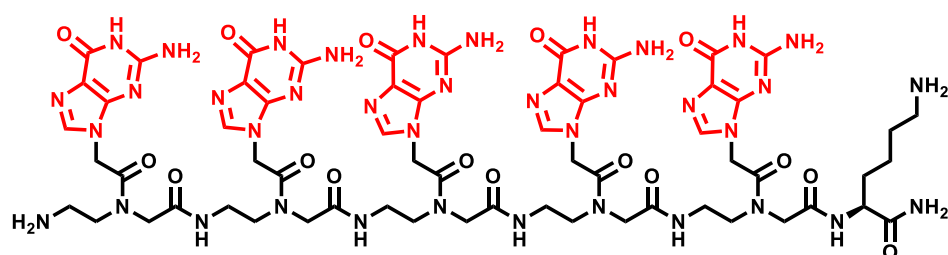


Figure 3.21 RP-HPLC and MALDI-TOF of *aeg*-PNA-G₅

3.7.9. *aeg*-M-PNA

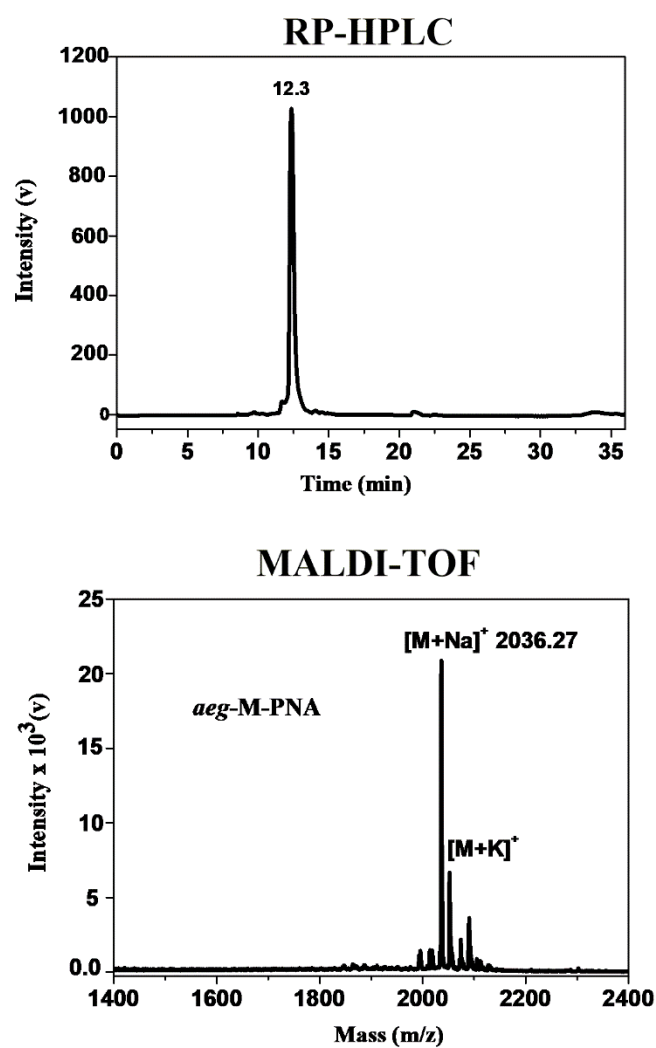
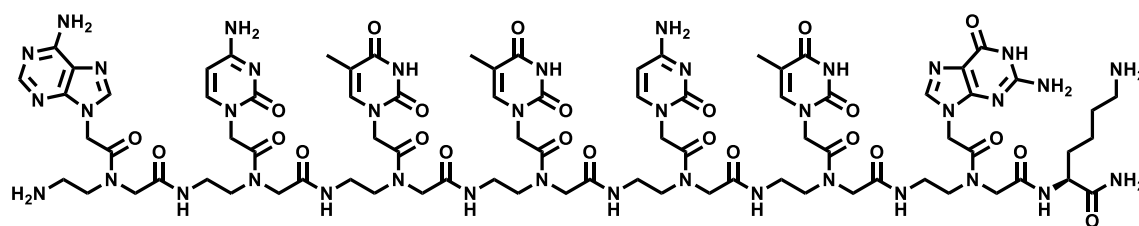


Figure 3.22 RP-HPLC and MALDI-TOF of *aeg*-M-PNA

Chapter 4

i-Motif formation from bimodal PNAs with all-Cytosine on one face

4.1 Introduction

The recognition that cytosine-rich DNA sequences can adapt unusual *i*-motif structures¹⁻⁴ at mildly acidic pH has fuelled numerous researches into their biological function⁵⁻⁸ and potential applications⁹⁻¹². An *i*-motif consists of two parallel-stranded duplexes held together by hemi-protonated C-H⁺:C base pairs interlaced with another set of C-H⁺:C base pairs held together in antiparallel orientated strands (Figure 4.1)

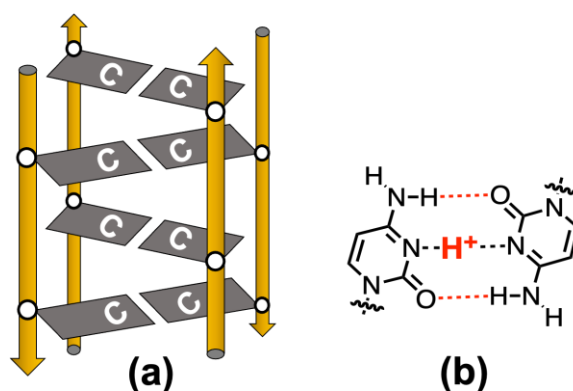


Figure 4.1 The DNA dTC₅ *i*-motif structure at acidic pH

Although the formation of *i*-motif DNA structures require slightly acidic conditions for protonation of C and so thought to be physiologically irrelevant, it has recently been realised that such structures can occur as well at neutral conditions.¹³⁻¹⁶ Their presence in human cell nuclei was shown by antibody binding and other experiments (Figure 4.2).^{17,18} Such C4-*i*-motif structure of DNA has not only been recognised for its role in biological function but also as attractive target for drug development⁵⁻¹² and as motifs for DNA based nanomaterials.¹⁸

The structural requirement of N3-protonation of C for *i*-motif formation (Figure 4.1b) points to the importance of its pK_a. The efficiency of *i*-motif formation is pH dependent and

may slightly change by chemical modifications in base and backbone or due to non-structural changes such as ionic strength, base sequence or the cellular microenvironment. The effect of various C5-substitutions, 2'-fluorination of ribose, phosphorothioate and LNA backbone modifications on *i*-motif stability have been studied.¹⁹⁻²⁴ One of the prime factors in stabilization of *i*-motif in DNA structures is the overall electrostatic balancing of the cationic protonated cytosines with the anionic phosphodiester backbone and the interbase interactions in the interdigitated C-H⁺:C base pairs.^{5,19}

The *i*-motif can also be formed by four strands of cytosine containing DNA (Figure 4a), two hairpin dimers of DNA strand (Figure 4.3b) or by a single DNA strand containing four cytidine stretches connected by a linker through intramolecular folding bringing together the four cytidine stretches. (Figure 4.2). The stoichiometries and topologies of *i*-motif thus depends on the linker present in the sequence.

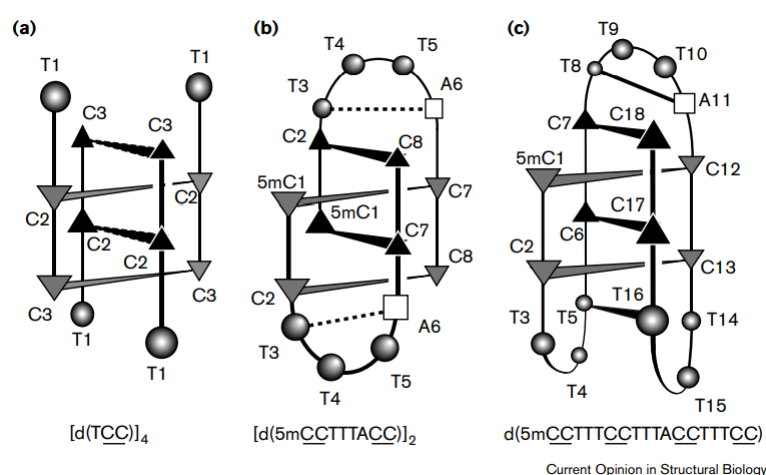


Figure 4.2 Topologies of DNA *i*-motif ⁵

The peptide nucleic acids (PNA) are acyclic DNA analogues (Figure 4.3) in which the nucleobases are (A/G/C/T) are linked to aminoethyl glycine polyamide backbone through a methylene carbonyl linker.²⁵⁻²⁷ In PNA, the bases are connected to a neutral backbone through an alkylamido linker (Figure 4.3a), unlike in DNA where the bases are attached to anion charged backbone via glycosidic bond (Figure 4.3b). Thus, the electronic properties of base pairs in PNA duplexes are likely to slightly differ from same the base pairs present in DNA duplex.²⁸ A plethora of chemical modifications on PNA backbone has been produced to improve both the hybridization properties of PNA as well as to equip them for improved biological properties.²⁹⁻³⁵ It is therefore interesting to study the *i*-motif formation by cytosine base pairs attached to PNA backbone.

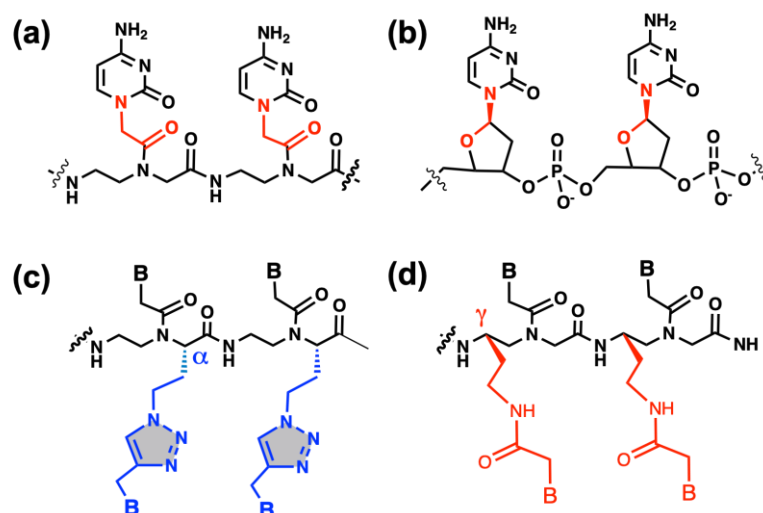


Figure 4.3. Structures of (a) *aeg*-PNA (b) DNA (c) bimodal- $C\alpha$ -tz-PNA (d) bimodal- $C\gamma$ -amide-PNA

Ganesh *et al* reported the formation of *i*-motif in PNA and found that at acidic pH 3.0, PNA-TC₈ formed *i*-motif with greater stability than DNA TC₈^{36,37} However, the stability of *i*-motif in PNA decreased with increase in pH and no *i*-motif was realised at pH > 5.0. The formation of PNA *i*-Motif at pH < 5.0 was also supported by mass spectroscopic studies.³⁸ A chiral version of PNA backbone derived from peptide *gly-ala* was also shown to form *i*-motif.

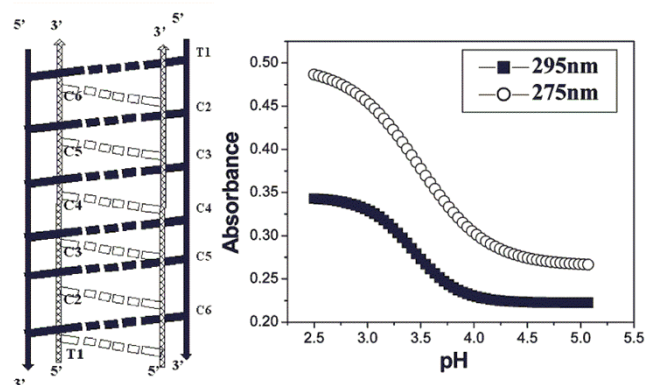


Figure 4.4 *aeg*-PNA TC₈ *i*-motif formation³⁶

The PNA-DNA hybrid composed of two DNA and two PNA strands showed *i*-motif structure derived from two intercalated PNA:DNA heteroduplexes with hemi-protonated C-H⁺:C base pairs.³⁹ The stability of the hybrid *i*-motif of DNA with backbone modified aminoethyl prolyl PNA⁴⁰ was enhanced due to electrostatic interaction between the cationic tertiary amine of proline on PNA with the anionic phosphodiester backbone.

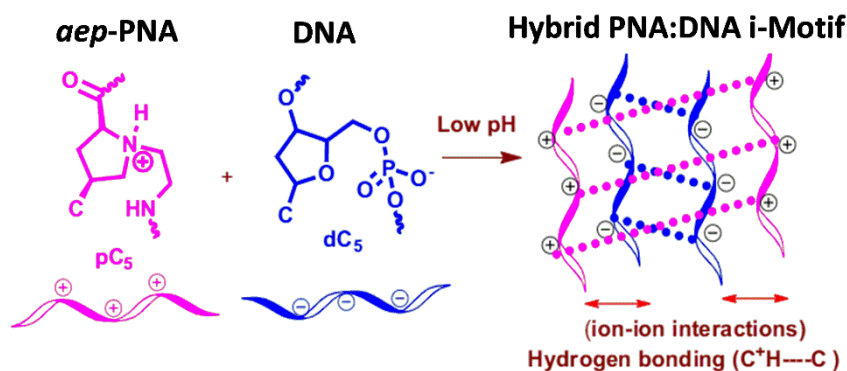


Figure 4.5 *aeg*-PNA:DNA hybrid i-motif formation⁴⁰

Ganesh et al have recently designed⁴¹⁻⁴⁴ a new class of PNAs termed “bimodal PNA” in which each aminoethylglycyl unit of *aeg*-PNA carries a second nucleobase linked at either C α through a triazole spacer (Figure 4.3c) or at C γ via an amide spacer chain (Figure 4.3d), in addition to the nucleobases linked by t-amide sidechain. The bimodal PNAs with mixed base sequences are endowed with structural features that enables them to bind simultaneously two complementary DNAs to form double duplexes with enhancement of stability.^{45,46} When the t-amide side carries a homopyrimidine T_n sequence that can form a triplex with dA₈, bimodal PNAs are shown to yield pentameric triplex of duplex, with triplex derived from dA₈ (*bm*-PNA-T₈:dA₈:T₈-*bm*-PNA) fused to duplex from mixed sequence on C α /C γ side with complementary DNA (*bm*-PNA:DNA).⁴⁷ Bimodal PNAs are thus good templates to organise multistrand nucleic acid assemblies. In this manuscript, we demonstrate that bimodal PNAs having all-C sequence either on triazole or t-amide face form a tetrameric *i*-motif at acidic pH and duplex at pH > 5.0. The duplex constituted at pH 7.0 can be further hybridized with DNA complementary to the other face to generate tetra stranded three conjoined duplexes (triple duplex). In this assembly two DNA:*bm*-PNA duplexes base paired by WC base H-bonds are welded by *bm*-PNA:*bm*-PNA duplex through C:C self-pairing. UV and CD spectroscopy are used to characterise the formation of different complexes through established signature properties

4.2 Rationale and Objective

For comparative studies of *i*-motif formation by different PNA variants were used in this study. The standard *aeg*-PNA-C₅ (Figure 4.6a) has five Cytosines (C) linked via t-amide to *aeg* backbone. The *iso*-PNA 1 (Figure 4.6b) has five Cytosines attached at C α of *aeg*

backbone by triazole spacer, without any nucleobases on t-amide side. The bimodal *bm*-PNA 1 has mixed base sequence on t-amide side and five Cytosines (C) on the triazole face (Figure 4.6c) similar to *iso*-PNA 1. *bm*-PNA 2 (Figure 4.6d) has reverse structure with poly C sequence on t-amide side and mixed base sequence at C $_{\alpha}$ on triazole face (reverse of *bm*-PNA 1). These were synthesised on solid phase as per earlier reported procedures,⁴⁵⁻⁴⁷ employing *aeg*-PNA-A/G/C/T monomers with C $_{\alpha}$ -azidoethyl side chain. The *iso*-PNA 1 (Figure 4.6b) having nucleobases only on C $_{\alpha}$ -azidoethyl sidechain without nucleobases linked by t-amide link (as in standard PNA) was also synthesised similarly to examine the ability of triazole linked cytosines to form *i*-motif. *aeg*-PNA-C₅ and dTC₅ (5'-TCCCCC-3') were used as references for comparison.

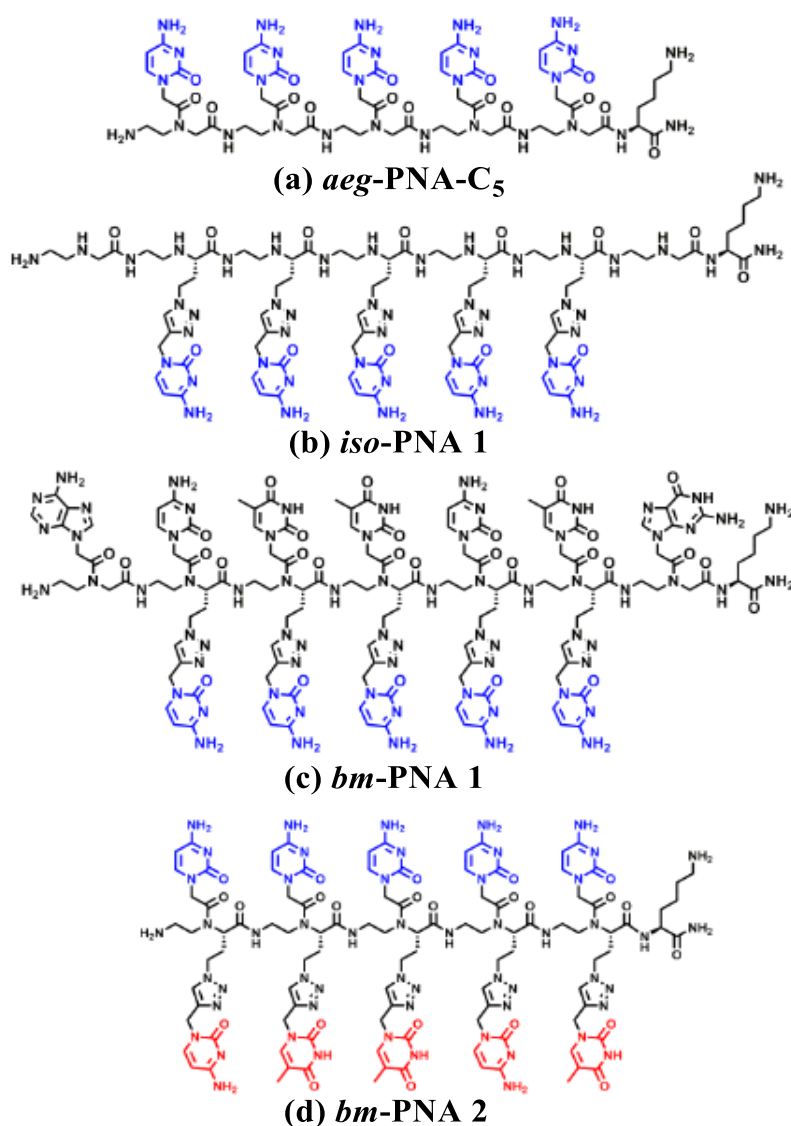


Figure 4.6. Structures of (a) *aeg*-PNA-C₅ (b) *iso*-PNA 1 (c) *bm*-PNA 1 and (d) *bm*-PNA 2 and DNA dTC₅ oligomers. (5'-TCCCCC-3')

4.3 Results and discussion

Section A: Self tetraplexes

Single stranded *bm*-PNA oligomers exist in aggregated form due to self-base stacking. When these single stranded oligomers are exposed to temperature dependent UV-absorbance, stacked bases will open and resulting in melting. In acidic conditions these exist as i-motif tetraplexes (295 nm band in UV spectra) and self-melting at acidic pH corresponds to i-motif melting.

4A.3.1. UV Spectra of dTC₅, *aeg*-PNA, *iso*-PNA 1, *bm*-PNA 1 and *bm*-PNA 2

It is known that *i*-motif formation results in *hyperchromic* effect at 265 nm and *hypochromic* effect at 295 nm, which is considered as hall marks of the presence of hemi protonated C••H⁺••C base pairs.^{3,45-47} The protonation of C in DNA is characterised by maximum wavelength in the region 275 nm to 300 nm at acidic pH, that shifts to a lower wavelength from 265 nm to 270 nm (for neutral C). Thus monitoring of absorbance changes in 270–295 nm range as a function of temperature is a nice way to study the stability of the *i*-motif at different pHs.⁴⁵⁻⁴⁷ The UV spectra of the five oligomers dTC₅, *aeg*-PNA-C₅, *iso*-PNA 1, *bm*-PNA 1 and *bm*-PNA 2 were recorded at pHs 3.6, 4.5, 5.5 and 7.3 (Figure 4.7). It was found that at lower pH of 3.6, the λ_{max} was at a higher wavelength (270 nm -275 nm) as compared to that at higher pH of 7.0 ($\lambda_{\text{max}} < 270$ nm).

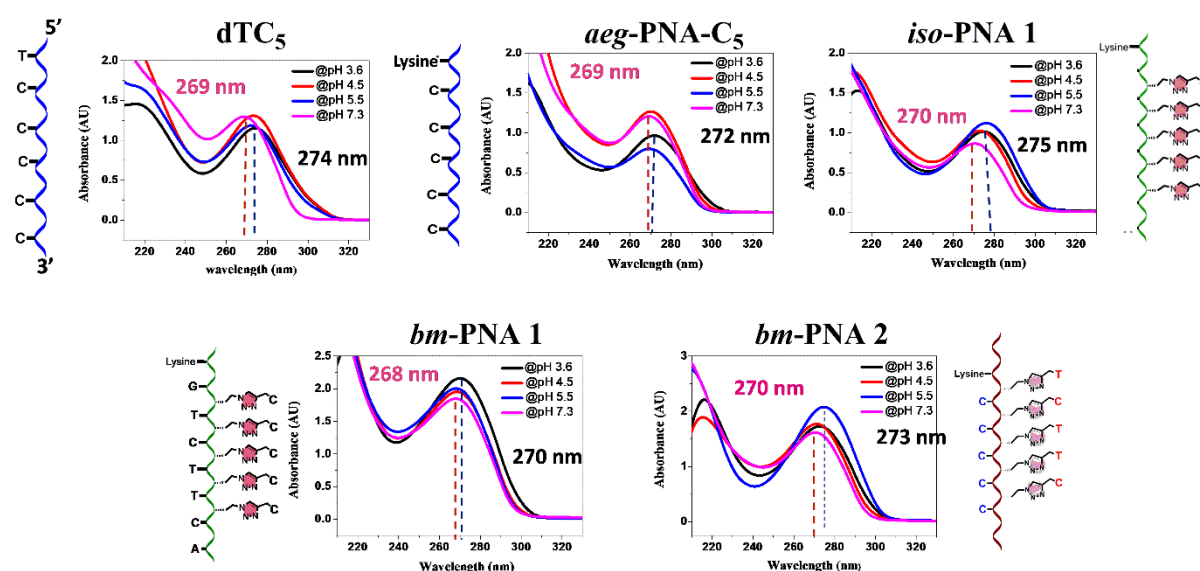


Figure 4.7. UV spectra of DNA dTC₅ and PNA oligomers at pH 3.6, 4.5, 5.5 and 7.3, with structures of dTC₅ and PNA oligomers in cartoon representation. Concⁿ of DNA/PNA oligomers, 10 μ M. Buffers: Sodium acetate, pH 3.6 – 4.5, 30 mM; Sodium cacodylate buffer, pH 5.5– 7.3, 30 mM)

4A.3.2. pKa determination of dC₆, *aeg*-PNA-C₅ and *iso*-PNA 1

The formation of *i*-motif requires protonation of N3 and hence it is pH dependent. and favourable at pHs lower than the pK_a of N3 of Cytosine (C). Compared to C in DNA which is linked via glycosidic bond, C in *aeg*-PNA is attached via t-amide linker and C present on side chain at C α in *iso*-PNA/*bm*-PNA is linked via an alkyl triazole spacer. Due to these structural differences, the Cs in DNA, *aeg*-PNA and *iso*-PNA/*bm*-PNA may have different pK_a values and altered abilities to form *i*-motif. Hence the comparative pK_a values of N3-H in DNA (dC₆), *aeg*-PNA-C₅ and *iso*-PNA 1 were determined by pH dependent UV absorbance at 295 nm. It was found that the pK_a of N3-C decreased in the order, dC₆ (4.03), *iso*-PNA 1 (3.81), *aeg*-PNA-C₅ (3.6), indicating that *aeg*/*iso*-PNAs require a lower pH (3.6) than DNA (4.0) for effective protonation needed for *i*-motif formation.

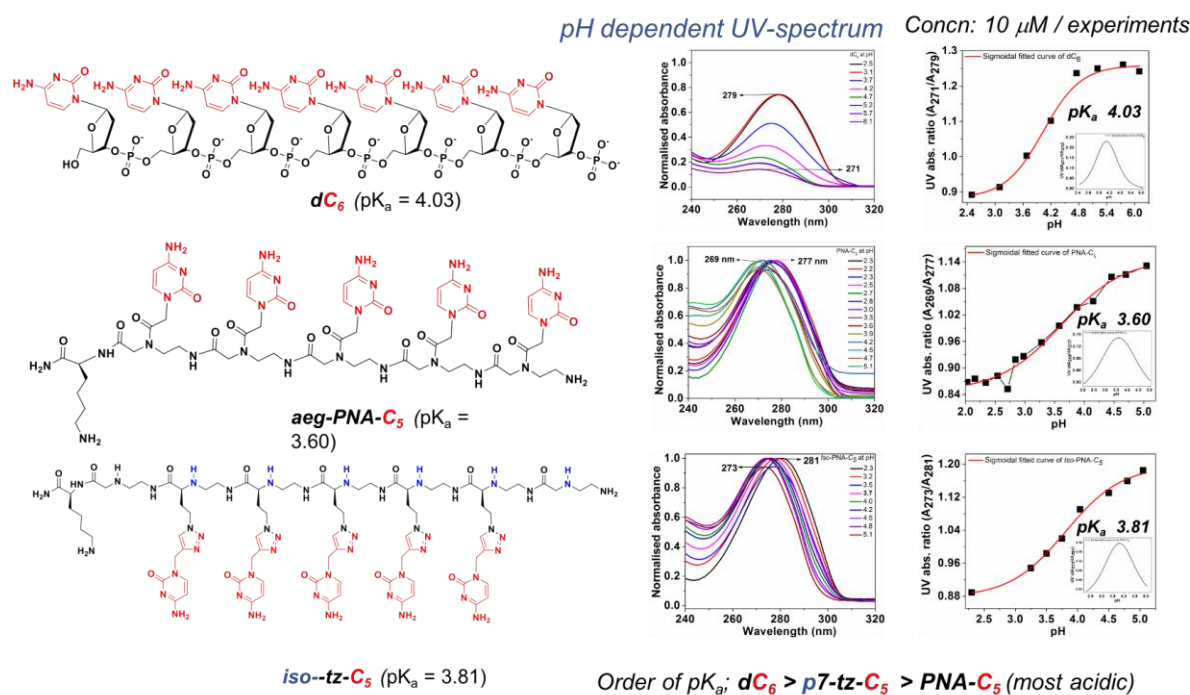


Fig.4.8. pKa determination of *aeg*-PNA-C₅, *iso*-PNA 1 and dC₆

4A.3.3. pH dependent UV-T plots of *i*-motifs of dTC₅/*aeg*/*iso*/*bm*-PNAs

4A.3.3a UV-T plots of dTC₅/*aeg*/*iso*/*bm*-PNAs @pH 3.6

The formation of *i*-motif from the five oligomers dTC₅, *aeg*-PNA, *iso*-PNA 1, *bm*-PNA-1 and *bm*-PNA-2 were characterized by temperature dependent UV absorbance changes and determination of T_m .^{48,49} The hallmark of *i*-motif tetraplex with N-3 protonated C is the observance of a negative sigmoidal transition at 295 nm (hypochromicity) and a positive

sigmoidal transition at 260 nm (hyperchromicity) in temperature dependent UV absorbance plots (UV-T plot).^{3,4,45-47} The duplex formation is evidenced by a positive sigmoidal transition at both 260 nm and 295 nm. Figure 4.9 shows the comparative UV-T plots of the oligomers dTC₅, *aeg*-PNA, *iso*-PNA 1, *bm*-PNA-1 and *bm*-PNA-2 at pH 3.6, followed at 295 nm and 260 nm. It is seen that at pH 3.6, the UV-T plots of all oligomers show a negative sigmoidal transition at 295 nm (Figure 5A-E) and a positive sigmoidal plot at 260 nm (Figure 5F-J). The stabilities of *i*-motifs (T_m) were obtained from the minimum in the each of the first derivative curves of the negative sigmoidal plots at 295 nm. It is seen that the T_m for oligomers decreased in the order dTC₅ (68.3 °C) > *bm*-PNA-2 (47.6) > *aeg*-PNA-C₅ (44.8 °C) > *bm*-PNA-1 (41.6 °C) > *iso*-PNA 1 (32.2 °C). These data pointed to the formation of *i*-motif tetraplexes by PNA oligomers at pH 3.6, similar to that of dTC₅. Notably, the cytosines linked via triazole spacer at C $_{\alpha}$ as in *iso*-PNA, *bm*-PNA-1 form tetraplexes at pH 3.6. similar to C5 on t-amide side as in *aeg*-PNA and the new *bm*-PNA-2 that is similar to *aeg*-PNA-C₅, but in addition has a mixed sequence on C $_{\alpha}$ -face.

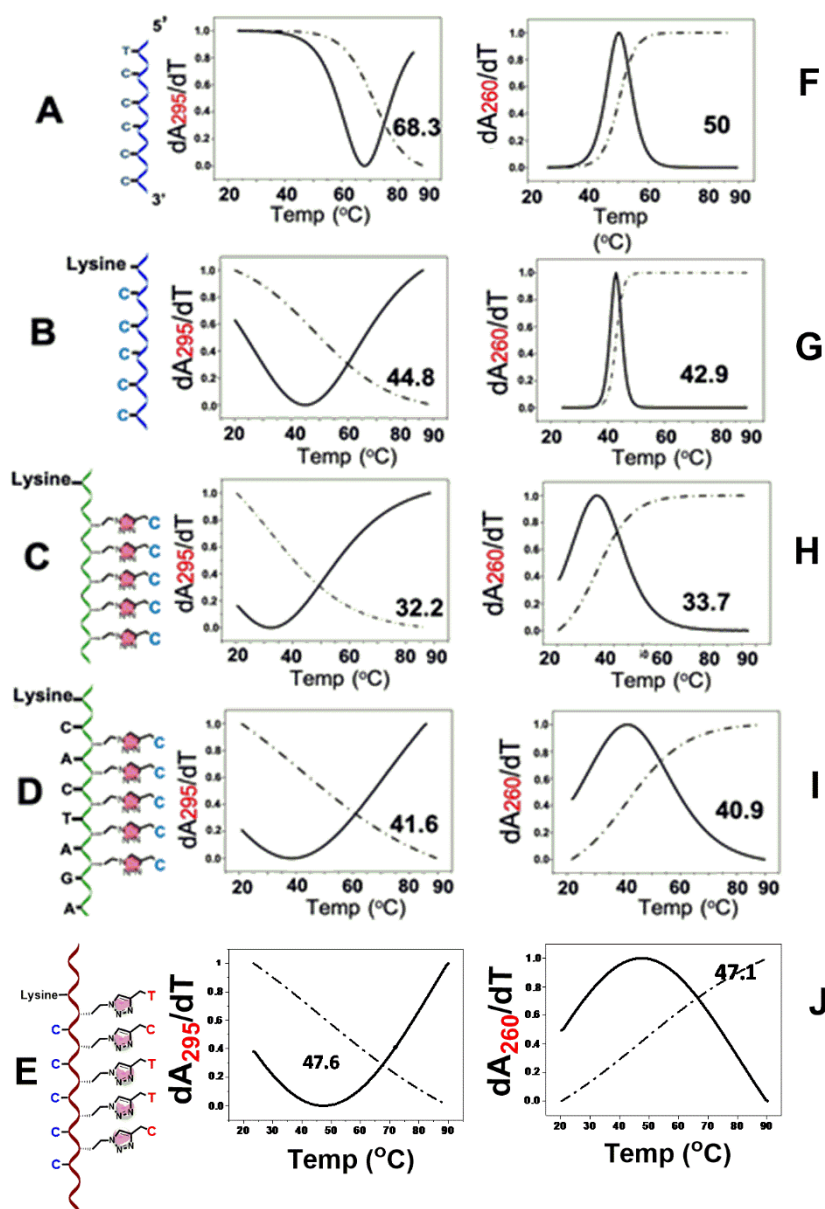


Figure 4.9 UV (λ_{295} nm) -T plots at pH 3.6 and first derivative curves of self-melting of (A) dTC₅, (B) *aeg*-PNA-C₅ (C) *iso*-PNA 1 (D) *bm*-PNA-1 and (E) *bm*-PNA-2. UV (λ_{260nm}) -T plots for (F) dTC₅, (G) *aeg*-PNA-C₅ (H) *iso*-PNA 1 (I) *bm*-PNA-1 and (J) *bm*-PNA-2. Conc'n of PNA, 5 μ M. Buffer: Sodium acetate, pH 3.6, 30 mM

The comparative T_m s for *i*-motifs of four oligomers obtained from UV-T plots at 260 nm (Figure 4.9F-J) are close to that obtained at 295 nm (the error margin of ± 0.5 °C), in agreement with the trends reported for *i*-motifs in DNA.⁴⁷ The slight difference in T_m s at 260 nm and 295 nm perhaps due to slight differences in absorbance of protonated and non-protonated Cs at 260 nm and their relative variation with temperature.

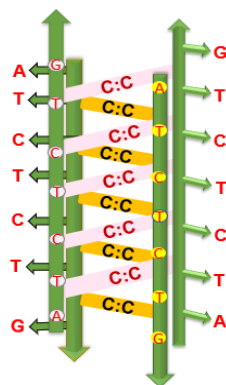


Figure 4.10 Cartoon presentation of *bm*-PNA *i*-motif structure

4A.3.3b UV-T plots of dTC₅/aeg/iso/bm-PNAs @pH 4.5

The UV-T plots were also recorded for the five oligomers at pH 4.5 and the results showed a similar behaviour with negative sigmoidal transition at 295 nm and positive transition at 260 nm, indicating successful realization of *i*-motif by all oligomers at pH 4.5. Stability order of *i*-motif at 295 nm as follows (Figure 4.11)- *aeg*-PNA-C₅ (59.7 °C) > dTC₅ (51.7 °C) > *bm*-PNA-1 (44.4 °C) > *iso*-PNA 1 (40.9 °C). > *bm*-PNA 2 (31.3 °C)

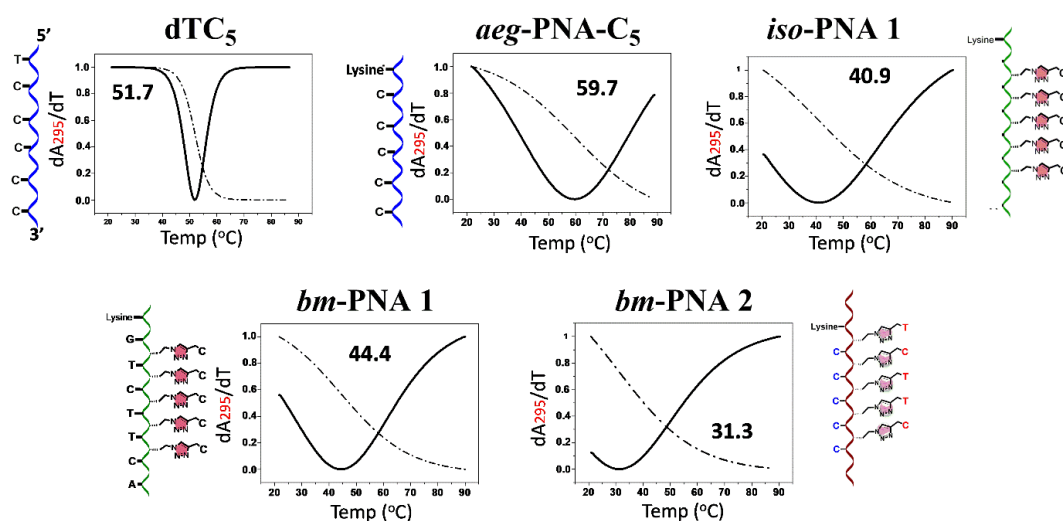


Figure 4.11 UV (λ_{295} nm) absorbance-T plots at pH 4.5 and first derivative curves of self-melting all oligomers. Concn of PNA, 5 μ M. Buffer: Sodium acetate, pH 4.5, 30 mM.

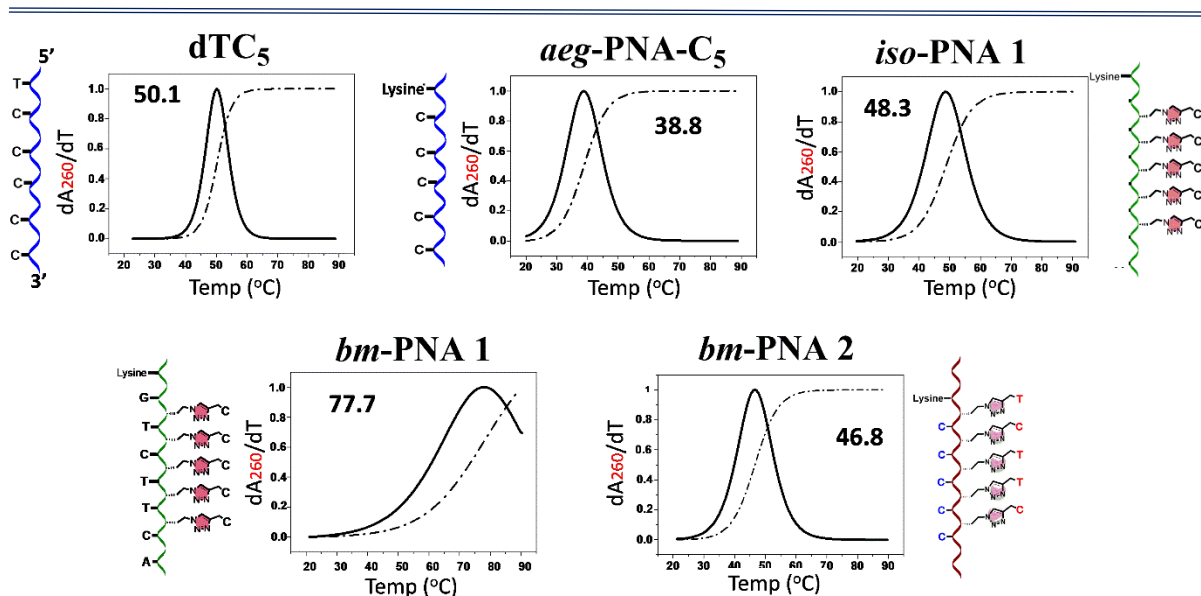


Figure 4.12 UV (λ_{260} nm) -T plots at pH 4.5 and first derivative curves of self-melting all oligomers. Conc'n of PNA, 5 μ M. Buffer: Sodium acetate, pH 4.5, 30 mM.

4A.3.3c UV-T plots of dTC₅/aeg/iso/bm-PNAs @pH 5.5

In contrast to the UV-T plots of the five oligomers at pHs 3.6 and 4.5, the corresponding plots at pH 5.5 exhibited positive sigmoidal transition at both 295 nm (Figure 4.13) and 260 nm (Figure 4.14). This suggested the absence of the *i*-motif structure for all four oligomers at pH 5.5, but the sigmoidal transitions indicated the presence of ordered structures, which may be duplexes arising either from either hemi-protonated or neutral C:C base pairs. The T_m values of such duplexes at pH 5.5 for all oligomers were generally higher than T_m of corresponding *i*-motif, with order of decreasing T_m : dTC₅ (79.7 °C) > bm-PNA-2 (75.1) > aeg-PNA-C₅ (64.8 °C) ~ bm-PNA-1 (65.1 °C) > iso-PNA 1 (47.5 °C).

4A.3.3d UV-T plots of dTC5/aeg/iso/bm-PNAs @pH 7.3

The UV λ_{max} of all oligomers at pH 7.3 was almost identical, lower than 270 nm and the sigmoidal transitions at both 295 nm and 260 nm were positive at pH 7.3. The T_{ms} from UV-T plots for duplexes monitored at 295 nm for pH 7.3 was in the order *bm*-PNA-1 (73.3 °C) > *bm*-PNA-2 (67.2) > *aeg*-PNA- C_5 (70.2 °C) > *iso*-PNA 1 (62.7 °C) > dTC₅ (52.3 °C).

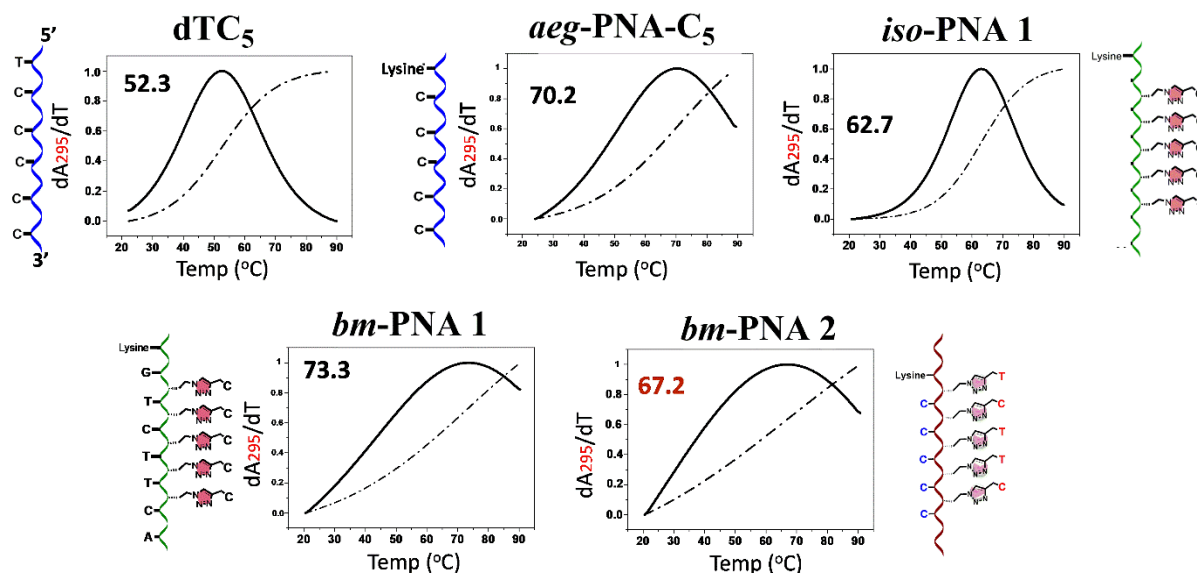


Figure 4.15 UV (λ_{295} nm)-T plots at pH 7.3 and first derivative curves of self-melting all oligomers. Concn of PNA 5 μM ; Buffer: Sodium cacodylate, pH 7.3, 30 mM.

The T_{ms} from UV-T plots for duplexes also monitored at 260 nm for pH 7.3 was in the order *bm*-PNA-2 (67.2) > *aeg*-PNA- C_5 (50.7 °C) ~ dTC₅ (49.4 °C) > *bm*-PNA-2 (47.1 °C) ~ *iso*-PNA 1 (46.5 °C), without much significant differences.

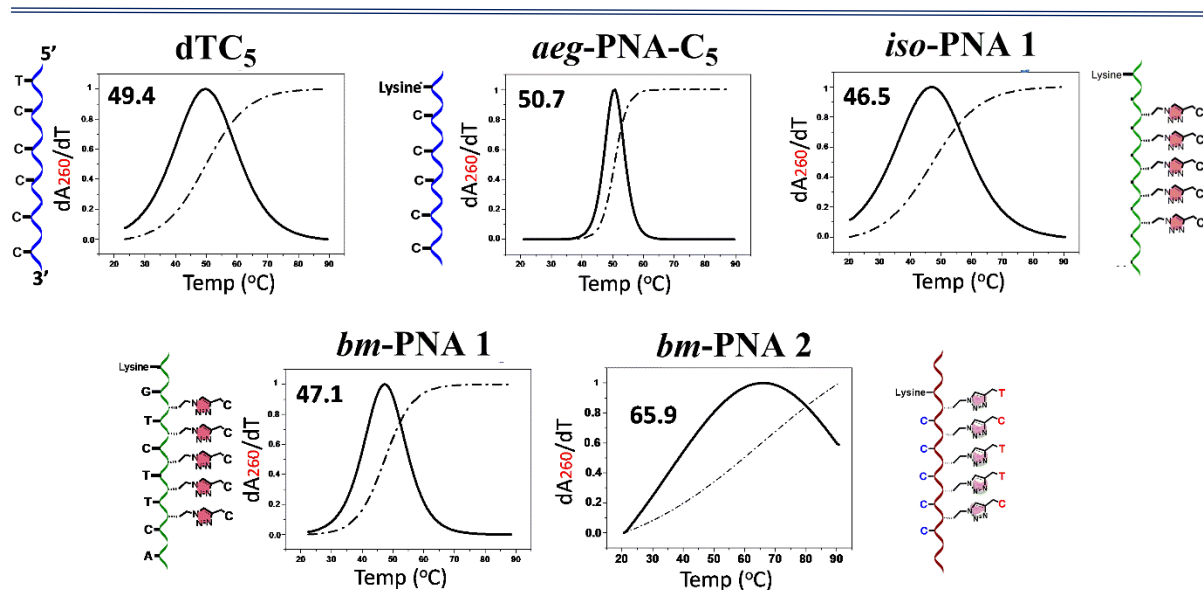


Figure 4.16 UV (λ_{260} nm) -T plots at pH 7.3 and first derivative curves of self-melting all oligomers. Conc'n of PNA 5 μ M; Buffer: Sodium cacodylate, pH 7.3, 30 mM.

4A.3.4. Circular dichroic spectra of bimodal PNA *i*-motifs

DNA *i*-motifs show characteristic CD signatures with an intense positive band at 285 nm and a negative band at 255 nm at acidic pH 3.6 which shifts to 270 nm and 250 nm respectively with increase in pH to (7.15).^{3,50,51} However, PNAs which lack chiral centre show very weak or negligible CD bands at all pHs.⁵¹ The *bm*-PNAs carry a chiral centre at C α with *S*-configuration and since the cytosine being far away from chiral centre only a weak induced CD signal is observed. At acidic pH (3.6) *bm*-PNA 1 exhibited a negative CD band at 282 nm, with a shoulder band at 295 nm (Figure 4.17). At pH 5.0, the 295 nm disappeared, and the 282 nm shifted slightly to lower wavelengths, while at pH 7.3 further broadening of bands were observed. While, CD band pattern in the *bm*-PNA 2 are not significant. Though, the CD bands are weak, pH dependent changes suggested that the *bm*-PNAs transit from an ordered *i*-motif (295 nm) at pH 3.6 to duplex state upon increase in pH to 7.3.

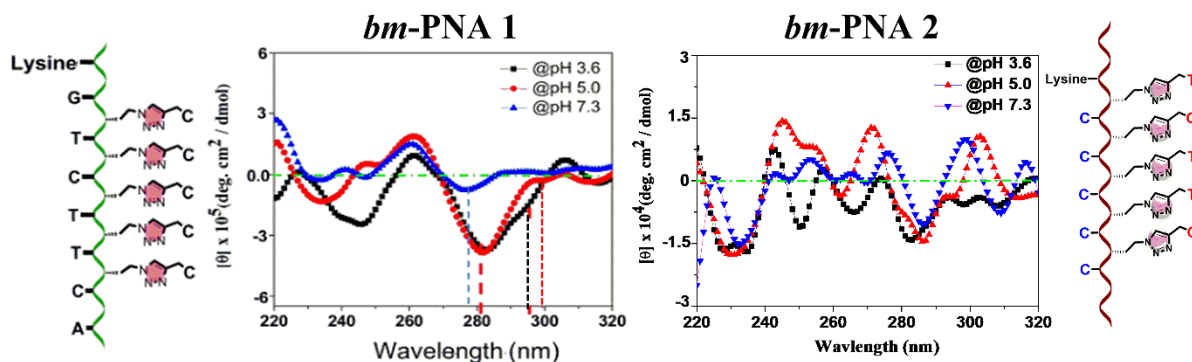


Figure 4.17. pH dependent CD spectra (a) *bm*-PNA-1 and (b) *bm*-PNA-2. Conc'n of *bm*-PNAs, 25 μ M. Buffer: Sodium acetate pH 3.6 (100 mM), Sodium cacodylate, pH 5-7.3, (100 mM).

4A.3.5. Summary of thermal stability of bimodal PNA *i*-motifs

Figure 4.19 shows the comparative pH dependent T_m data for PNA oligomers dTC₅, *aeg*-PNA-C₅, *iso*-PNA 1, *bm*-PNA 1 and *bm*-PNA 2 at different pHs. The T_m s at pH 3.6 and 4.5 correspond to tetraplex formation followed at 295 nm, while at pHs 5.5 and 7.3 correspond to duplex formation followed at 260 nm. The slightly increased T_m s for *iso*-PNA and *bm*-PNA oligomers at higher pH (7.3) suggests that the duplexes are more stable than tetraplexes. For PNA *i*-tetraplexes T_m at pH 3.6 is lower than at 4.5 due to the increased protonation of N3 at lower pH 3.6 caused by enhanced electrostatic repulsion. This is unlike in DNA which has the anionic phosphate to compensate repulsion by partial charge neutralization. However the presence of a mixture of *i*-motif tetraplex and duplex at certain pHs cannot be ruled out.

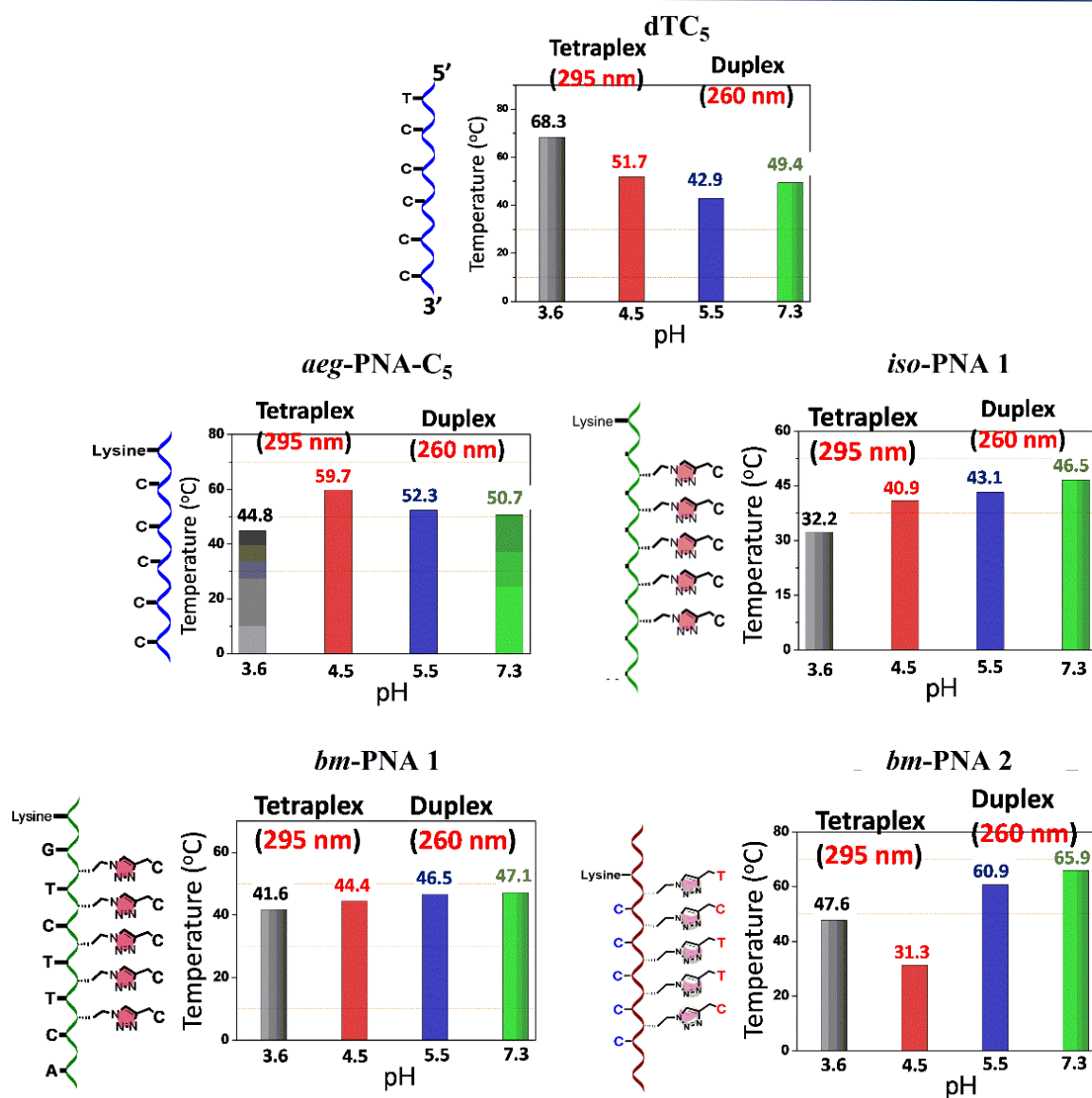


Figure 4.18. Summary of UV-T plots of dTC₅, aeg-PNA-C₅, iso-PNA 1, bm-PNA-1 and bm-PNA-

2

Section B: Hybrid tetraplexes

Previous section described i-tetraplexes formed from self-complexation of different *bm*-PNAs with all cytosine sidechains. However, when mixed with complementary dTC₅, these can form hybrid tetraplexes with 1:1 stoichiometry. At acidic pH, it will result in two interdigitated PNA:DNA duplexes seen in case of PNA:DNA hybrid motifs. In which, duplexes formed from two *aeg*-PNA-C₅ strands can interdigitate with duplexes formed from two dTC₅ strands.³⁹ These will have different UV T_{ms} and the following section illustrates such possibilities.

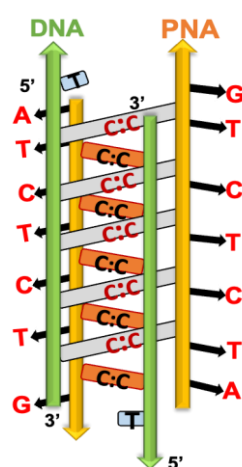


Figure 4.19 Cartoon presentation of dTC₅:*bm*-PNA hybrid i-motif structure. Amber colour denotes *bm*-PNA strand while green represents DNA.

4B.3.1. UV Spectra of dTC₅:*aeg*-PNA-C₅, dTC₅:*iso*-PNA 1 and dTC₅:*bm*-PNA-1 hybrid tetraplexes

UV absorbance of hybrid complexes such as dTC₅:*aeg*-PNA, dTC₅:*iso*-PNA 1, and dTC₅:*bm*-PNA 1 showed maximum wavelength in the region 275 nm to 300 nm at acidic pH, that shifts to a lower wavelength from 265 nm to 275 nm at higher pH indicates presence of tetraplex structure. (Figure 4.20)

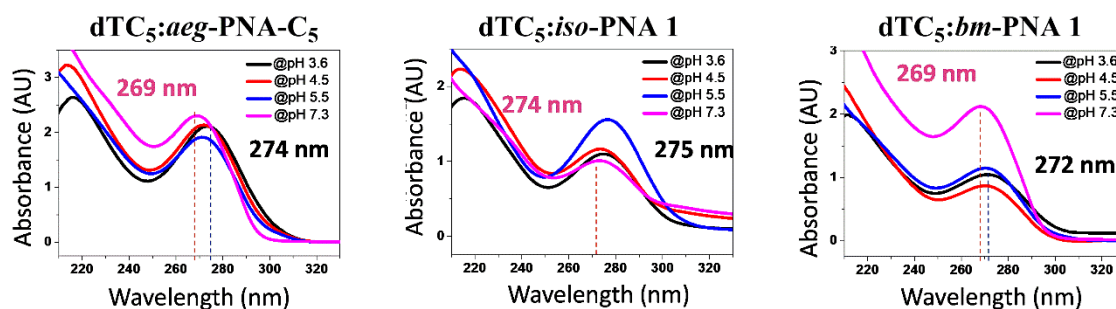


Figure 4.20. UV spectra of hybrid DNA dTC₅:PNA complexes at pH 3.6, 4.5, 5.5 and 7.3. Conc'n of DNA/PNA oligomers, 10 μ M. per strand Buffers: Sodium acetate, pH 3.6 – 4.5, 30 mM; Sodium cacodylate buffer, pH 5.5– 7.3, 30 mM).

4B.3.2. pH dependent UV-T plots of hybrid *i*-motifs of dTC₅:*aeg/iso/bm*-PNAs

4B.3.2a UV-T plots of hybrid dTC₅:*aeg/iso/bm*-PNAs @pH 3.6:

All three hybrid complexes showed inverse sigmoidal transition at 295 nm in the UV T_m indicates *i*-motif formation, while at 260 nm, the curves appeared positive sigmoidal in nature which supports to *i*-motif formation (Figure 4.21). The stability order at 295 nm- dTC₅:*aeg*-PNA-C₅ (62.8 $^{\circ}$ C) > dTC₅:*iso*-PNA 1 (46.8 $^{\circ}$ C) > dTC₅:*bm*-PNA-1 (45.0 $^{\circ}$ C).

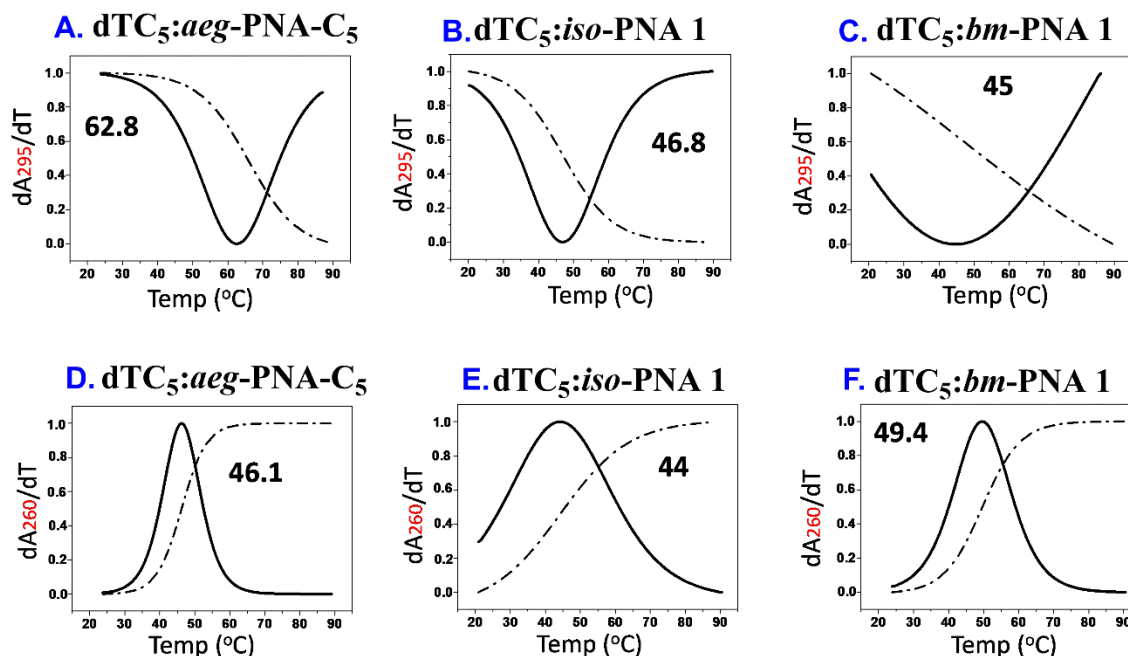


Figure 4.21 UV (λ_{295} nm) absorbance-T plots at pH 3.6 and first derivative curves of hybrid tetraplexes of (A) dTC₅:*aeg*-PNA-C₅ (B) dTC₅:*iso*-PNA 1 (C) dTC₅:*bm*-PNA 1 and UV (λ_{260} nm) absorbance -T plots for (D) dTC₅:*aeg*-PNA-C₅ (E) dTC₅:*iso*-PNA 1 (F) dTC₅:*bm*-PNA 1 Conc'n of PNA/DNA, 5 μ M. Buffer: Sodium acetate, pH 3.6, 30 mM.

4B.3.2b UV-T plots of hybrid dTC₅:*aeg/iso/bm*-PNAs @pH 4.5

Similarly, the UV-T plots at pH 4.5 closely resembled to that at pH of N3 3.6. It is observed that these hybrid complexes are able to form *i*-motif structure at this pH 4.5. The stability order at 295 nm: dTC₅:*aeg*-PNA-C₅ (55.6 $^{\circ}$ C) > dTC₅:*bm*-PNA-1 (43.8 $^{\circ}$ C) > dTC₅:*iso*-PNA 1 (33.9 $^{\circ}$ C)

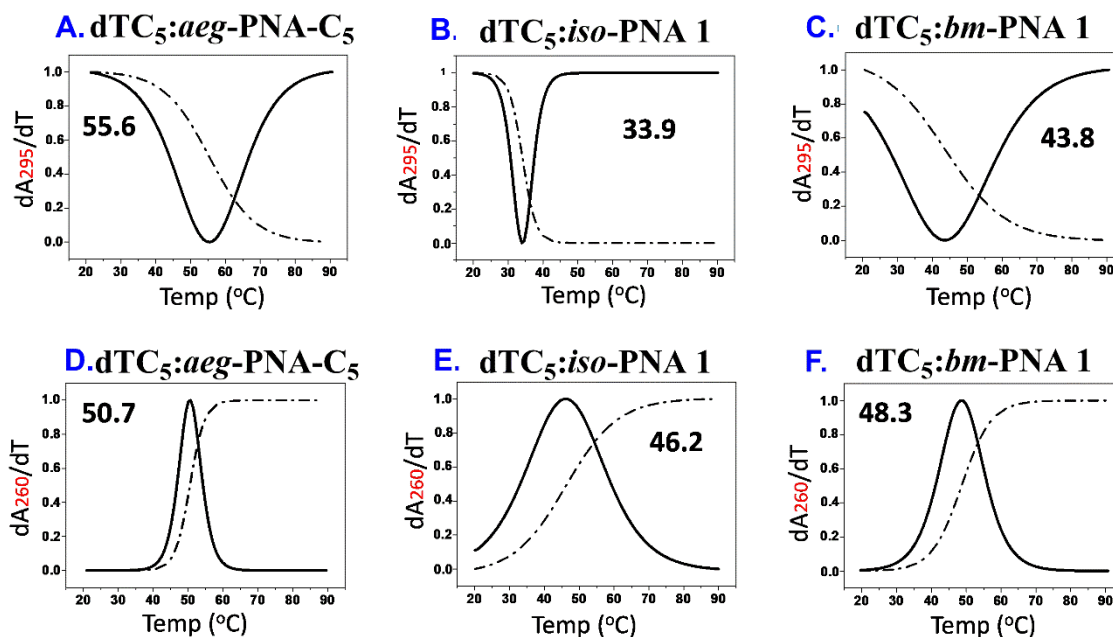


Figure 4.22 UV (λ_{295} nm) absorbance-T plots at pH 4.5 and first derivative curves of hybrid tetraplexes of (A) dTC₅:aeg-PNA-C₅ (B) dTC₅:iso-PNA **1** (C) dTC₅:bm-PNA **1** and UV (λ_{260} nm) absorbance -T plots for (D) dTC₅:aeg-PNA-C₅ (E) dTC₅:iso-PNA **1** (F) dTC₅:bm-PNA **1** Conc'n of PNA/DNA, 5 μ M. Buffer: Sodium acetate, pH 4.5, 30 mM.

4B.3.2c UV-T plots of hybrid dTC₅:aeg/iso/bm-PNAs @pH 5.5

i-Motif formation of different hybrids was monitored at pH 5.5 and interestingly both at both wavelengths 260 nm and 295 nm, the transitions appeared positive sigmoidal in nature. Such positive sigmoidal transition is characteristic for duplex formation, indicating that at pH 5.5 all three hybrids form C:C duplex structure. The stability order of duplex monitored at 260 nm dTC₅:iso-PNA **1** (61.9 °C) > dTC₅:aeg-PNA-C₅ (51.1 °C) > dTC₅:bm-PNA-**1** (43.1 °C) .

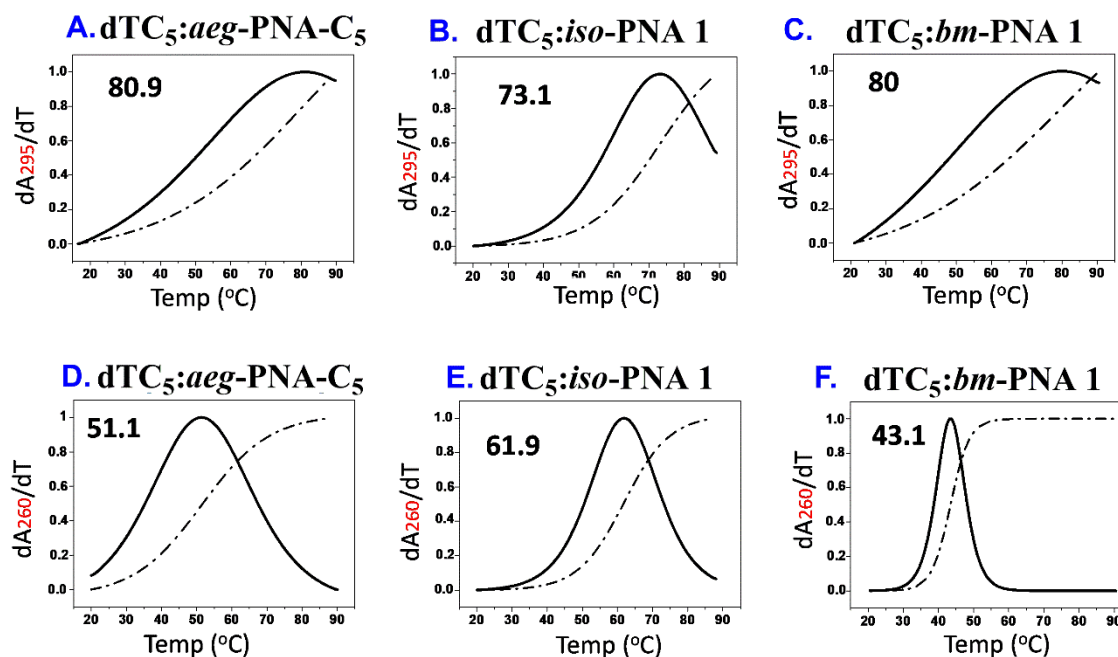


Figure 4.23 UV (λ_{295} nm) absorbance-T plots at pH 5.5 and first derivative curves of hybrid tetraplexes of (A) dTC₅:aeg-PNA-C₅ (B) dTC₅:iso-PNA 1 (C) dTC₅:bm-PNA-1 and UV ($\lambda_{260\text{nm}}$) absorbance -T plots for (D) dTC₅:aeg-PNA-C₅ (E) dTC₅:iso-PNA 1 (F) dTC₅:bm-PNA-1 Conc'n of PNA/DNA, 5 μM . Buffer: Sodium cacodylate, pH 5.5, 30 mM

4B.3.2d UV-T plots of hybrid dTC₅:aeg/iso/bm-PNAs @pH 7.3

At both 295 nm and 260 nm wavelength positive sigmoidal transitions were observed at pH 7.3, indicating the formation of C:C duplex structure. The observance only one transition indicates the presence of only the hybrid duplexes DNA:aeg-PNA / DNA:iso-PNA and DNA:bm-PNA and not the possible self-duplexes DNA:DNA / iso-PNA:iso-PNA / bm-PNA:bm-PNA. The stability of the hybrid duplex at 260 nm followed the order dTC₅:iso-PNA 1 (58.8 °C) > dTC₅:bm-PNA-1 (51.4 °C) > dTC₅:aeg-PNA-C₅ (50.4 °C).

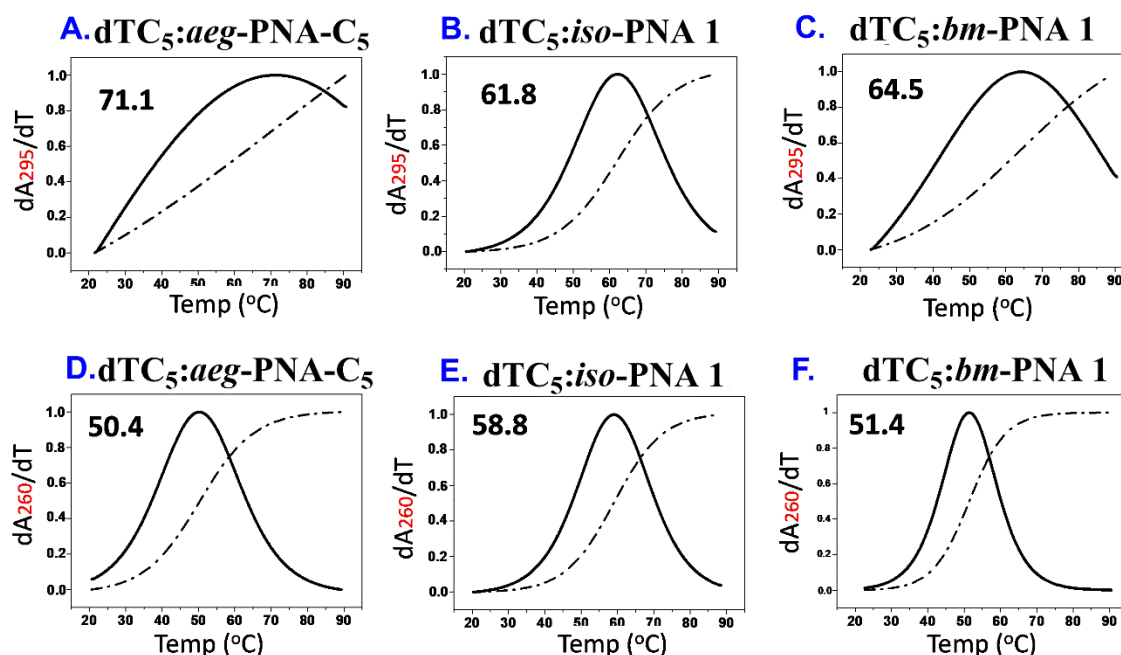


Figure 4.24 UV (λ_{295} nm) -T plots at pH 7.3 and first derivative curves of hybrid tetraplexes of (A) dTC₅:aeg-PNA-C₅ (B) dTC₅:iso-PNA 1 (C) dTC₅:bm-PNA-1 and UV ($\lambda_{260\text{nm}}$) -T plots for (D) dTC₅:aeg-PNA-C₅ (E) dTC₅:iso-PNA 1 and (F) dTC₅:bm-PNA-1. Conc of PNA/DNA, 5 μM . Buffer: Sodium cacodylate, pH 7.3, 30 mM.

4B.3.3. Circular dichroic spectra of hybrid *i*-motifs.

In the CD spectra, DNA TC₅ *i*-motif shows a positive maximum near 285 nm, and negative band centered near 265 nm.⁵⁰ In hybrid *i*-motifs, the basic features of hybrid CD profiles retained the CD band pattern of DNA *i*-motif, but the relative intensities of bands change³⁹

4B.3.3a pH dependent CD spectra of dTC₅. DNA dTC₅ showed positive maximum at 287 nm and negative trough at 265 nm at acidic pH 3.6, indicating *i*-motif structure. As pH increased, the intensity of these bands decreased and both disappeared at neutral pH 7.3, with appearance of a positive band at 275 nm.

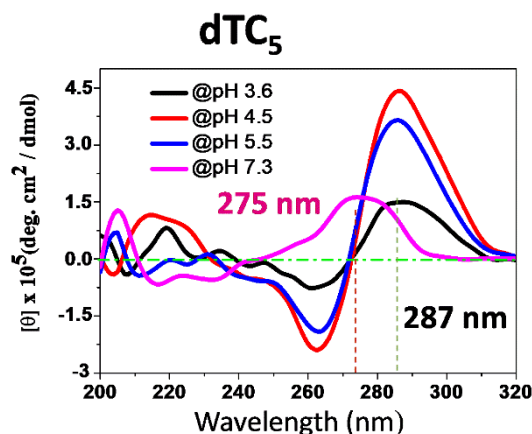


Figure 4.25 CD spectra of dTC₅ from pH 3.6 to pH 7.3

4B.3.3b pH dependent CD spectra of dTC₅:*ae*g-PNA-C₅. The dTC₅:*ae*g-PNA-C₅ hybrid complex is similar to the CD profile of DNA *i*-motif. At neutral pH 7.3 both positive band at 287 nm and negative band at 265 nm bands disappeared and 287 nm with positive band band shifting to 278 nm.

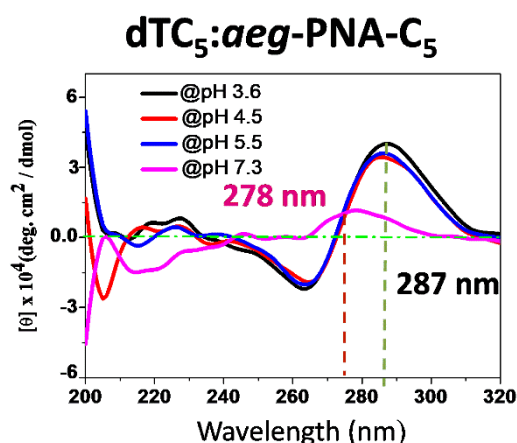


Figure 4.26 CD spectra of dTC₅:*ae*g-PNA-C₅ from pH 3.6 to pH 7.3

4B.3.3c pH dependent CD spectra of dTC₅:*iso*-PNA 1. The dTC₅:*iso*-PNA 1 hybrid complex forms *i*-motif at acidic pH 3.6, with CD bands at 300 nm. As pH increased to pH 4.5, the pattern did not change much. At 5.5 and pH 7.3, the positive band shifted to lower wavelength 295 nm, indicating formation of mixture of duplex structures (Figure 4.27).

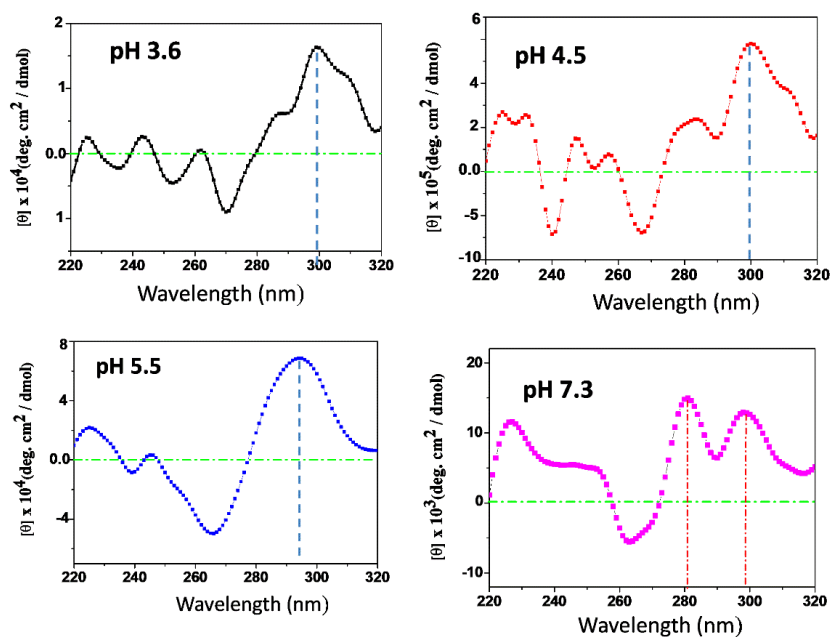


Figure 4.27 Isolated CD plots of dTC₅:iso-PNA **1** at pH 3.6, 4.5 and pH 7.3.

4B.3.3d pH dependent CD spectra of dTC₅:bm-PNA-1. dTC₅:bm-PNA-1 hybrid complex forms *i*-motif under acidic pHs 3.6 and 4.5 conditions as the CD plots at pH 3.6 and pH 4.5 showed maxima around 286 nm nm. However at neutral pH only positive bands are seen indicating a mixture of duplex structures. (Figure 4.28)

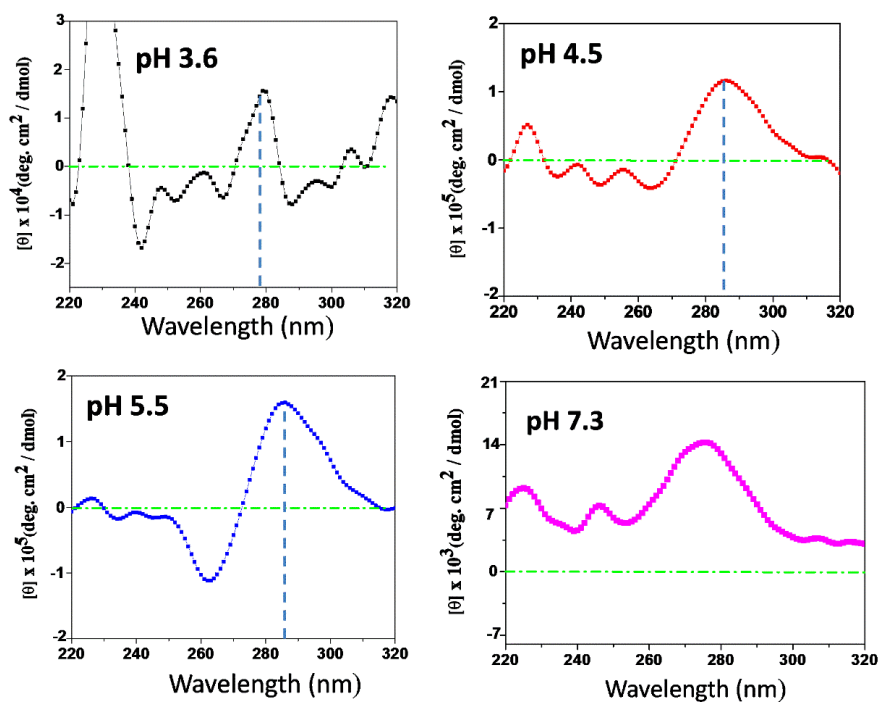


Figure 4.28 CD plots of dTC₅:bm-PNA **1** at pH 3.6, 4.5 and pH 7.3

4B.3.4. Summary of thermal stability of hybrid *i*-motifs

In summary, the pH dependent thermal stability of hybrid complexes as seen from their T_{ms} at pH 3.6 and 4.5 correspond to tetraplex formation depicted by positive band at 295 nm, while at lower pHs of 5.5 and 7.3, only duplex formation is seen by shift of positive band to lower wavelengths. dTC₅:*aeg*-PNA hybrid tetraplex seem to be more stable at acidic pH condition while dTC₅:*iso*-PNA hybrid duplex more stable at neutral pH. The hybrid complex dTC₅:*bm*-PNA-1 has moderate stability from pH 3.6 to pH 7.3. The trend in T_{ms} suggests that the duplexes seen at neutral pH correspond to DNA:PNA hybrids and not the possible self-duplexes DNA:DNA and PNA:PNA. These systems are complex for analysis by UV-T data alone and hence it is not possible to clearly rule out the possibility of presence of homo duplex / tetraplex structures from the present results.

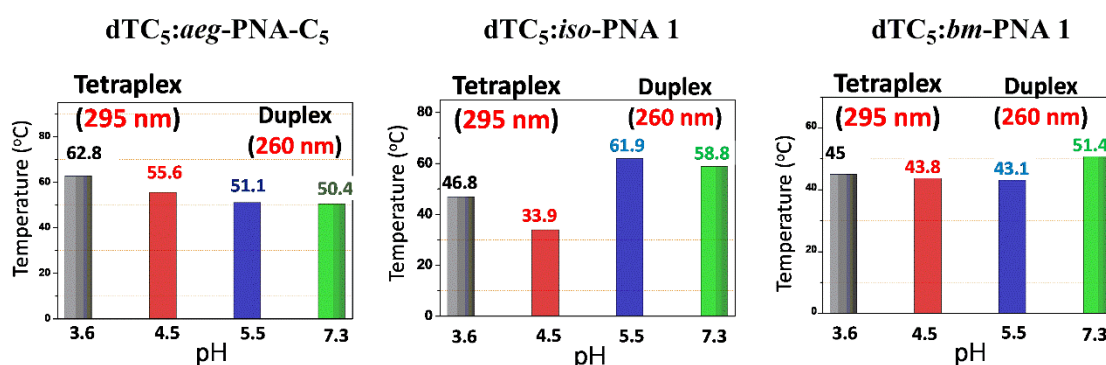


Figure 4.29. Summary of UV-T plots of dTC₅:*aeg*-PNA-C₅, dTC₅:*iso*-PNA 1, and dTC₅:*bm*-PNA-1

4B.4 Discussion: Base pairing in C:C duplexes

It is seen from the above results that *iso*-PNA and bimodal PNAs endowed with all-Cytosines on the triazole face can form *i*-motif structure at pH lower than 5.0 as seen from characteristic negative transition in UV-T plots monitored at 295. At pH > 5.0 (above pK_a) where the Cs are hemi or non-protonated, the triazole face Cytosines self-associate to form [*bm*-PNA]₂ duplexes. The T_m of such duplexes are slightly higher than that of *i*-motif tetraplex seen at lower pH (Figure 4.29). The C:C base pairs constituting such duplexes can be the resultant of two types of H-bond pairing (i) protonated hydrogen bonding: N3•••H⁺•••O2 and N4-H•••N3 (Figure 4.30a, antiparallel strands) or (ii) non-protonated pair of H-bonds: N4-H•••N3 and N3•••H-N4 (Figure 4.30b, parallel strands). Both types of base paired C:C duplexes have been detected in crystal structures.⁵²⁻⁵⁴ Since C is perhaps non-protonated at pHs

5.5 and 7.0 (as seen from UV spectra), the C:C duplex formation seen here may happen predominantly through scheme shown in Figure 4.30b. However, our present results cannot distinguish these two possibilities. It should be pointed out that in case of DNA, *i*-motif tetraplexes have been observed even under neutral conditions.¹³⁻¹⁶

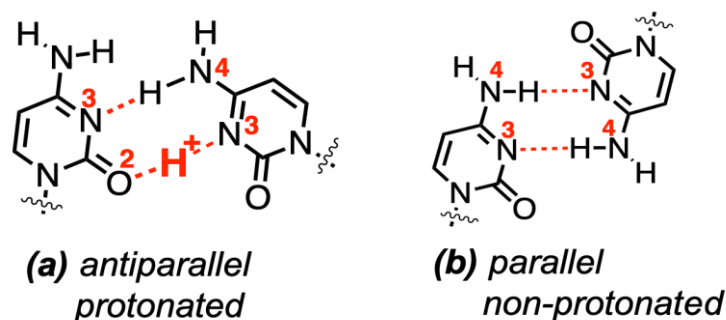


Figure 4.30. Hydrogen bonding schemes for C:C duplex formation¹⁹

4B.5 Conclusions

It is demonstrated that pK_a of N3-H of Cytosines in different structural environment as in DNA (N-glycosidic), *aeg*-PNA (N-acetamido) and *iso*-PNA and *bm*-PNA (N-methylene triazolyl) are different and this has effect on their pH dependent ability to form *i*-motif tetraplex structure. *aeg*-PNA with all-C sequence on t-amide side is well known to form *i*-tetraplexes at low pH that are more stable than analogous *i*-tetraplexes from DNA.^{36,37,39} It is demonstrated that *iso*-PNA and bimodal PNAs that have all-Cytosines on triazole face and being more flexible than Cytosines anchored on t-amide link, also form *i*-motif with similar stability. The pH dependent UV spectra and T_m studies indicated that *aeg*-PNA, *iso*-PNA and *bm*-PNA form *i*-motif at pH below 5.0, while at higher pH they form more stable C:C duplexes. Such C:C duplexes constituted from Cytosines on triazole face in *bm*-PNA and having mixed base sequence on t-amide face can create two outer duplexes with complementary DNA. This leads to assembly of triple duplex with a combination of two outer PNA:DNA duplexes (WC base pairs) fused to a central PNA:PNA duplex held by self-base pairing of Cytosines. Our present studies extend the adaptability of *bm*-PNAs to form multistranded double duplex^{15a,b} and triplex of duplex^{15c,d} to constituting triple duplexes. Work is in progress to constitute and characterise tetraduplexes derived from *bm*-PNA-G4 tetraplexes to take the versatility of *bm*-PNAs to the next level. Such new ways to form supramolecular assemblies from predesigned PNA (*bm*-PNA) and related peptide structures that are programmable have great potential in tuning

PNA⁵⁵⁻⁵⁸ for functional molecular assemblies and molecular nanotechnology for both therapeutics and diagnostics.⁵⁹⁻⁶²

4.6 EXPERIMENTAL SECTION

The syntheses of monomers and oligomers were done following the reported procedures.⁴³ The chemicals used were of laboratory or analytical grade. All the solvents used were distilled or dried to carry out different reactions. Reactions were monitored by thin layer chromatography (TLC). Usual workup involved sequential washing of the organic extract with water and brine followed by drying the organic layer over anhydrous sodium sulphate and evaporation of solvent under vacuum. TLCs were carried out on pre-coated silica gel GF₂₅₄ sheets (Merck 5554). TLCs were analysed under UV lamp, iodine spray and by spraying with ninhydrin solution, followed by heating of the plate. Column chromatographic separations were performed using silica gel (60-120 or 100-200 mesh).

4.5.1 pK_a determination by pH dependent UV-measurements

The UV absorbance of each oligomer (dC₆, *aeg*-PNA-C₅ and *iso*-PNA 1) were determined at 10 μ M concentrations at various pHs (pH 3.6 – 4.5, sodium acetate buffer, 30 mM; pH 5.5-pH 7.3 sodium cacodylate buffer, 30 mM). UV measurements were done on Shimadzu UV-2100 spectrophotometer at each pH at 25 °C. The ratios intensities of absorbance maxima (protonated) vs minima (non-protonated) of cytosine for each oligomer were plotted against the pH. The points obtained were fitted (Boltzmann) using Origin 8.5 software, and by first derivative of the fitted curve obtained the pK_a value.

4.5.2 Temperature dependent UV absorbance and determination of T_m

UV-melting experiments were carried out on Varian Cary 300 UV spectrophotometer equipped with a peltier heating-cooling system. For i-motif the samples for T_m measurements were prepared as DNA/PNA [5 mM], Buffers Sodium acetate (30 mM), pH 3.6-4.5, Sodium cacodylate buffer (30 mM), pH 5.5-7.3. The samples were annealed by heating at 90 °C for 10 min followed by slow cooling to room temperature for at least 8-10 h and then refrigerated for at least 12 to 24 h. The samples (500 μ l) were transferred to quartz cell and equilibrated at the starting temperature for 5 min. The OD at 260 nm was recorded in steps from 20-90 °C with temperature increment of 0.5 °C. 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 of normalized absorbance with respect to temperature and is accurate to ± 1.0 °C. The data were processed using OriginPro 8.5, data fitted by sigmoidal curve, function - Boltzmann for one face and biphasic dose response for two face binding. The concentrations of all oligonucleotides were calculated on the basis of absorbance from the molar extinction coefficients of the corresponding nucleobases i.e. T = 8.8 cm²/μmol; C = 6.6 cm²/μmol; G = 11.7 cm²/μmol and A = 13.7 cm²/μmol.^{48,49}

4.5.3 Circular Dichroism (CD) spectroscopy

CD spectra were recorded on JASCO J-815 spectropolarimeter connected with a peltier. the samples for CD spectra were prepared as *bm*-PNA [10 μM] in buffers consisting of aq.sodium acetate (100 mM), pH 3.6 and aq. sodium cacodylate buffer (100 mM), pH 5.5-7.3. The samples were annealed by heating at 90 °C for 10 min. followed by slow cooling to room temperature for at least 8-10 h. The cooled samples were transferred to refrigerator for at least 8 to 12 h. To record the CD spectra the temperature was maintained at 10 °C. The CD spectra were recorded as an accumulation of 3 scans from 300 to 190 nm using 1 mm quartz cell, a resolution of 0.1 nm, band-width of 1 nm, sensitivity of 2 m deg, response of 1 sec and a scan speed of 100 nm/min.

4.6 References

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Chapter 5

Silver and Mercury mediated Supramolecular assemblies of *bm*-PNAs

5.1 Introduction

Peptide nucleic acids (PNA) are achiral mimics of nucleic acids composed of a polyamide backbone with recurring units of 2-aminoethylglycine (*aeg*) to which nucleobases A, C, T and G are linked via a tertiary amide linkage (Figure 5.1a).¹ The sequence-specific complementary base pairing between PNA and DNA/RNA yield duplexes that have superior specificity and stability over that of natural DNA:DNA/RNA duplexes.² Such attributes of PNA are favourable in their applications for in vitro diagnostics and antisense therapeutics.³ A number of efforts on structural editing of PNA backbone has led to a wide assortment of cyclic, acyclic, chiral and cationic backbones that have shown improved properties of PNA desirable for their successful applications.⁴⁻⁹ These studies have revealed that substitutions on *aeg* backbone at C $_{\alpha}$, C $_{\beta}$, and C $_{\gamma}$ were not deleterious for hybridization with cDNA/cRNA.¹⁰⁻¹⁸

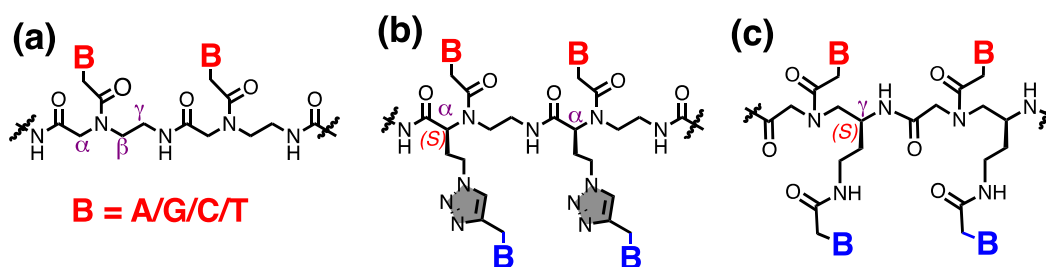


Figure 5.1. (a) *aeg*-PNA (b) *bm*-C $_{\alpha}$ -triazole PNA (c) *bm*-C $_{\gamma}$ -amide PNA

Taking cognizance of the simple attributes of PNA backbone, we designed bimodal PNA (*bm*-PNA),⁷ in which a second nucleobase was linked in each *aeg* unit of PNA backbone at C $_{\alpha}$ via a triazole linker (Figure 5.1b) or at C $_{\gamma}$ through an amide linker (Figure 5.1c), in addition to the nucleobases on t-amide face as in standard *aeg*-PNA. This enabled *bm*-PNA to

complement with another DNA/RNA strand to form a second duplex sharing backbone with the first duplex from t-amide face. Instead of the standard PNA₂:DNA triplexes, the bimodal PNAs form *bm*-PNA:DNA₂ double duplexes¹⁹ and *bm*-PNA₂:DNA₃ triplex of double duplex,⁸ employing standard WC / Hoogsteen base pairing. Such multistranded base-paired assembly of bimodal PNA with DNA is programmable, since just one step mixing of the appropriate components leads to the same final complex.²⁰

In comparison with the canonical A:T and C:G hetero base pairing, the pyrimidine nucleobases C and T are known to form homo base pairing (self-pairing) assisted by proton²¹ or a metal ion (Figure 5.2).²²⁻²⁵ It was shown that specific C:C mismatch pairing is stabilised through mediation of Ag⁺ (C:Ag⁺:C), while Hg²⁺ specifically stabilizes T:T mismatch (T:Hg²⁺:T) in DNA duplexes.²⁶⁻²⁸

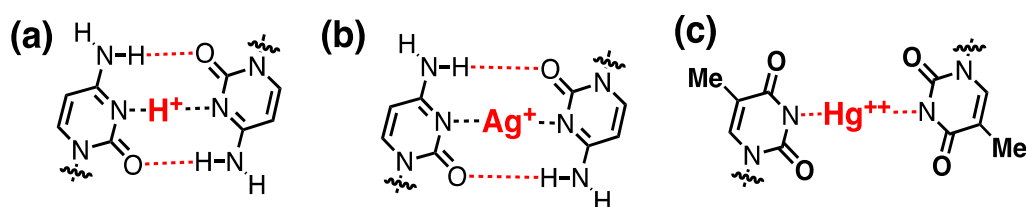


Figure 5.2. C:C base pairs via (a) H⁺ and (b) Ag⁺ (c) T-T base pair via Hg²⁺

Muller *et al*²⁹ showed that Ag⁺ mediated imidazole base pairing (Figure 5.3). In the presence of Ag(I) ions, the DNA oligomer containing three consecutive imidazole bases forms stable duplex by forming imidazole:Ag⁺:imidazole pairing and self-hairpin structure in the absence of Ag⁺.

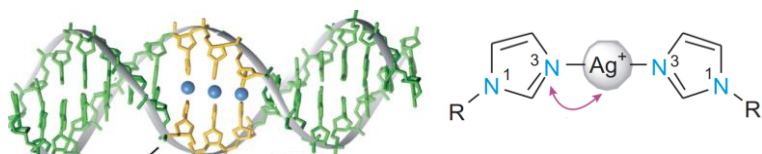


Figure 5.3. imidazole:Ag⁺:imidazole base pairing²⁹

There are several reports available on artificial metal-mediated base pairs, such as Cu²⁺ mediated hydroxypyridone base pair. Shionoya *et al*³⁰ showed incorporation of Ag⁺ in a double or triple stranded DNA, significantly increases the stability of DNA duplex or triplex structure (Figure 5.4).

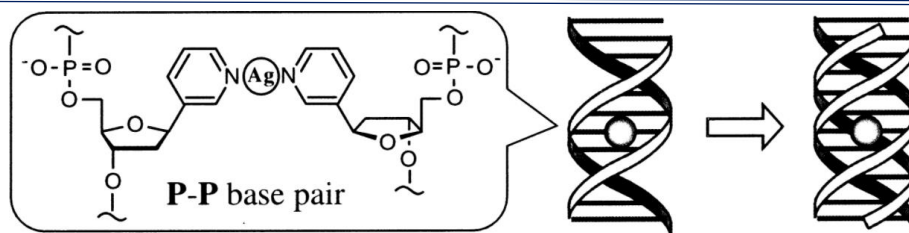


Figure 5.4. Ag^+ in the DNA duplex and triplex³⁰

As in pyrimidines, silver mediated purine base pairing reports are available. Gan *et.al* first reported G- Ag^+ -G pair in a short 8mer DNA strand³¹. Silver mediated G-duplexes formed preferentially over G-quadruplexes.³² The addition of Ag^+ increased the stability of triplex structure by forming C: Ag^+ :G:C base triplet structure.³³

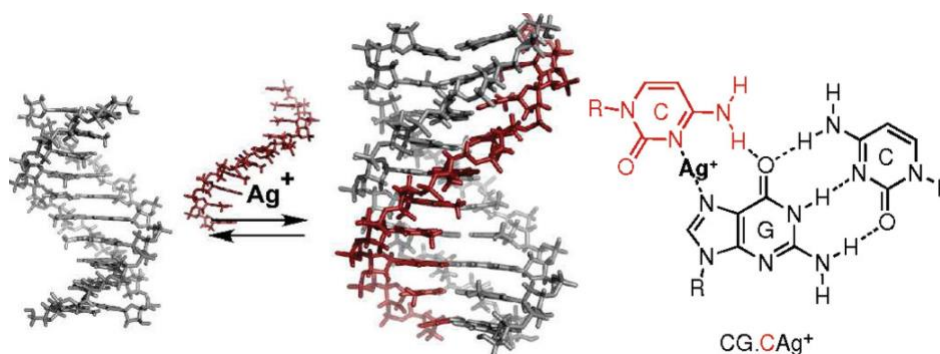


Figure 5.5. Ag^+ mediated C: Ag^+ :G:C base triplet³³

Using branched PNA with poly C in all the 3-arms attached to chiral 4-amino proline scaffold, we recently demonstrated that in presence of Ag^+ , the branched PNA self assembles through C: Ag^+ :C base pair into 4-pointed nano star architecture.³⁴

5.2 Rationale and Objective

In this Chapter, we describe a novel multistrand assembly of bimodal PNAs with poly C sequence on either triazole or t-amide face and a mixed sequence on other face to form triple duplexes in presence of Ag^+ . The mixed base derived duplex is held by WC H-bonded base pairing, while the poly C face duplex is held by Ag^+ ion. Figure 5.6 shows the different PNA constructs used in this study. The standard *aeq*-PNA- C_5 (Figure 5.6a) has 5 cytosines (C) linked via t-amide to *aeq* backbone. The *iso*-PNA 1 (Figure 5.6b) has 5 Cytosines attached at C_α of *aeq* backbone by triazole spacer, without any nucleobases on t-amide side. The bimodal *bm*-PNA 1 has mixed base sequence on t-amide side and 5 cytosines (C) on the triazole face (Figure 5.6c) similar to *iso*-PNA 1. The *bm*-PNA 2 (Figure 5.6d) has reverse structure with all-C

sequence on t-amide side and mixed base sequence at C α on triazole face (reverse of *bm*-PNA **1**). Multistrand assemblies can be assembled in which two from *bm*-PNA strands are held as duplexes by complexation of poly C sequence present either on triazole face or t-amide face with Ag⁺ or Hg⁺⁺ and the outer mixed base sequences are hybridized with complementary DNA. This would generate triple duplexes as a single complex held composed by two types of base pairing: one central duplex with metal mediation and two outer duplexes with WC H-bonds.

5.3 Results and discussion

The target *aeg*, *iso* and *bm*-PNA **1** and *bm*-PNA **2** were synthesised on solid support using appropriate monomers following procedures reported in Chapter 2 and Chapter 3. These were purified by HPLC and characterised by Nano ESI mass spectrometry. The DNA **1**, DNA **2** and dTC₅ (Table 5.1) which are complementary to mixed base sequence on the t-amide face on *bm*-PNA **1** and triazole face in *bm*-PNA **2** respectively, were obtained from commercial sources.

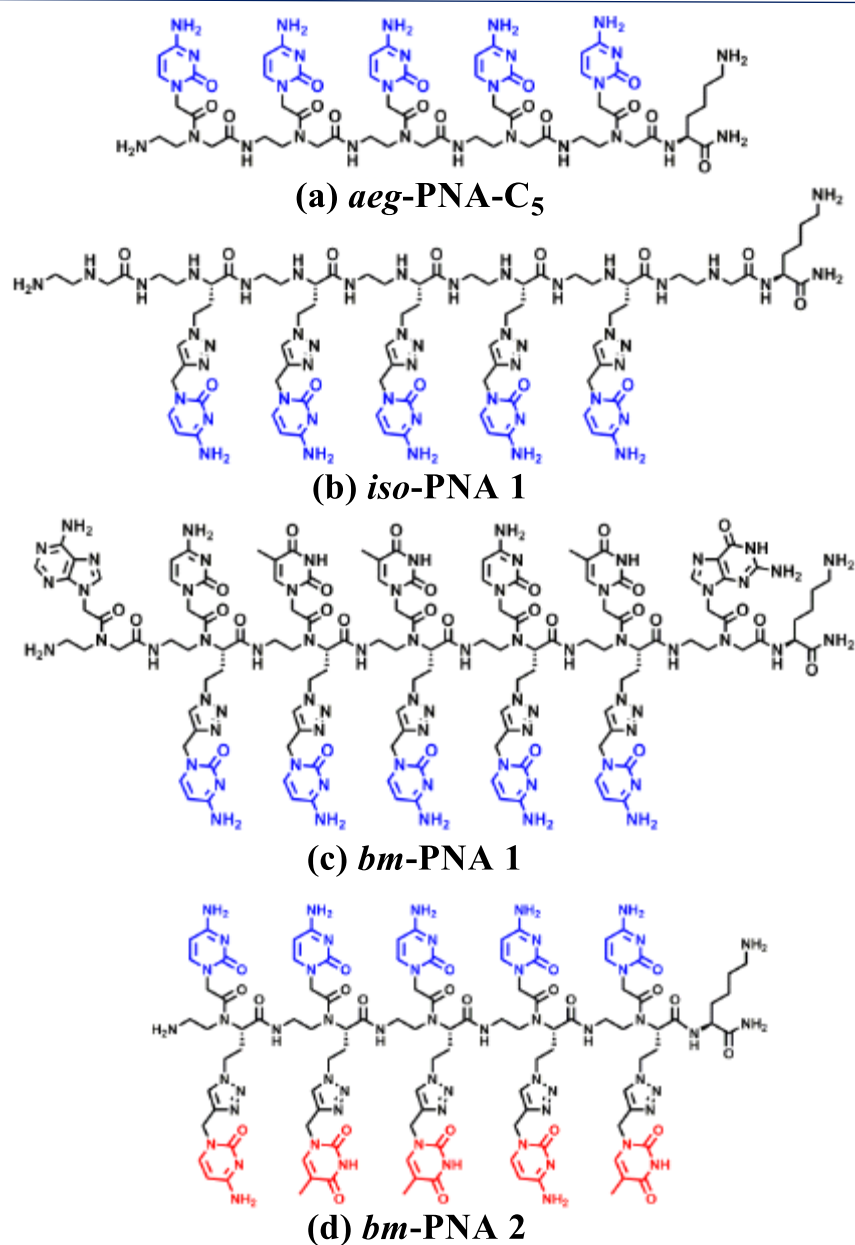


Figure 5.6. Structures of (a) *aeg*-PNA-C₅ (b) *iso*-PNA 1 (c) *bm*-PNA 1 and (d) *bm*-PNA

Table 5.1 Complementary DNAs used for metal assisted base pairing

Entry	DNA	Sequence (5'-3')
01	DNA1	CAGAAGT
02	DNA2	TAGAAG
03	dTC ₅	TCCCCC

For biophysical studies, various complexes were assembled by mixing *bm*-PNA **1** / *bm*-PNA **2** with respective complementary DNA **1** / DNA **2** (2.5 μ M) in stoichiometric ratios in the presence or absence of AgNO₃ (12.5 μ M) or HgCl₂ (12.5 μ M) in MOPS (10 mM, pH 7.0) and NaNO₃ (10 mM). The samples were dissolved in buffer and annealed at 90 $^{\circ}$ C by heating and cooling to room temperature. These were used in variable temperature UV absorbance (UV-T), CD experiments and for imaging by scanning electron microscopy (SEM). Successful formation of duplexes were indicated by observance of sigmoidal UV-T plots and the T_m s for each duplex in the complex were obtained by the maxima in first derivative curves of UV-T plots.^{35, 36}

Section A: Ag⁺ assisted base pairing

5A.3.1 Thermal stability of dTC₅, *aeg*-PNA-C₅ and *iso*-PNA **1** silver duplexes by UV-T

The standard DNA dTC₅ and *aeg*-PNA-C₅ were examined for formation of duplexes in the presence and absence of Ag⁺ in buffer. Both showed sigmoidal transitions due to self-melting with T_m s of 64.1 and 48.8 $^{\circ}$ C respectively (Figure 5.7a and Figure 5.8a). In the presence of Ag⁺, both dTC₅ and *aeg*-PNA-C₅ showed increase of their T_m s to 80 $^{\circ}$ C (Figure 5.7b; ΔT_m +15.9 $^{\circ}$ C) and 71 $^{\circ}$ C (Figure 5.8b; ΔT_m +22.2 $^{\circ}$ C) respectively. This indicated the formation of duplexes mediated by silver ion (C:Ag⁺:C). Thus compared to *i*-motif (C:H⁺:C) tetraplex held by protonated C at acidic pH, the silver mediated duplexes are realised at neutral pH. In DNA, the C is linked to sugar-phosphate backbone by glycosidic bond, while in *aeg*-PNA, C is linked to *aeg* backbone by t-amide bond via methylene carbonyl linker. Nevertheless, both C's can form complex with Ag⁺, with poly C sequence of *aeg*-PNA forming more stable duplex with than the one from DNA.

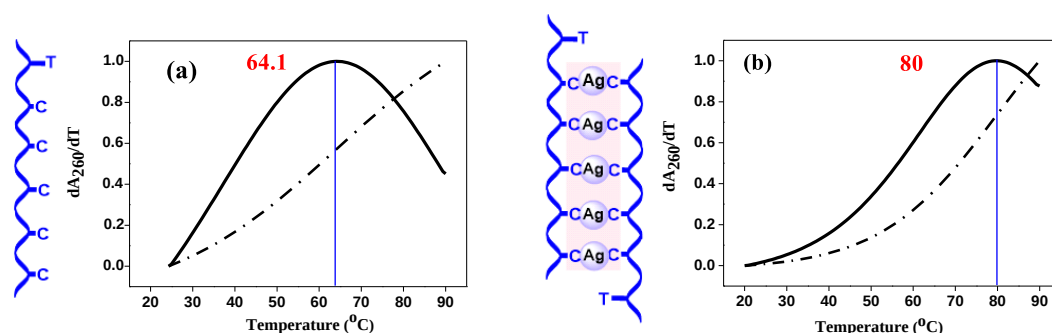


Figure 5.7 UV-T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm of (a) dTC₅ self-melting and (b) dTC₅:Ag⁺:dTC₅.

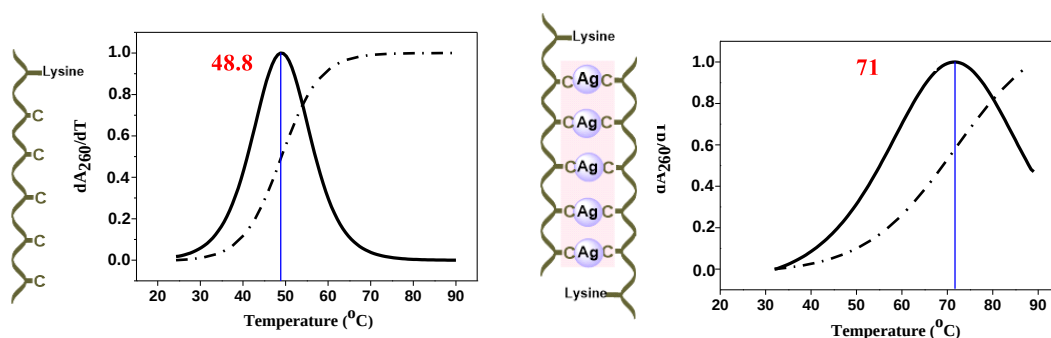


Figure 5.8 UV- T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm (a) *aeg*-PNA-C₅, self-melting (b) *aeg*-PNA-C₅:Ag⁺:*aeg*-PNA-C₅.

Similar UV-T experiments were done with *iso*-PNA **1** in which poly C is present on a sidechain at C α , linked at N1 by alkyl triazole link (triazole face). The self-melting T_m of *iso*-PNA **1** 60 °C was enhanced to 73.9 °C (Figure 5.9; ΔT_m +13.9 °C) in presence of silver. This indicated that poly C sequences on triazole face also form self-duplexes and Ag⁺ induced complexes with higher stability, similar to poly C on t-amide face in *aeg*-PNA-C₅. However, the extent of stabilization (ΔT_m) for triazole linked C (*iso*-PNA **1**) was slightly lower than that for t-amide linked C (*aeg*-PNA-C₅).

N3 of cytosine has pK_a of 4.5 and it stabilises N3-Ag⁺-N3 bonding. In the *iso*-PNA the pK_a of secondary NH in the backbone is around 10.5 and hence is protonated at physiological pH, with Ag interaction with backbone amine is less likely. In literature³⁷ it is reported that binding of silver to triazole alone is weak and needs a second stronger binding component such as O or S. UV-T_m studies of silver complexes of *bm*-PNA in presence of excess AgNO₃ suggests that at higher concentrations the purines can also bind.³⁸ Further, the EDAX analysis of *bm*-PNA complexes suggest a stoichiometry expected from Ag binding to N3 of C at lower concentrations of AgNO₃.

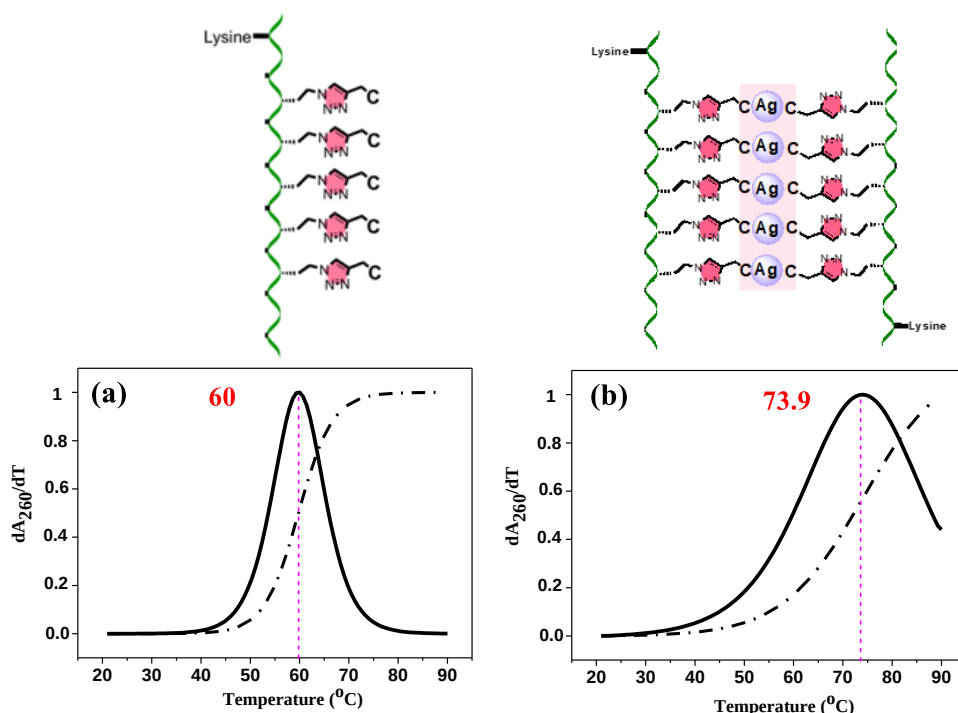


Figure 5.9 UV- T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm (a) *iso*-PNA- C_5 alone and (b) *iso*-PNA **1**: Ag^+ :*iso*-PNA **1**

5A.3.2 UV-T studies of Ag^+ mediated duplex of *bm*-PNA **1**

The bimodal *bm*-PNA **1** formed the expected WC duplex from mixed base sequence on t-amide face with complementary DNA **1** with T_m of 36 °C (Figure 5.11a). The *bm*-PNA **1** alone also undergoes self-melting with T_m of 30 °C (Figure 5.10) and when complexed with Ag^+ with poly C chain from triazole side formed silver mediated duplex (*bm*-PNA **1**: Ag^+ :*bm*-PNA **1** Figure 5.11b) analogous to *iso*-PNA **1**, which exhibited single transition with T_m of 68.3 °C. Compared to its self-melting, T_m of [*bm*-PNA **1**] $_2$: Ag^+ complex showed a higher stability with T_m of 68.3 °C (ΔT_m +38.3 °C). While poly C on triazole face of *bm*-PNA **1** is complexed with Ag^+ , it has mixed nucleobase sequence available on the the two-outer t-amide faces. This can base pair with complementary DNA **1** via WC H-bonding to form additional duplexes (Figure 5.10d). The silver complex *bm*-PNA **1**: Ag^+ :*bm*-PNA **1** was mixed with two equivalents of complementary DNA **1**. The UV-T plot of the resulting complex interestingly showed double sigmoidal transition with T_m s at 49.3 °C and 81.5 °C. This suggests the presence of two melting species in solution, perhaps with formation of the complex DNA **1**:*bm*-PNA **1**: Ag^+ :*bm*-PNA **1**:DNA **1** (Figure 5.11c). This is composed of two duplexes of DNA **1**:*bm*-PNA **1** fused with Ag^+ from poly C face. The two T_m s in principle can arise from melting of these two duplexes, DNA **1**:*bm*-PNA **1** and the [*bm*-PNA] $_2$: Ag^+ in the complex. However, the

two T_m s in the complex are higher by ~ 13.2 °C than that of isolated constituent duplexes of the complex. A priori, the lower transition at 49.3 °C may result from the WC duplex melting, while the higher transition at 81.5 °C may arise from the melting of the silver complex. The fact that both T_m s are higher suggests that each of the duplexes do not melt independently and synergistically stabilise the other duplex as they are conjoined with a common backbone of *bm*-PNA **1**.

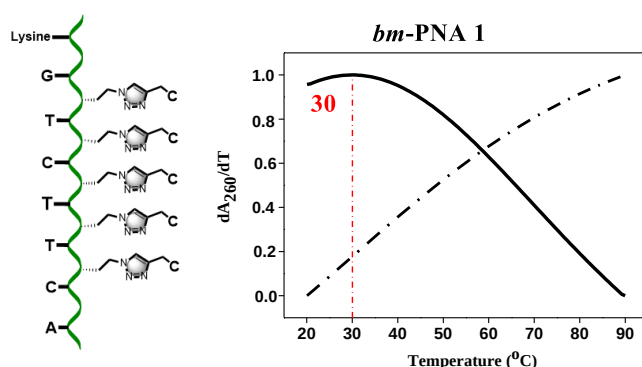


Figure 5.10 UV-T plot of *bm*-PNA **1** (Self melting-30 °C)

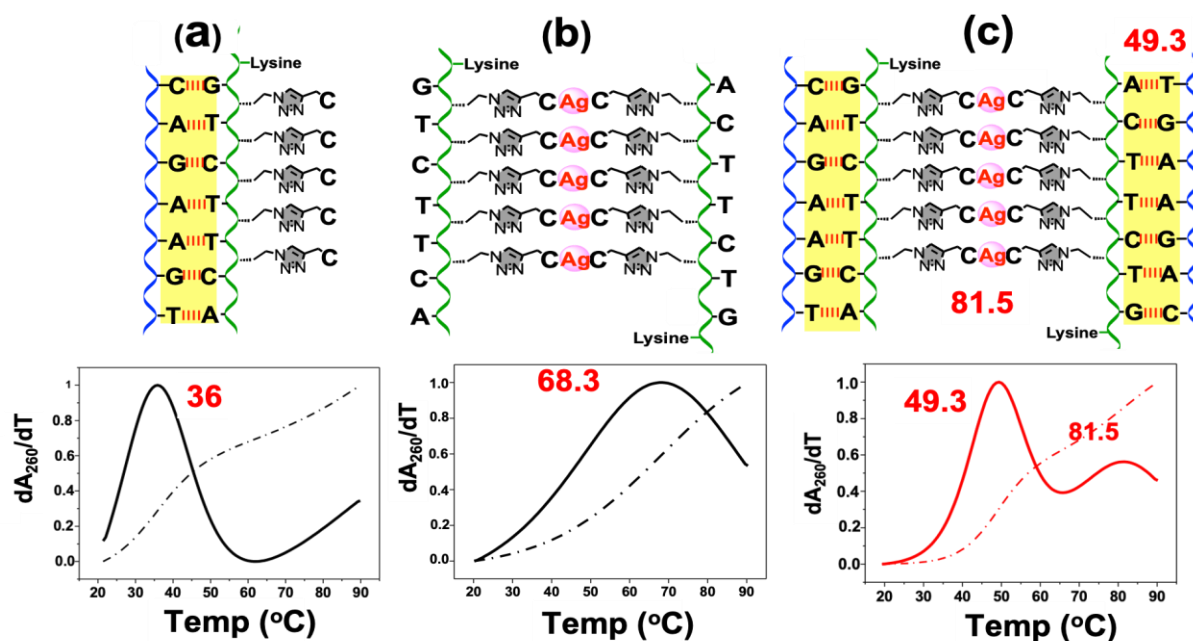


Figure 5.11 UV-T plots of *bm*-PNA **1** with all-C on triazole face (a) duplex DNA **1**: *bm*-PNA **1** (b) silver complex [*bm*-PNA **1**]₂:Ag⁺ and (c) silver complex of double duplex [DNA **1**: *bm*-PNA **1**]₂:Ag⁺.

The UV-tm experiments of each complex were repeated thrice and results are consistent. The possibility of racemization during solid phase synthesis using the HBTU-HOBT as coupling

conditions without preactivation is minimal/ruled out since HOBt is a well-known suppressor of racemization.³⁹⁻⁴² Further racemizations would lead to diastomeric oligomers which can be seen as mixture in HPLC, which was not the case. PNA:DNA melting transitions are broad sigmoidal and hence first derivative curves are used to obtain accurate and reliable T_m s.

5A.3.3 UV-*tm* studies of Ag^+ mediated *bm*-PNA with poly C on amide face: double duplexes of *bm*-PNA 2

To examine the generality of triple duplexes from *bm*-PNA, in presence of DNA and Ag^+ , *bm*-PNA 2 was constructed by reversing the sequences on two faces, with poly C present on t-amide face and mixed base sequence on triazole side. It is seen that individual face duplexes showed a single transition in the UV-T plot, with T_m of 45.6 °C for triazole face DNA duplex (Figure 5.13a) and T_m of 67.1 °C for the t-amide face poly C silver complex (Figure 5.13b). The mixed sequence triazole face DNA duplex (Figure 5.13a) was slightly more stable than analogous mixed face DNA duplex from t-amide face (Figure 5.11a) in spite of having more number of bases in the sequence. The T_m of t-amide poly C silver duplex was almost equal to that of triazole poly C silver duplex (Figure 5.13b). In presence of Ag^+ , the DNA:*bm*-PNA duplex DNA 2:*bm*-PNA 2 dimerized with t-amide face poly C chains held by Ag^+ to form a single complex comprising of three duplexes (Figure 5.13c) DNA 2:*bm*-PNA 2: Ag^+ :*bm*-PNA 2:DNA 2. This complex also exhibited double transition with T_m s at 38.4 °C and 75.4 °C, with lower DNA:*bm*-PNA duplex transition slightly destabilised and the higher transition stabilised over isolated silver complex. However, both transitions were slightly destabilised compared to the analogous triple duplex from *bm*-PNA 1 (Figure 5.11c). The reversed amplitude of first and second transitions in the complexes of *bm*-PNA 1 (Figure 5.11c) and *bm*-PNA 2 (Figure 5.13c) suggests differences in the relative extents of base stacking from the t-amide and triazole faces in the two complexes. Nevertheless, the results confirmed the generality of formation of triple duplexes from *bm*-PNAs when poly C is present either at t-amide or triazole face. The T_m results are summarised in Table 5.2. When the sequence of addition is reversed, first with AgNO_3 to *bm*-PNA followed by addition of DNA 1, identical T_m s were obtained. This is similar to that demonstrated earlier with triplex of duplexes,^{19, 20} wherein the assembly was realised, irrespective of the order of additions. Thus, the assembly process is programmable in nature.

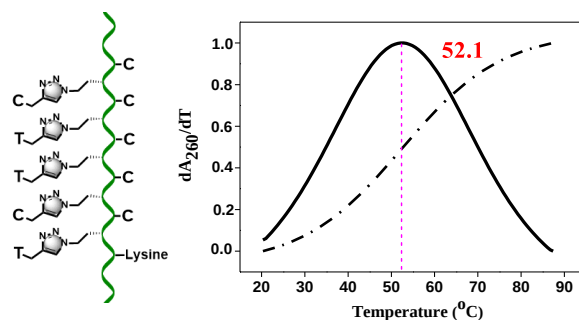


Figure 5.12 UV-T plot of *bm*-PNA 2 (Self melting-52.1 °C)

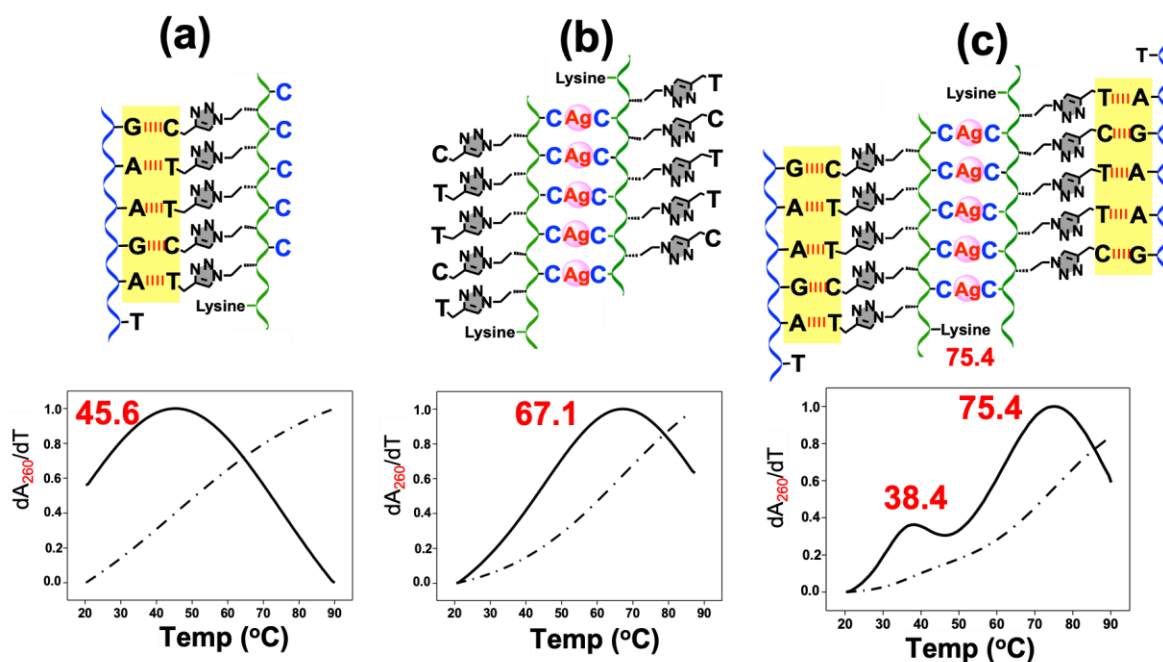


Figure 5.13 UV-T plots of *bm*-PNA 2 with poly C on t-amide face (a) duplex DNA 2: *bm*-PNA 2 (b) silver complex [*bm*-PNA 2]₂:Ag⁺ and (c) silver complex of duplex [DNA 2: *bm*-PNA 2]₂:Ag⁺.

Table 5.2. T_m (°C) of PNA oligomers and duplexes in the presence and absence of Ag⁺

Entry	Oligomer	In buffer	With Ag ⁺	ΔT_m
01	dTC ₅	64.1	80.0	15.9
02	<i>aeg</i> -PNA-C ₅	48.8	71.0	22.2
03	<i>iso</i> -PNA 1	60	73.9	13.9
04	<i>bm</i> -PNA 1	30	68.3	38.3
05	<i>bm</i> -PNA 2	52.1	67.1	15.0

06	DNA 1: <i>bm</i> -PNA 1	36	49.3,81.5	13.3, 13.2
07	DNA 2: <i>bm</i> -PNA 2	45.6	38.4,75.4	-7.2, 8.3

The T_m values are average of three experiments. Values in brackets indicate stabilization compared to buffer. Entry 6 and 7, Values in square brackets are with respect to corresponding T_m values in entry 4 and 5 in presence of Ag^+

In each *bm*-PNA/*iso*-PNA complexes, Ag^+ stabilizes the duplexes. The higher melting phenomenon of both duplexes in *bm*-PNAs cannot be explained as consequence of sequential melting process. We have attributed the first transition it to arise from a pre-melting process that involves partial unstacking of both duplexes, keeping the complex intact. This is followed by simultaneous dissociation of both strands in one step leading to the second higher transition. Such a mechanism would explain synergistic stabilization of both duplexes.

5A.3.4. Comparative thermal stability of Ag^+ mediated hybrid duplexes and Watson Crick duplex

Instead of silver induced self-duplexes, the corresponding hybrid duplex formation with DNA-dTC₅ was attempted and their stabilities examined by UV-T and CD experiments.

5A.3.4a UV-T studies of *bm*-PNA 1: Ag^+ :dTC₅ and *bm*-PNA 1:dTG₅. Silver mediated C:C duplex formation were investigated for hybrid complex of *bm*-PNA 1 with dTC₅ for comparison with homo silver complexes of dTC₅ and *bm*-PNA 1. The hybrid silver complex comprised from the *bm*-PNA 1 and dTC₅ (*bm*-PNA 1: Ag^+ :dTC₅ Figure 5.14) showed T_m of and *bm*-PNA 1:dTC₅ is 35.2 and 31 °C respectively. Ag^+ mediated C:C mismatch duplex showed increase in T_m than C:G duplex i.e. $\Delta T_m +4.2$ °C indicating metal mediated base pairs are more stable than canonical WC base pairing. (Figure 5.14 a and Figure 5.14 b.)

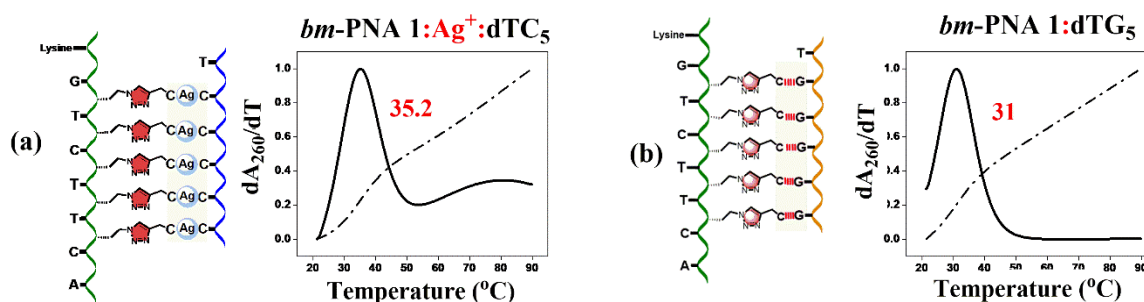


Figure 5.14 UV-T plots of metal mediated and WC duplexes (a) Silver duplex *bm*-PNA1: Ag^+ :dTC₅ (b) WC duplex *bm*-PNA 1:dTG₅

5A.3.4b UV-T studies of DNA 1: *bm*-PNA 1: Ag^+ :dTC₅ and DNA 1: *bm*-PNA 1:dTG₅

The formation of Ag^+ induced hybrid assembly of three components having double duplexes was examined. The pre-formed DNA: *bm*-PNA **1** Watson Crick duplex (Figure 5.15a) treated with dTC₅ and Ag^+ for inducing silver mediated heterostrand base pairing (*bm*-PNA **1**:dTC₅, Figure 5.15b) on triazole side, in addition to the canonical WC base pairing (DNA **1**: *bm*-PNA **1**) from t-amide face. The DNA **1** complementary to t-amide face forms stable duplex DNA **1**: *bm*-PNA **1** with T_m 36 °C. In presence of dTC₅ and Ag^+ added the hybrid double duplex DNA **1**: *bm*-PNA **1**: Ag^+ :dTC₅ was obtained as shown by double sigmoidal transition (Figure 5.15c) with T_{ms} 35.9 °C and 77.4 °C. Similarly, complementary DNA dTG₅ added to the DNA **1**: *bm*-PNA **1** duplex to result in double duplex of DNA **1**: *bm*-PNA **1**:dTG₅ (Figure 5.15d) with T_{ms} of 35.2 °C and 80.1 °C. (Figure 5.15e)

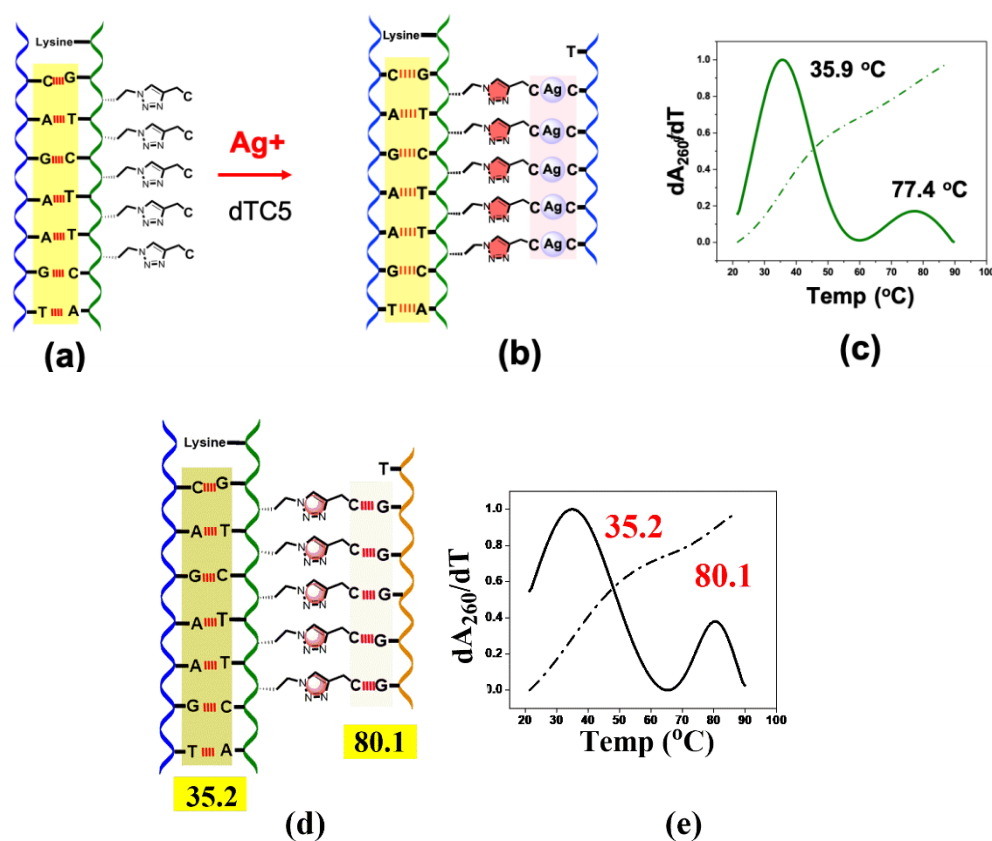


Figure 5.15 UV-T plots of double duplexes (c) silver complex DNA **1**: *bm*-PNA **1**: Ag^+ :dTC₅ and (e) (a) Wc duplex DNA **1**:*bm*-PNA **1**:dTG₅

5A.3.5. Circular Dichroism (CD) spectroscopy of Ag^+ -mediated complexes

5A.3.5a CD studies of DNA **1: *bm*-PNA **1**: Ag^+ : *bm*-PNA **1**:DNA **1**.** The CD spectra of *iso*-PNA **1** and *bm*-PNA **1** do not show any significant bands in spite of their chiral nature, as the nucleobases are far removed from the chiral centre. However, upon complexation to form DNA **1**: *bm*-PNA **1** duplex, the CD spectrum shows a broad negative band at 283 nm and a positive

band at 263 nm (Figure 5.16, curve **b**) which is different from the CD spectrum of DNA alone (Figure 5.16, curve **a**). Adding AgNO_3 to the above duplex generated the complex DNA **1**: *bm*-PNA **1**: Ag^+ : *bm*-PNA **1**:DNA **1** and the CD showed appearance of a large negative band with maxima at 290 nm (Figure 5.16 curve **c**).

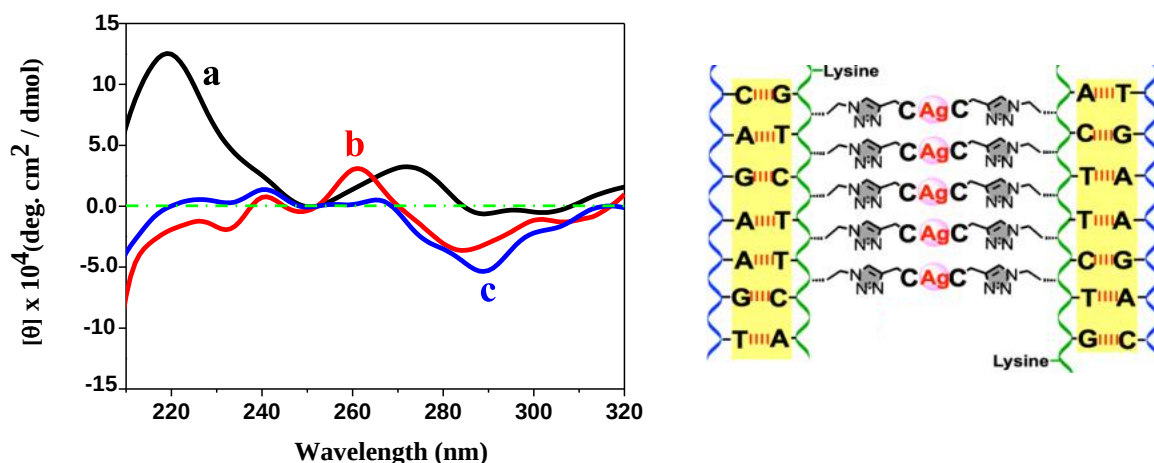


Figure 5.16. CD spectra of *bm*-PNA: DNA complexes, single stranded DNA **1** (a), duplex DNA **1**: *bm*-PNA **1** (b) and its complex with silver (c).

5A.3.5b CD studies of DNA 2: *bm*-PNA 2: Ag^+ : *bm*-PNA 2:DNA 2. The reverse complex with all-C on t-amide side *bm*-PNA **2** showed only marginal changes in CD bands (Figure 5.17). The CD spectra of triple duplexes (Figure 5.17c). are weak due to the fact that the two *bm*-PNA **2**:DNA **2** duplexes are perhaps oriented in opposite directions, mutually cancelling the signals. In conjunction with UV-T results, they support the formation of the double duplex with successive addition of *bm*-PNAs and AgNO_3 to complementary DNA **2**.

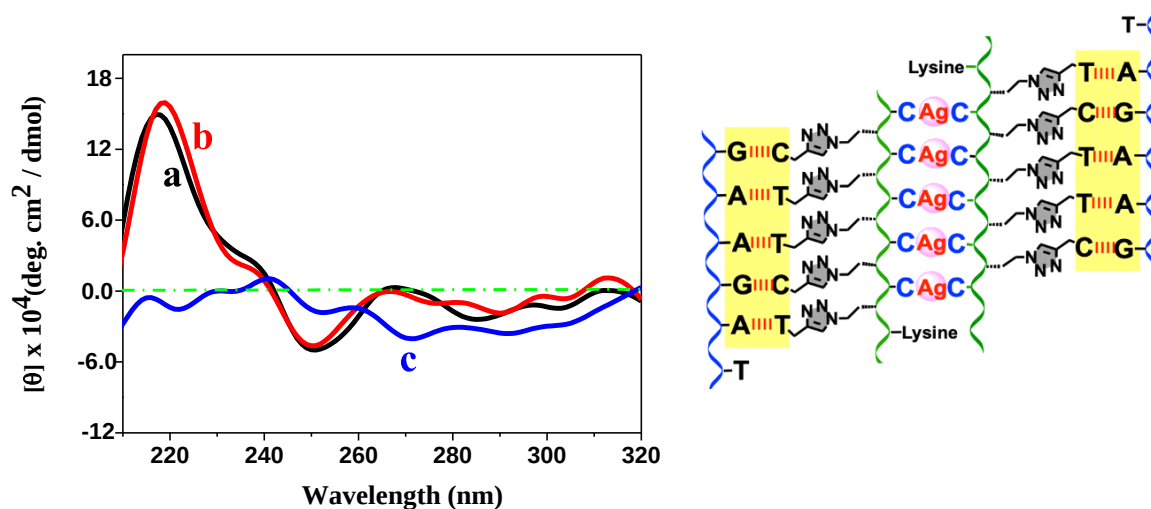


Figure 5.17. CD spectra of *bm*-PNA:DNA complexes, single stranded DNA **2** (a), duplex DNA **2**: *bm*-PNA **2** (b) and its complex with silver (c).

5A.3.5c. CD studies of metal mediated hybrid double duplex DNA **1: *bm*-PNA **1**:Ag⁺:dTC₅.** Upon complexation of *bm*-PNA **1** with dTC₅ to form *bm*-PNA **1**:dTC₅ WC duplex (Figure 5.18), it showed a broad positive band at 278 nm and a larger negative band at 263 nm (red curve) which is different from the CD spectrum of dTC₅ alone (Figure 5.18, black curve). Addition complementary DNA **1** to the t-amide face followed by AgNO₃ addition to the above duplex to generates the complex DNA **1**: *bm*-PNA **1**:Ag⁺:dTC₅ that exhibited double sigmoidal transition in UV-T plot (Figure 5.15). In CD it showed near disappearance of the positive peak at 278 nm, with appearance of a large negative band with maxima at 260 nm and a broad shoulder at 285 nm (Figure 5.18). Though individual bands cannot be assigned at this stage, such negative bands in CD are characteristic of silver complexes of DNA and PNA⁴³. The CD spectra support the sequential formation of the final silver complex from dTC₅ with successive additions of *bm*-PNA **1** and AgNO₃.

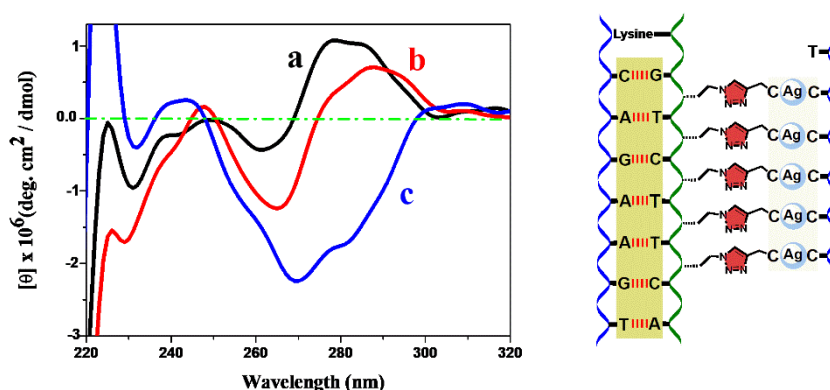


Figure 5.18. CD spectra of hybrid complexes, single stranded dTC₅ (a) duplex *bm*-PNA **1**:dTC₅ (b) DNA **1**: *bm*-PNA **1**:Ag⁺:dTC₅ (c)

5A.3.5d CD studies of WC double duplex DNA **1: *bm*-PNA **1**:dTG₅.** The CD spectrum of DNA dTG₅ shows broad positive band at 265 nm, a positive hump at 295 nm and negative band at 240 nm (Figure 5.19, curve **a**) with addition of *bm*-PNA **1** band pattern remains with additional negative band at 230 nm but intensity of 265 nm reduced for complex *bm*-PNA **1**:dTG₅ (Figure 5.19, curve **b**). Finally, after adding complementary DNA **1** to the amide face in the above duplex generated the complex DNA **1**: *bm*-PNA **1**:dTG₅ and the CD showed decrease in band intensity at 295 nm (Figure 5.19, curve **c**).

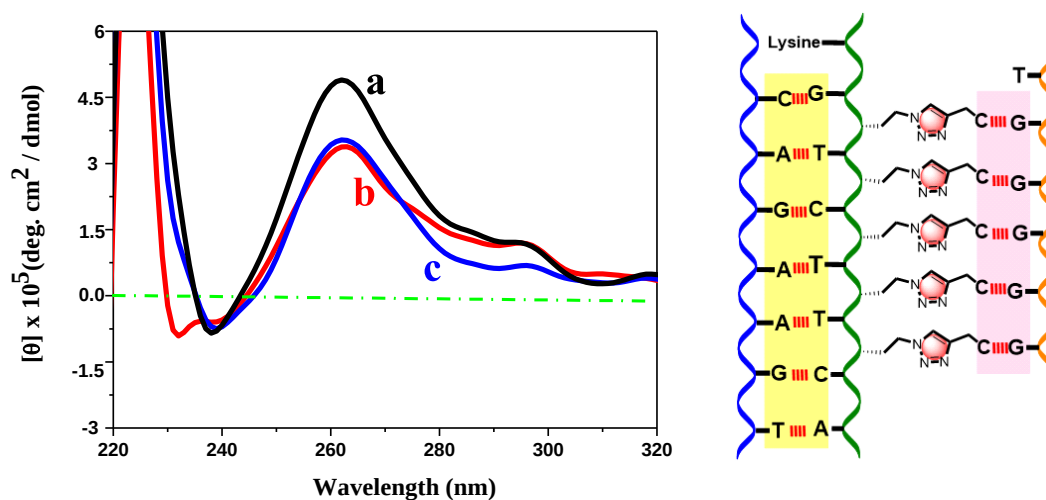


Figure 5.19. CD spectra of hybrid complexes, single stranded dTG₅ (a) duplex *bm*- *bm*-PNA **1**:dTG₅ (b) DNA **1**: *bm*-PNA **1**:dTG₅

5A.3.6 Morphological study by Field Emission Scanning Electron Microscope (FESEM) and Elemental composition by EDAX analysis

The various duplexes, double duplexes and triple duplexes described in earlier section give rise to different supramolecular assemblies. Such features will be reflected in their morphological features and these were examined by their FESEM images (Figure 5.20).

5A.3.6a *iso*-PNA **1:Ag⁺:*iso*-PNA **1** complex:** The *iso*-PNA **1** showed spherical nanoparticle heterosize aggregates of 100-150 nm size (Figure 5.20A). After addition of Ag⁺, dimerization of *iso*-PNA leads to formation of silver complex *iso*-PNA **1**:Ag⁺: *iso*-PNA **1** which showed fusion of spherical aggregates into rod shapes with sizes in the range of 100 nm (Figure 5.20B). Energy Dispersive X-ray (EDAX) analysis of this complex had elemental composition shown in Figure 5.20C showing the presence of stoichiometric silver (1.41 weight %), thus confirming the presence of silver in the duplex.

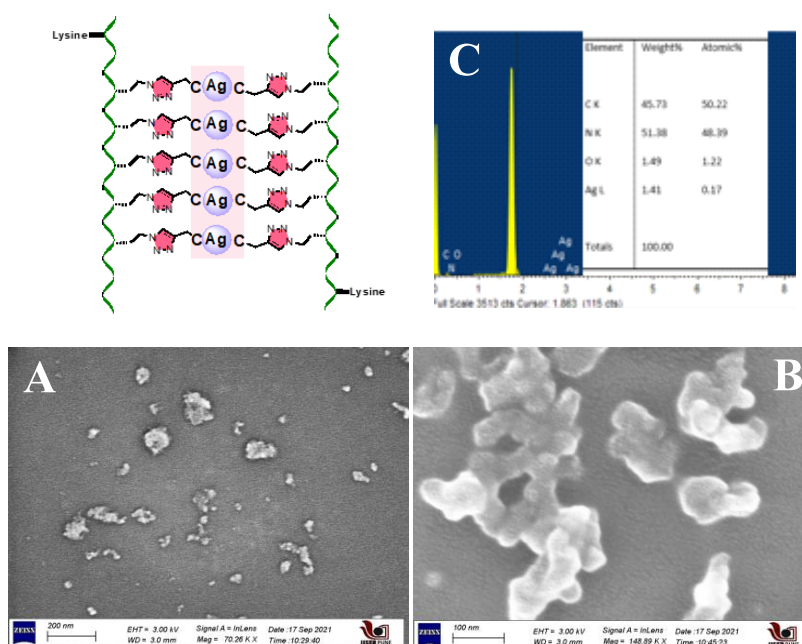


Figure 5.20. FESEM and EDAX of *iso*-PNA **1** complexes

5A.3.6b. DNA **1: *bm*-PNA **1**:Ag⁺: *bm*-PNA **1**:DNA **1** complex.** *bm*-PNA **1** alone showed well defined homo spherical nanoparticles of size 100 nm-200 nm (Figure 5.21A). When hybridized duplex DNA **1**: *bm*-PNA **1** the sample showed an irregular large sticky mass (Figure 5.21B). Upon complexation of *bm*-PNA **1** with Ag⁺, the resulting [*bm*-PNA **1**]₂:Ag⁺ duplex exhibited alignment of spherical nanoparticles into a linear arrangement (Figure 5.21C). Upon hybridization of this silver complex with DNA **1**, the resulting complex [DNA **1**: *bm*-PNA **1**]₂:Ag⁺ transformed into a larger conglomerate mass networked held through a high density fibrous network (Figure 5.21D) of spherical nanoparticles (Figure 5.21D). Thus while PNAs adapt regular shaped nanoparticle morphology, combination with DNA leads to sticky and fibrous materials. The presence of Ag⁺ in the tetra-stranded complex was confirmed by EDAX data (Figures 5.21E and 5.21F) in which the mass proportion of silver roughly corresponded to the molecular composition of the complexes [*bm*-PNA **1**]₂:Ag⁺ and [DNA **1**: *bm*-PNA **1**]₂:Ag⁺.

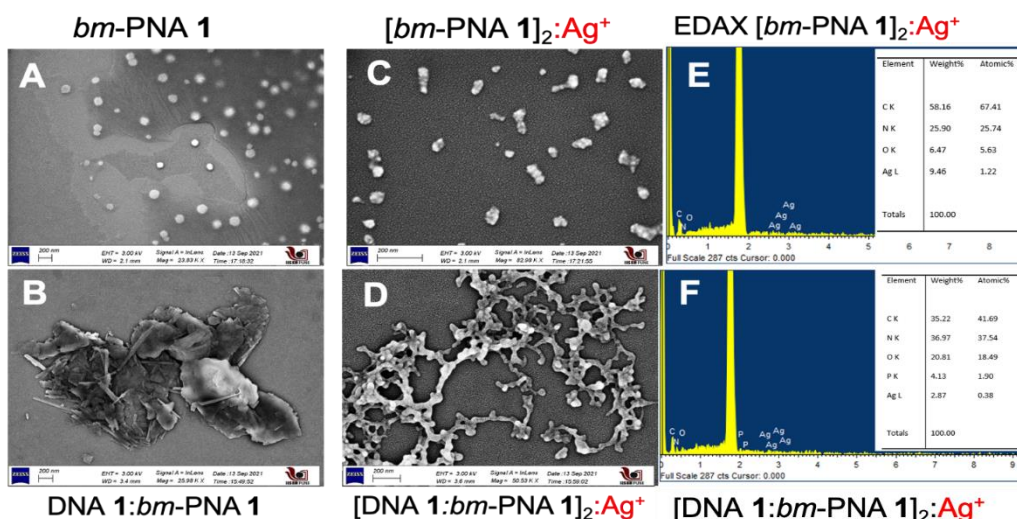


Figure 5.21. FESEM and EDAX of *bm*-PNA 1 complexes

5A.3.6c *bm*-PNA 1:Ag⁺:dTC₅ hybrid complex The duplex *bm*-PNA 1:dTC₅ is spherical particle in nature (Figure 5.22A), which after addition of Ag⁺ resulted in the hybrid complex *bm*-PNA 1:Ag⁺:dTC₅. This showed a highly networked fibrillar structure (Figure 5.22B). Edax analysis confirmed the presence of Ag⁺ (Figure 5.22C) with weight % of 2.87.

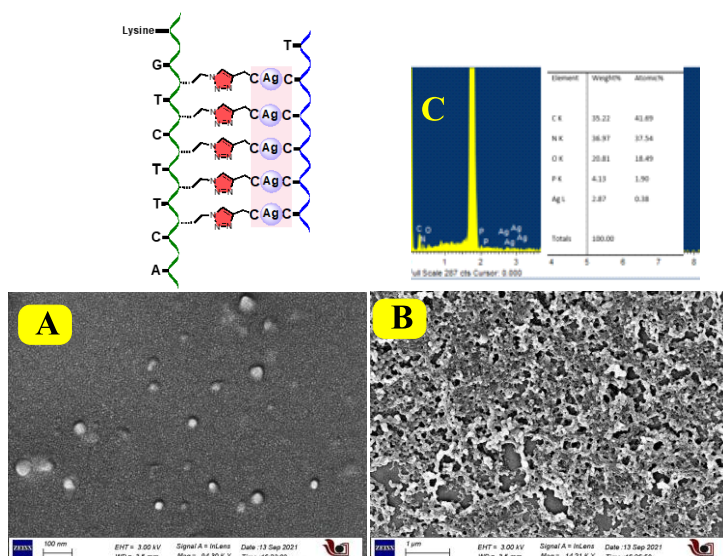


Figure 5.22. FESEM and EDAX of *bm*-PNA 1:Ag⁺:dTC₅ complex

5A.3.6d. DNA 1: *bm*-PNA 1: Ag⁺:dTC₅ complex. In the absence of Ag⁺, the complex DNA 1: *bm*-PNA 1:dTC₅ has rice bead like morphology (Figure 5.23A). After addition of Ag⁺ the resulting hybrid silver complex DNA 1: *bm*-PNA 1:Ag⁺:dTC₅ starts aggregating into fibrous

network structure (Figure 5.23B). EDAX analysis of this complex confirmed presence of Ag^+ (Figure 5.23C) of 1.21 weight %.

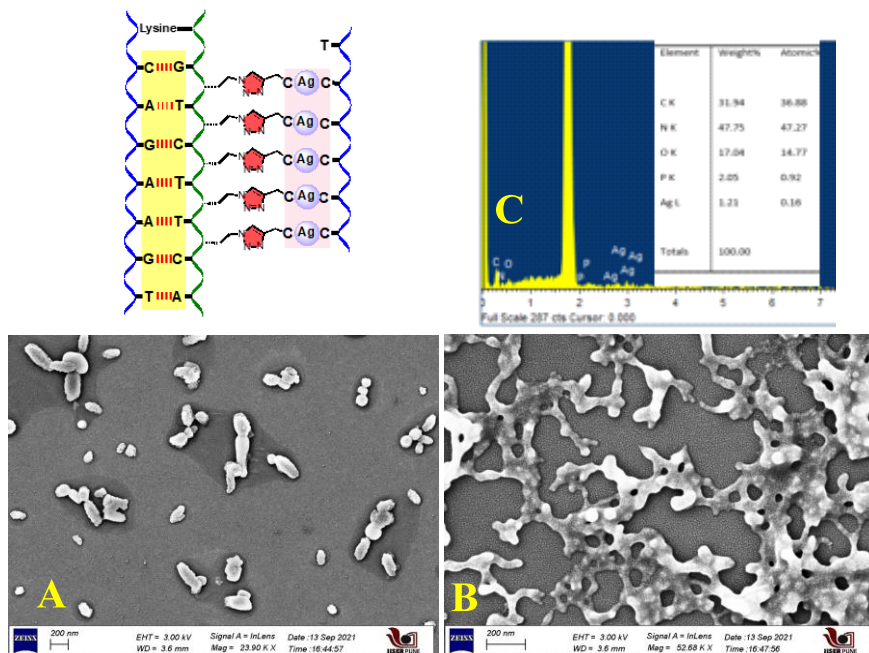


Figure 5.23. FESEM and EDAX of DNA 1: *bm*-PNA 1: Ag^+ :dTC₅ complex

5A.3.6f *bm*-PNA 1:dTC₅ duplex. The oligomer *bm*-PNA 1 showed spherically shaped nanoparticle (Figure 5.24A), while DNA dTC₅ alone gave elongated twisted fibres (Figure 5.24B) of 400 nm – 800 nm in length. Upon hybridization of *bm*-PNA poly C chain on triazole face with complementary DNA dTC₅, the resulting duplex *bm*-PNA 1:dTC₅ showed very nice hollow spheres (Figure 5.24C) of 400 nm diameter with holes of 200 nm diameter. The diameter to central hollowness was generally about 50%.

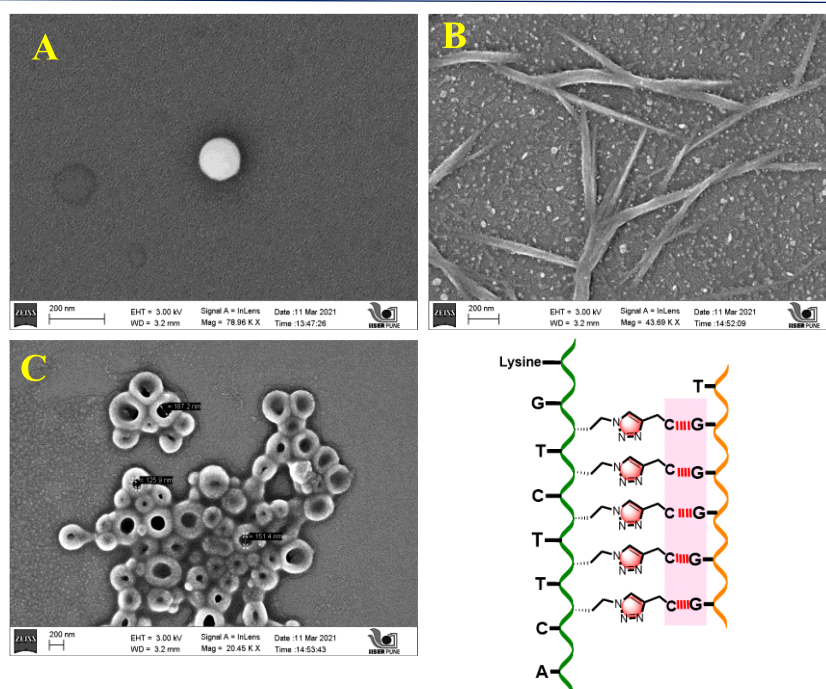


Figure 5.24. FESEM of (A) *bm*-PNA **1** (b) dTG₅ oligomer and (C) *bm*-PNA **1**:dTG₅ duplex

5A.3.6g The double duplex DNA **1: *bm*-PNA **1**:dTG₅.** The WC duplex DNA **1**: *bm*-PNA **1** was irregular sticky mass (Figure 5.26A) while the double duplex complex DNA **1**: *bm*-PNA **1**:dTG₅ was an aggregate of spherical nanoparticles particles (Figure 5.26B).

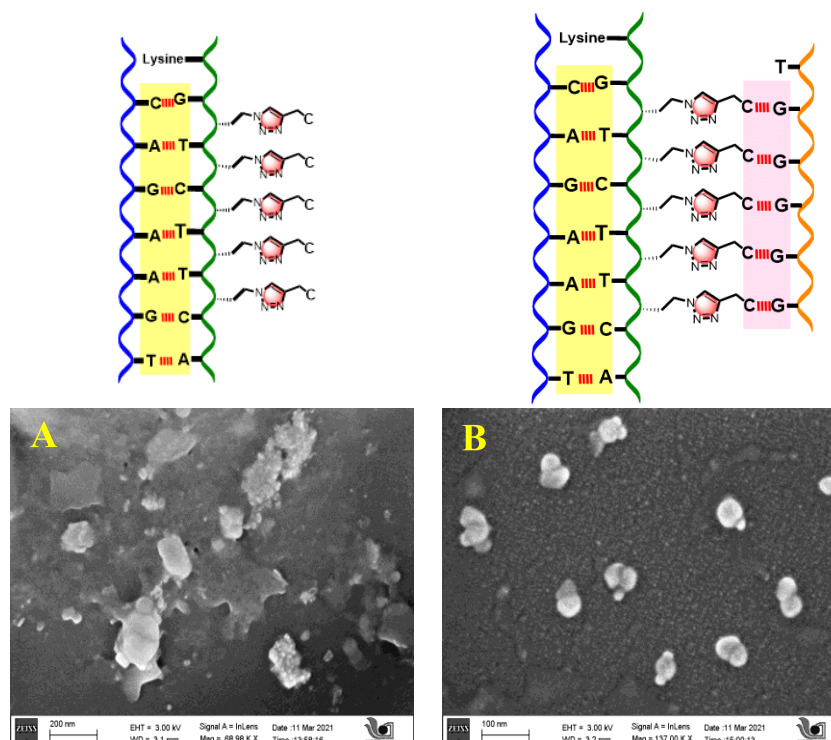


Figure 5.26. FESEM of (A) duplex DNA **1**: *bm*-PNA **1** and (B) double duplex DNA **1**: *bm*-PNA **1**:dTG₅

Section B: Hg^{++} assisted base pairing

5B.3.1 Thermal stability of dTC₅, *aeg*-PNA-C₅ and *iso*-PNA 1 mercury mediated duplexes by UV-T

Similar to silver mediated base pairing, mercury ions also formed stable duplexes in the DNA dTC₅ and *aeg*-PNA-C₅. Both showed sigmoidal transitions due to self-melting with T_m s of 64.1 and 48.8 °C respectively (Figure 5.27a and Figure 5.28a). In the presence of Hg^{++} , both dTC₅ and *aeg*-PNA-C₅ showed increase of their T_m s to 81.2 °C (Figure 5.27b; $\Delta T_m +17.1$ °C) and 51.4 °C (Figure 5.28b; $\Delta T_m +2.6$ °C) respectively. This indicated the formation of duplexes mediated by mercury ion ($\text{C}:\text{Hg}^{++}:\text{C}$). Thus mercury formed $\text{C}-\text{Hg}^{++}-\text{C}$ duplex like silver at neutral pH.

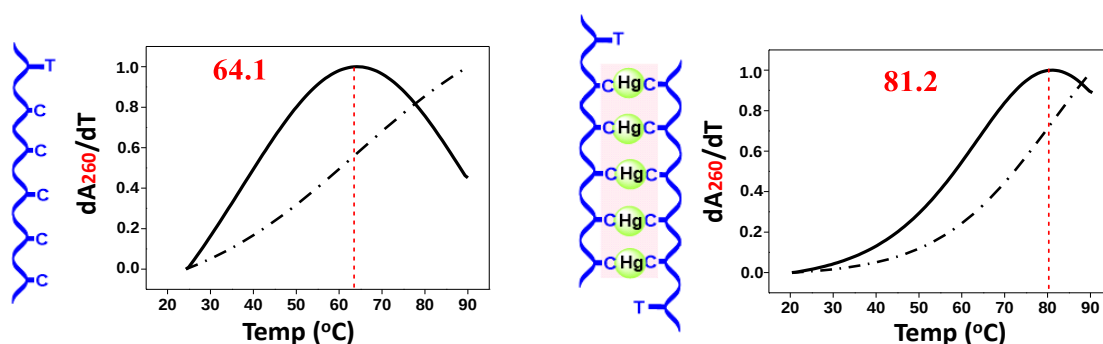


Figure 5.27 UV- T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm of (a) dTC₅ self-melting and (b) dTC₅: Hg^{++} :dTC₅.

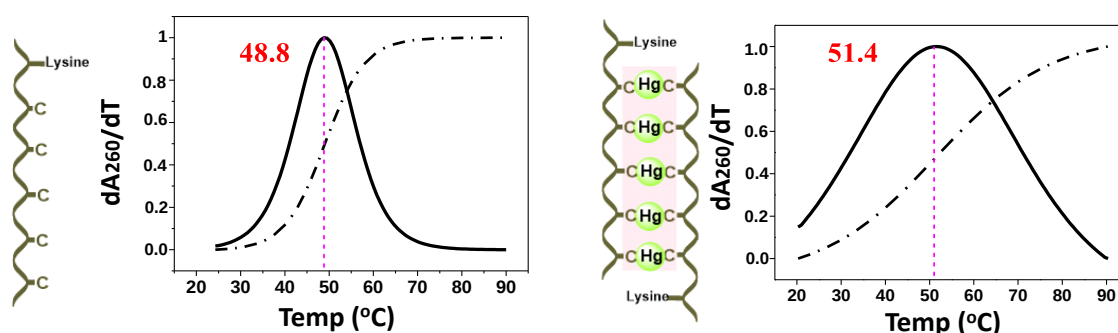


Figure 5.28 UV- T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm (a) *aeg*-PNA-C₅, self-melting (b) *aeg*-PNA-C₅: Hg^{++} :*aeg*-PNA-C₅.

Mercury can base pair in the *iso*-PNA 1 in which poly C is present on a sidechain at C α , linked at N1 by alkyl triazole link (triazole face). The self-melting T_m of *iso*-PNA 1 60 °C was enhanced to 71.1 °C (Figure 5.29; $\Delta T_m +11.1$ °C) in presence of mercury.

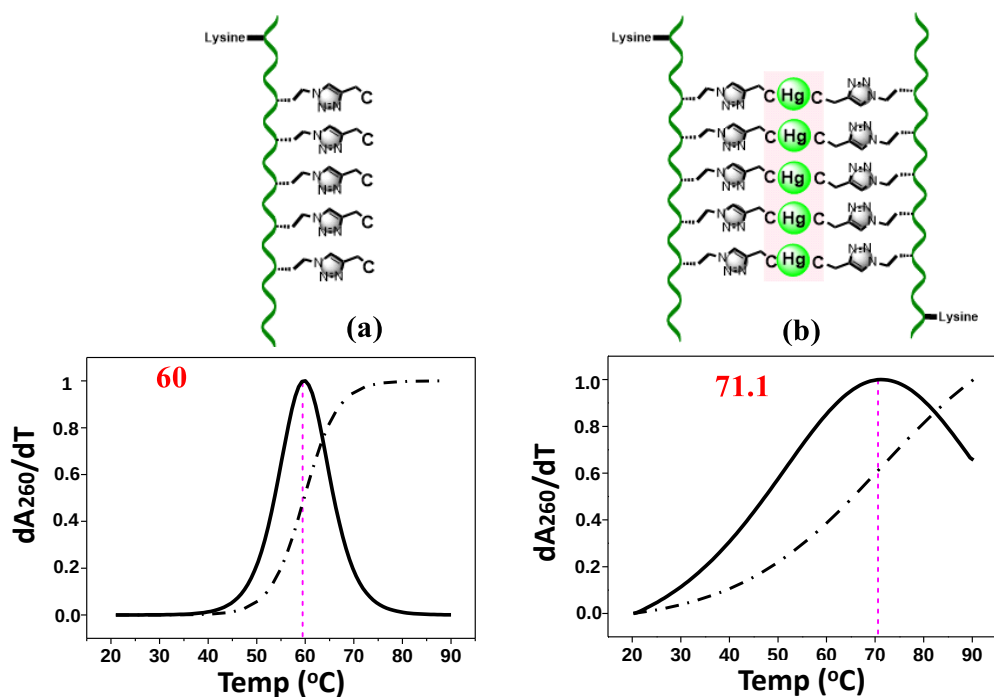


Figure 5.29 UV- T_m plot (dotted curve) and first derivative plot (bold curve) recorded at 260 nm (a) *iso*-PNA **1**, self-melting (b) *iso*-PNA **1**: Hg^{++} :*iso*-PNA **1**.

Table 5.3. T_m (°C) of PNA oligomers and duplexes in the presence of Ag^+ and Hg^{++}

Entry	Oligomer	In buffer	With Ag^+	With Hg^{++}
01	dTC ₅	64.1	80.0	81.2
02	<i>aeg</i> -PNA-C ₅	48.8	71.0	51.4
03	<i>iso</i> -PNA 1	60	73.9	71.1

5B.3.2 UV-T studies of Hg^{++} mediated double duplex of *bm*-PNA **1**

The self-melting of *bm*-PNA **1** was of 30 °C (Figure 5.30a) and when complexed with Hg^{++} with poly C chain from triazole side formed mercury mediated duplex (*bm*-PNA **1**: Hg^{++} :*bm*-PNA **1** Figure 5.30b) analogous to *iso*-PNA **1**, which exhibited single transition with T_m of 65.3 °C. ($\Delta T_m + 35.3$ °C)

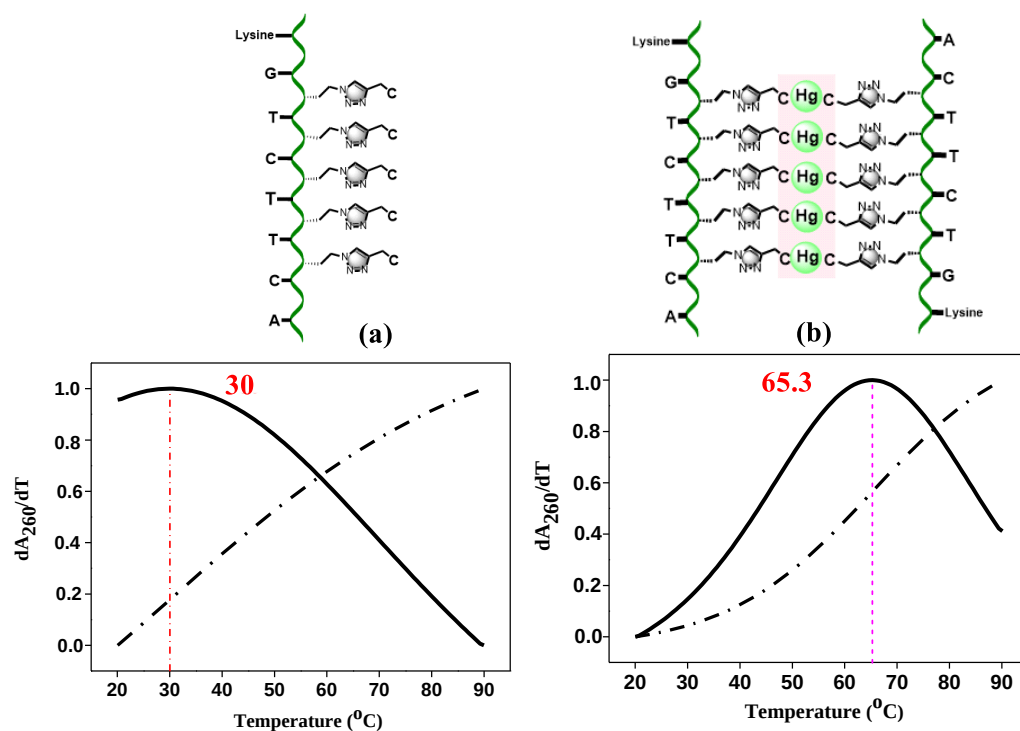


Figure 5.30 (a) UV-T plot of *bm*-PNA **1** (Self melting-30 $^{\circ}\text{C}$) (b) mercury complex $[bm\text{-PNA } 1]_2:\text{Hg}^{++}$

The performed WC duplex on t-amide face i.e DNA **1**: *bm*-PNA **1** (Figure 5.31a) was mixed with desired concentration of mercury solution.. The UV-T plot of the resulting complex interestingly showed double sigmoidal transition with T_m s at 45.5 $^{\circ}\text{C}$ and 75.9 $^{\circ}\text{C}$. This indicates formation of the double duplex of DNA **1**:*bm*-PNA **1**: Hg^{++} :*bm*-PNA **1**:DNA **1** (Figure 5.31b). The two T_m s in principle can arise from melting of these two duplexes, DNA **1**: *bm*-PNA **1** and the $[bm\text{-PNA } 1]_2:\text{Hg}^{++}$ in the complex. However, the T_m of WC duplex is higher by 9.5 $^{\circ}\text{C}$ and mercury mediated duplex by 10.6 $^{\circ}\text{C}$ than that of isolated constituent duplexes of the complex.

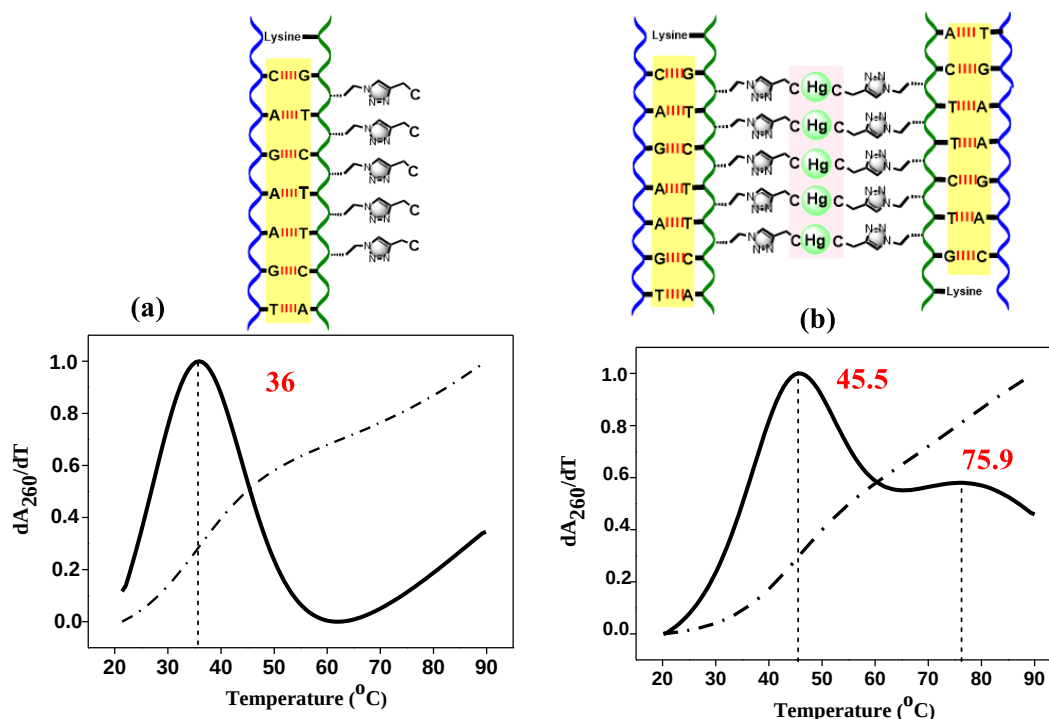


Figure 5.31 UV-T plots of *bm*-PNA **1** with poly C on triazole face (a) duplex DNA **1**: *bm*-PNA **1** (b) mercury complex of double duplex $[\text{DNA 1:bm-PNA 1}]_2:\text{Hg}^{++}$.

Table 5.4. T_m ($^{\circ}\text{C}$) of PNA oligomers and duplexes in the presence of Ag^+ and Hg^{++}

Entry	Oligomer	In buffer	With Ag^+	With Hg^{++}
01	<i>bm</i> -PNA 1	30	68.3	65.3
02	DNA 1 : <i>bm</i> -PNA 1	36	49.3, 81.5	45.5, 75.9

5B.3.3 UV-T studies of Hg^{++} mediated double duplex of *bm*-PNA 2

We have investigated mercury mediated base pairing on amide face also. The *bm*-PNA **2** was constructed by reversing the sequences on two faces, with poly C present on t-amide face and mixed base sequence on triazole side. The self-melting T_m of *bm*-PNA **2** was 52.1 $^{\circ}\text{C}$ (Figure 5.32a). In the presence of mercury ions T_m UV-T plot shifts to 57.7 $^{\circ}\text{C}$ (Figure 5.32b).

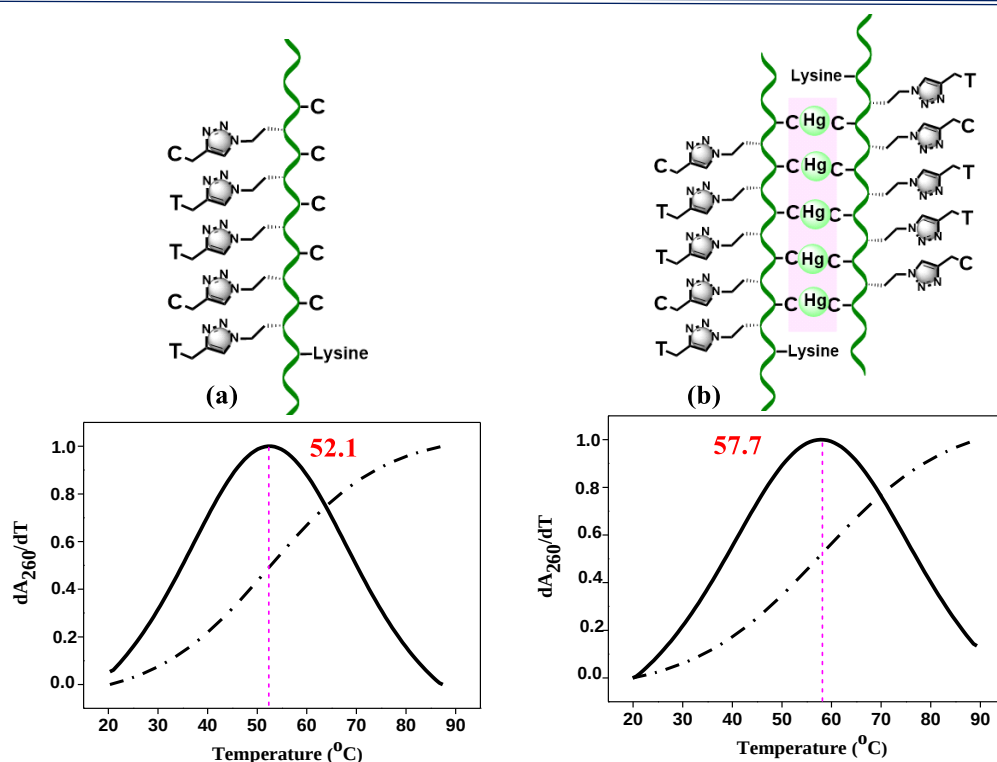


Figure 5.32 UV-T plot of *bm*-PNA **2** (Self melting-52.1 °C) (b) mercury complex [*bm*-PNA **2**]₂:Hg⁺⁺

The triazole face mixed base sequence of *bm*-PNA **2** formed duplex with complementary DNA **2** of T_m 45.6 (Figure 5.33a) The amide face has free cytosines available for base pairing. After mixing with mercury solution resulting complex interestingly showed double sigmoidal transition in temperature dependent UV-T with T_m s at 46.1 °C and 78.6 °C. This indicates formation of the double duplex of DNA **2**: *bm*-PNA **2**:Hg⁺⁺: *bm*-PNA **2**:DNA **2** (Figure 5.33b). The two T_m s in principle can arise from melting of these two duplexes, DNA **2**: *bm*-PNA **2** and the [*bm*-PNA **2**]₂:Hg⁺⁺ in the complex.

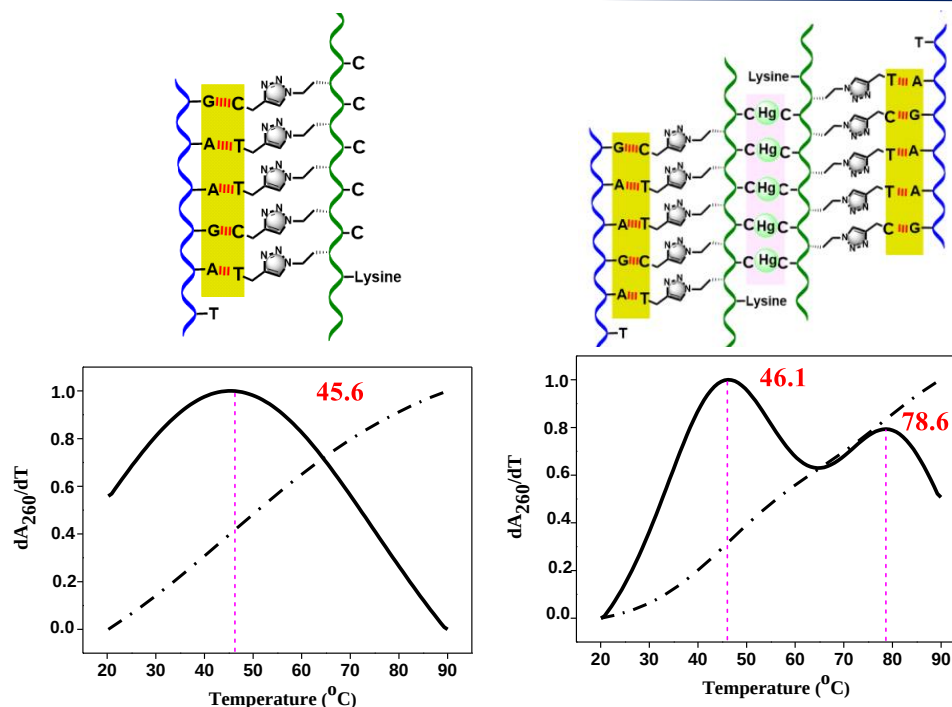


Figure 5.33 UV-T plots of *bm*-PNA 2 with poly C on t-amide face (a) duplex DNA 2: *bm*-PNA 2 (b) mercury complex of duplex [DNA 2: *bm*-PNA 2]₂:Hg⁺⁺.

Table 5.5. T_m (°C) of PNA oligomers and duplexes in the presence of Ag⁺ and Hg⁺⁺

Entry	Oligomer	In buffer	With Ag ⁺	With Hg ⁺⁺
01	<i>bm</i> -PNA 2	52.1	67.1	57.7
02	DNA 2: <i>bm</i> -PNA 2	45.6	38.4, 75.4	46.1, 78.6

5B.3.4 Morphological study by Field Emission Scanning Electron Microscope (FESEM).

Mercury mediated supramolecular assembly formation monitored by FESEM imaging analysis. The different kinds of duplexes, double duplexes and single strand morphological change discussed in this section.

5B.3.4a *iso*-PNA 1:Hg⁺⁺:*iso*-PNA 1 complex: The *iso*-PNA 1 showed spherical nanoparticle aggregates cluster of 200 nm size (Figure 5.34A). After addition of Hg⁺⁺, dimerization of *iso*-PNA leads to *iso*-PNA 1:Hg⁺⁺: *iso*-PNA 1 which showed spherical aggregates in to bulk with increase in size (Figure 5.34B).

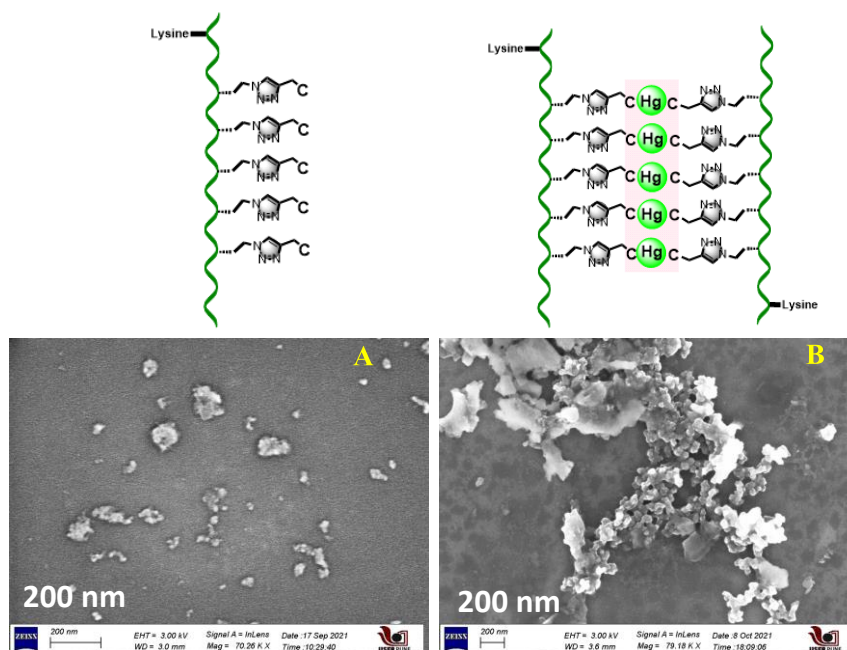


Figure 5.34. FESEM of *iso*-PNA **1** complexes

5B.3.4b. *bm*-PNA **1:Hg⁺⁺: *bm*-PNA **1** complex:** The *bm*-PNA **1** in water showed well defined homo spherical nanoparticles of size 100 nm-200 nm (Figure 5.35A). After addition of Hg⁺⁺ the resulting complex [*bm*-PNA **1**]:Hg⁺⁺ spherical nanoparticles aggregated in to bigger spheres (Figure 5.35B).

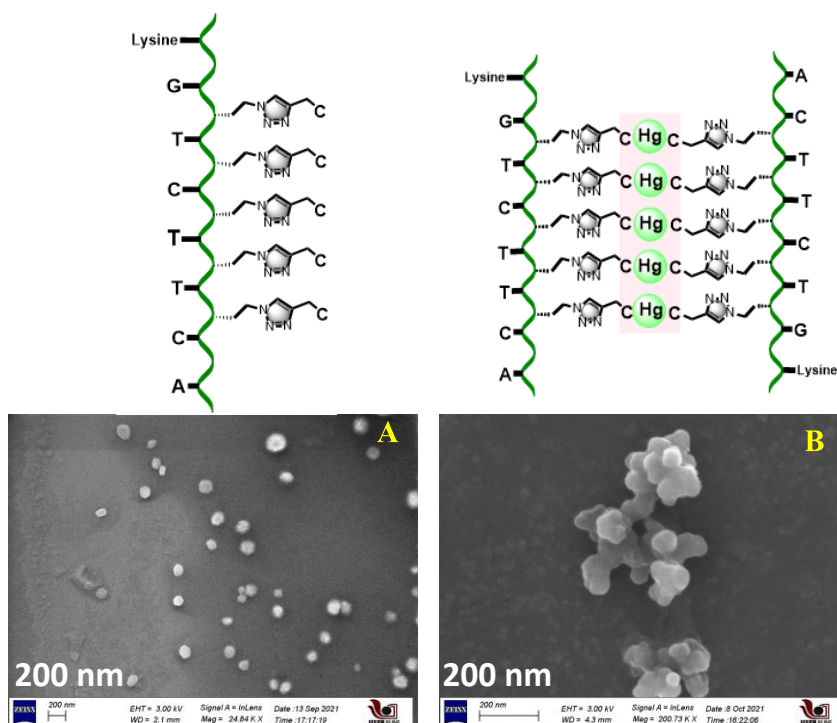


Figure 5.35. FESEM of *bm*-PNA **1** complexes

5B.3.4c. DNA 1: *bm*-PNA 1: Hg^{++} : *bm*-PNA 1:DNA 1 complex: The duplex DNA 1: *bm*-PNA 1 the sample showed an irregular large sticky mass (Figure 5.36A). Upon addition of Hg^{++} to the duplex, the resulting complex $[\text{DNA 1: } *bm*\text{-PNA 1}]_2:\text{Hg}^{++}$ transformed into a uniform particles (Figure 5.36B)

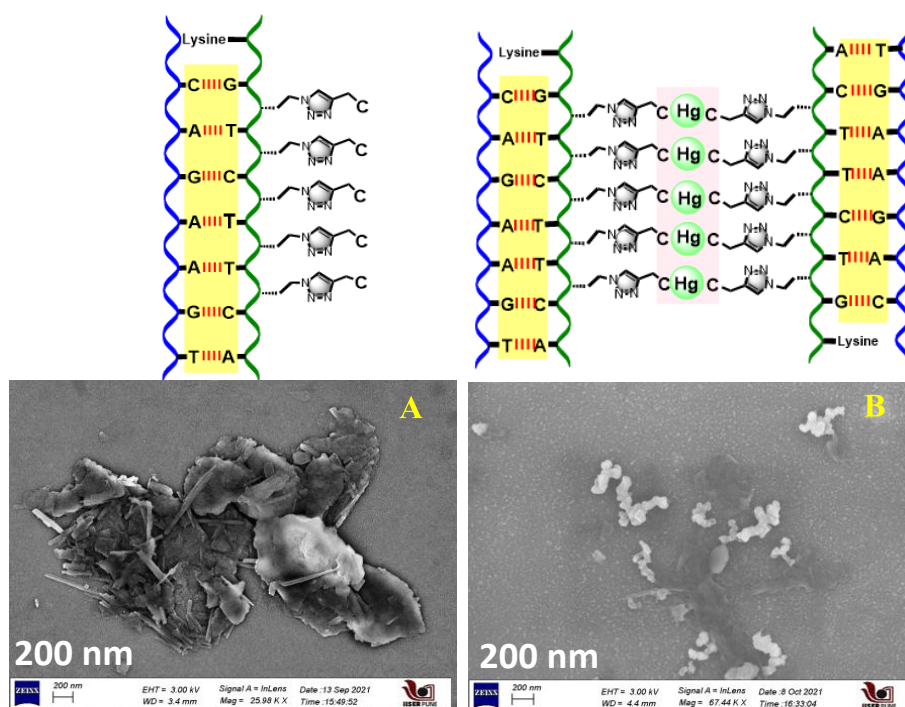


Figure 5.36. FESEM of DNA1: *bm*-PNA 1 complexes

5B.3.4d $\text{dTC}_5:\text{Hg}^{++}:\text{dTC}_5$ and *aeg*-PNA- $\text{C}_5:\text{Hg}^{++}:\text{aeg}-PNA- C_5 complexes$ The standard DNA dTC_5 and *aeg*-PNA- C_5 both can form metal mediated base pairing which are showed in UV-T plots. Morphological change of these duplexes supports the duplex formation in the presence of Hg^{++} . The oligomer dTC_5 in water are aggregated particles in nature (Figure 5.37A) while addition of Hg^{++} transforms to hallow rods morphology (Figure 5.37B). Similarly, *aeg*-PNA- C_5 has non-uniform spherical-particle nature (Figure 5.37C). In the presence of Hg^{++} it results the duplex *aeg*-PNA- $\text{C}_5:\text{Hg}^{++}:\text{aeg}$ -PNA- C_5 which showed aggregated nanoparticles (Figure 5.37D).

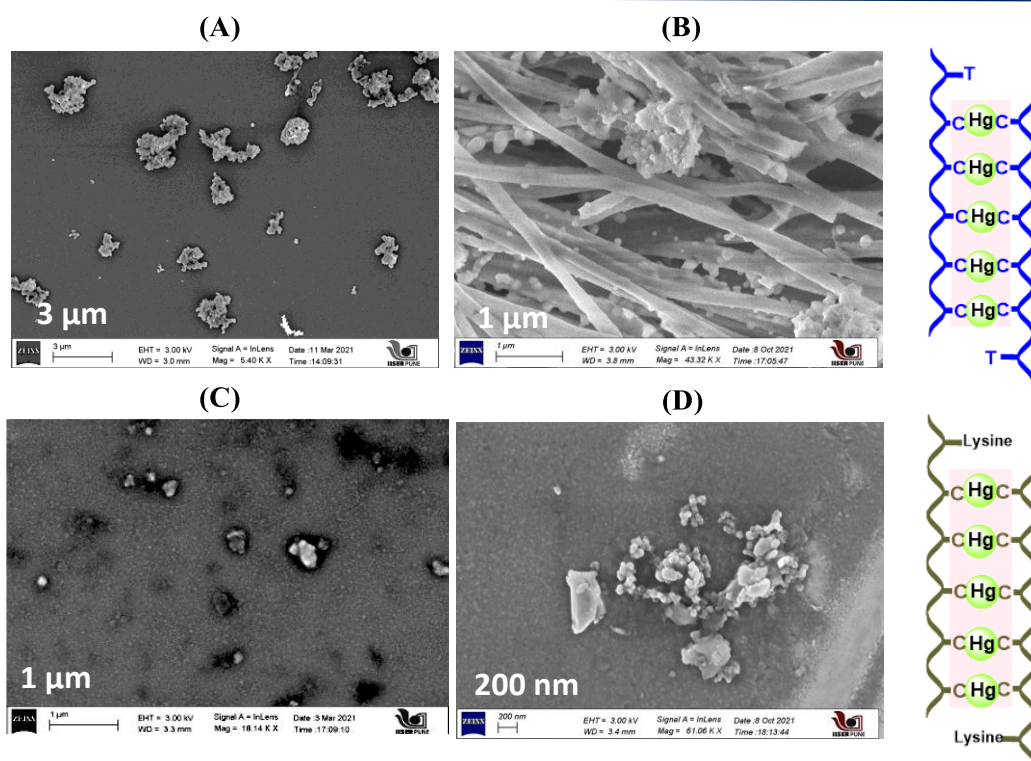


Figure 5.37. FESEM of (A) dTC₅ (B) dTC₅:Hg²⁺:dTC₅ (C) aeg-PNA-C₅ (D) aeg-PNA-C₅:Hg²⁺:aeg-PNA-C₅ complexes

5.4 Conclusions

This chapter establishes the construction of hitherto unknown unique hetero triple duplexes derived bimodal PNAs (*bm*-PNA **1**) with poly C on triazole or t-amide face and mixed sequence on other face with complementary DNA in presence of Ag⁺. Two WC H-bonded *bm*-PNA **1**: DNA duplexes are welded together by Ag⁺ or Hg²⁺ to yield fused triple duplexes [DNA: *bm*-PNA **1**]₂:Ag⁺/Hg²⁺ in a single assemblage. Such a complex exhibits two transitions with higher stabilities compared to single transition in respective isolated duplexes and the two conjoined synergistically stabilise each other. The sequential assembly of *bm*-PNA **1** with DNA **1** and Ag⁺/Hg²⁺ also lead to distinct changes in morphology with increasing order of the assembly.

The strategy of using metal ion to hold two duplexes can be extended to fuse triplexes and quadruplexes. When employed with Cγ- *bm*-PNA **1** having amide spacer,²⁰ it may lead to other interesting morphological assemblies. PNAs on their own and in combination with DNA are attracting interest in material and biomedical sciences through their assembling properties.⁴⁴⁻⁴⁶ Compared to the anionic backbone of DNA/RNA, the neutral polyamide

backbone in PNAs are more versatile in inducing self-assembly into nanofibres^{47, 48} and related nanostructures.^{49, 50} Metal mediated assemblies also have potential for design of molecular conducting wires.^{51, 52} Since T-T base pairs can be stabilised by Hg^{2+} the present idea has scope for creating base specific mixed metal assemblages, even with use of modified bases to enhance specificities and as molecular sensors for detecting metal ions or redox environments.⁵³

5.5 Experimental procedures

Chemicals: DNA oligonucleotides were obtained commercially from Integrated DNA Technologies (IDT). Salts and reagents used in buffer preparation such as MOPS, NaNO_3 , AgNO_3 , HgCl_2 etc. were obtained from Sigma-Aldrich. The pH of the buffer solutions was adjusted using HCl, from Sigma Aldrich.

5.5.1. Temperature-UV Absorbance Measurement

UV-melting experiments were carried out on Varian Cary 300 UV spectrophotometer equipped with a peltier. The samples for T_m measurement were prepared by mixing the calculated amounts of respective oligonucleotides in the stoichiometric ratio (1:1, duplex) in MOPS buffer (10 mM) pH 7 and NaNO_3 (10 mM); to achieve a final strand concentration of 2.5 μM for each strand. The samples were annealed by heating at 90 °C for 10 min. followed by slow cooling to room temperature for 8 to 10 h and then kept at 4°C for 12 h. The samples (500 μL) were transferred to quartz cell and equilibrated at the starting temperature for 5 min. The OD at 260 nm was recorded in steps from 20-90 °C with temperature increment of 1 °C. Each melting experiment was repeated at least twice. The normalized absorbance at 260 nm was plotted as a function of the temperature. The data were fitted by sigmoidal curve, function Boltzmann for one face and bidose response for two face binding, where R square value in range of 0.96 to 0.99. The T_m was determined from the first derivative of normalized absorbance with respect to temperature and is accurate to ± 1.0 °C. The data were processed using Origin Pro 8.5, [The concentration of all oligonucleotides were calculated on the basis of absorbance from the molar extinction coefficients of the corresponding nucleobases i.e. T = 8.8 $\text{cm}^2/\mu\text{mol}$; C = 6.6 $\text{cm}^2/\mu\text{mol}$; G = 11.7 $\text{cm}^2/\mu\text{mol}$ and A = 13.7 $\text{cm}^2/\mu\text{mol}$].

5.5 2. Circular Dichroism

CD spectra were recorded on JASCO J-815 spectropolarimeter connected with a peltier. The concentrations of DNA/*bm*-PNA each strand (10 μM), MOPS buffer (10 mM) pH 7. and NaNO_3 (10 mM); used for each experiment. The CD experiments were performed without

annealing. First after base line correction spectra recorded for only DNA, then added complementary strand and kept for 10 min then recorded spectra. Finally, after addition of silver kept for 10 min and recorded spectra. To record the CD spectra of PNA:DNA duplexes and single stranded PNAs, the temperature of circulating water was kept at 25 °C. The CD spectra were recorded as an accumulation of 3 scans from 320 to 200 nm using 2 mm cell, a resolution of 0.1 nm, band-width of 1 nm, sensitivity standard DIT 1 Sec and a scan speed of 100 nm/min.

5.5.3. Field emission scanning electron microscopy (FESEM) studies

A freshly prepared AgNO₃ solution was added to an aqueous solution of PNA, to get complex with a desired concentration of cytosine and Ag⁺. From this prepared solution, 2 µl aliquot then drop cast on to clean silicon wafer. The solvent from the drop was allowed to evaporate under high vacuum and the films were then left to stand for 8 h under ambient conditions. Before FESEM imaging, the samples were coated with gold prior to imaging with a ZEISS ULTRA PLUS electron microscope.

5.6 References

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Chapter 6

Synthesis and characterization of C5 Uracil substituted PNA monomers, their oligomerization and biophysical study

6.1 Introduction

Peptide nucleic acids (PNAs) are nucleic acid analogues pioneered by Buchardt and Nielsen in 1991,¹ where the negatively charged sugar-phosphate backbone of DNA is replaced with a pseudo peptide backbone (2-aminoethyl-glycine unit), and the nucleobases are attached to the backbone through a conformationally rigid tertiary acetamide linker. Unmodified PNA has some disadvantages such as poor cell permeability, low aqueous solubility and ambiguity in orientation of DNA binding. Several modifications of the PNA backbone have been attempted to overcome these limitations. Apart from a number of acyclic substitutions on *aeg*-backbone of PNA, conformationally constrained cyclic PNA analogues have been designed towards structural preorganization of backbone to address favorable attributes of PNA for selective hybridization with DNA/RNA.² Specifically, chiral PNA monomers have been developed to include modifications at the α -, β - and γ -sites on backbone, with unique synthetic routes and properties of the derived oligomers.³

Replacement of achiral glycine component of *aeg*-backbone with chiral amino acid having L- or D- form leads to C α -chiral PNAs. The oligomers synthesized from D-amino acids (C α -S) show higher affinity towards complementary DNA than the L-form (C α -R)⁴. Gem dimethyl substitution at C α position of PNA monomer not only achiral but also increases binding stability towards cDNA than cRNA⁵.

The major and minor grooves in nucleic acids differ in their polarity. The minor groove of DNA:DNA duplexes is hydrophobic in nature whereas major groove is hydrophilic in nature.⁶ Proteins recognise DNA via specific interactions in the major grooves.^{7, 8} while small DNA binders intercalate to the DNA or interact in the minor grooves^{9, 10} (Figure 1). Ganesh *et*

al.^{6, 11} characterized the polarity of major groove by covalently incorporating micro environment sensitive dansyl fluorphore on the nucleobase of DNA oligomers.

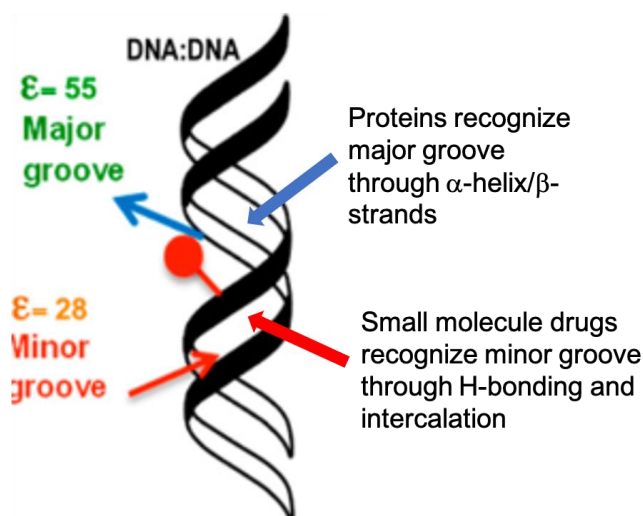


Figure 6.1 Schematic representation of properties of major and minor groove in DNA¹⁰

DNA has wide applicability in biological, medicinal and material chemistry in the form of duplexes with RNA/PNA/LNA. Earlier work from Ganesh et al¹² demonstrated that polarity of major grooves changes with structural diversity of PNA:DNA/RNA/PNA duplex structure.. This was done by employing fluorescent probe strategy to measure the polarity of the major groove of PNA:DNA/RNA/PNA duplexes (Figure 6.2). It was revealed that major groove in PNA:PNA duplexes are more hydrophobic than PNA:DNA, PNA:RNA and DNA:DNA duplexes. This is due to the nonpolar character of PNA backbone.

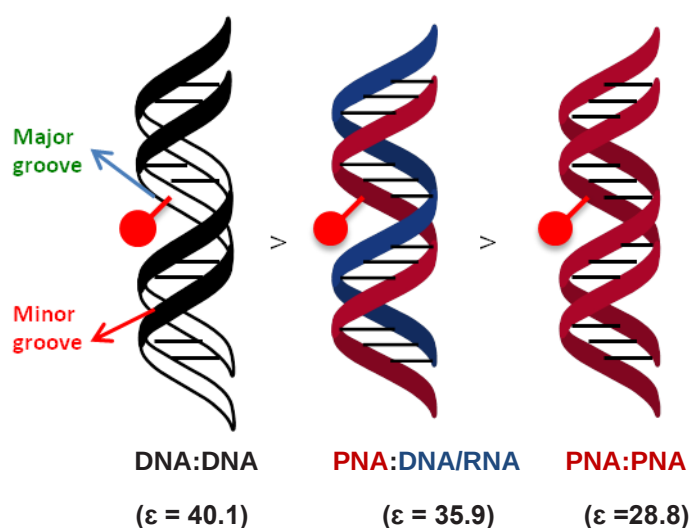


Figure 6.2 Representation of the DNA:DNA, PNA:RNA/DNA and PNA:PNA duplexes, where one strand is modified with fluoroprobe.¹²

In DNA, C5-modified deoxy uridine derivatives have been studied to improve the properties of nucleic acids such as the affinity to their complementary RNAs and the nuclease resistance.¹³⁻²⁶ Similarly, to improve solubility, hybridisation properties, and cell membrane permeability PNA presents a versatile scaffold for chemical modifications on base, backbone and side chain (Figure 6.3)²⁷⁻⁴⁰

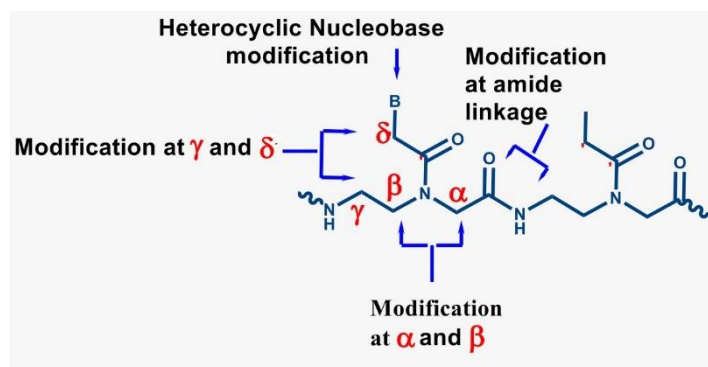


Figure 6.3 A Modifications sites of PNA oligomers

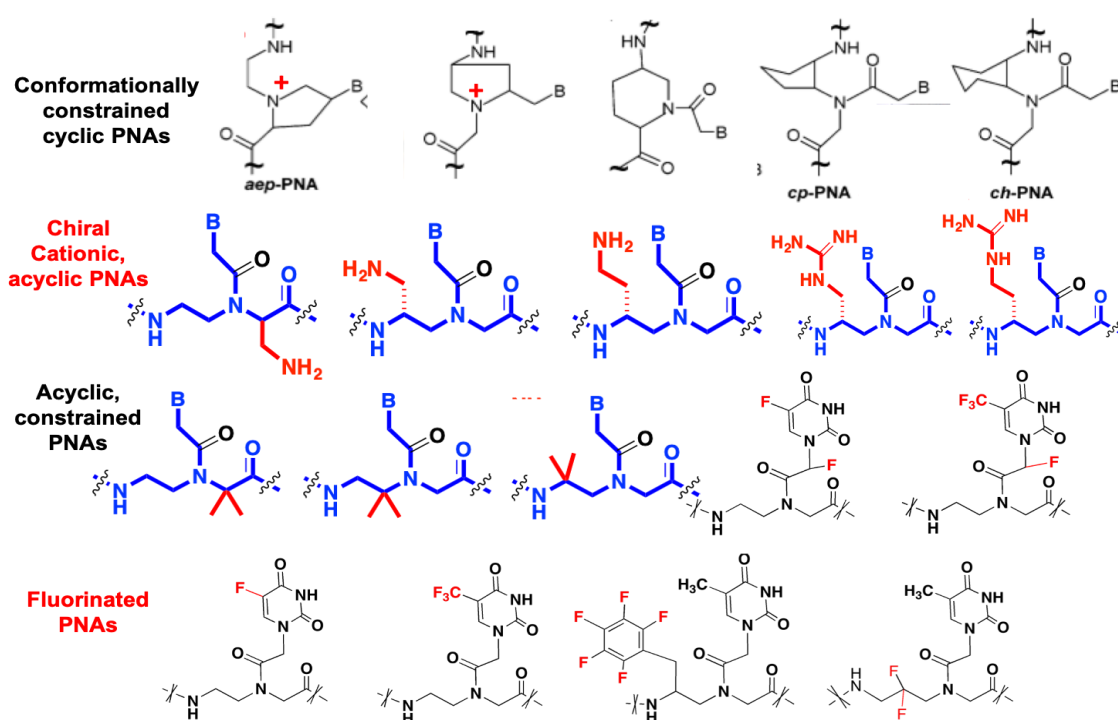


Figure 6.3 B Some representative chemical modifications of PNA at backbone, sidechain and base from Ganesh *et al*^{29, 31, 41-46}

6.2 Rationale

The C5-H/Me in pyrimidines Cytosine (C) and thymine (T) are projected into the major groove of DNA in DNA:DNA duplexes and DNA:RNA duplexes. Hence C5 substitutions are capable of altering the major groove characteristics and thereby the stability of duplexes. While the effect of various C5 substitutions on DNA:DNA duplexes is well known, such is not the case with PNA:DNA duplexes. In this context, the aim of this chapter is to investigate the effect of various, hydrophobic (H), hydrophilic (NH_2) polar hydrophobic (CF_3) substitutions on PNA:DNA/RNA duplex stability. This chapter presents synthesis of C5-modified uracil PNA monomers, their incorporation into PNA oligomers and complementation studies with DNA/RNA and PNA.

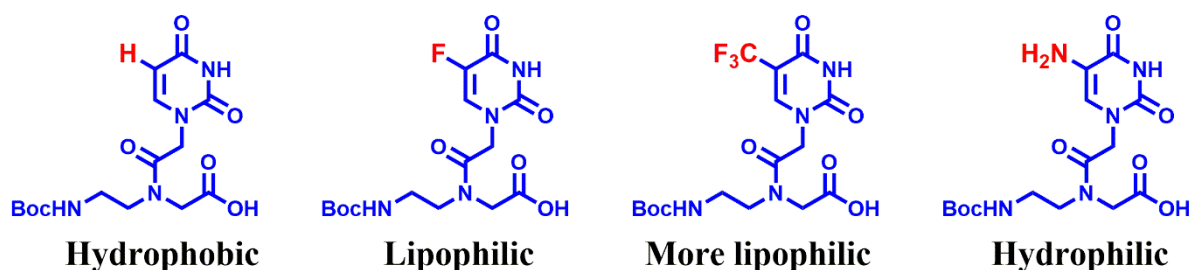


Figure 6.4 Various C5-substituted Uracil PNA monomers

6.3 Aim of the present work

1. Synthesis and characterization of C5-Uracil substituted PNA monomers (Figure 6.5)

- 5-Fluorouracil monomer (1, U^{F})
- 5- CF_3 Uracil monomer (2, U^{CF_3})
- 5- NH_2 Uracil monomer (3, U^{NHBz})

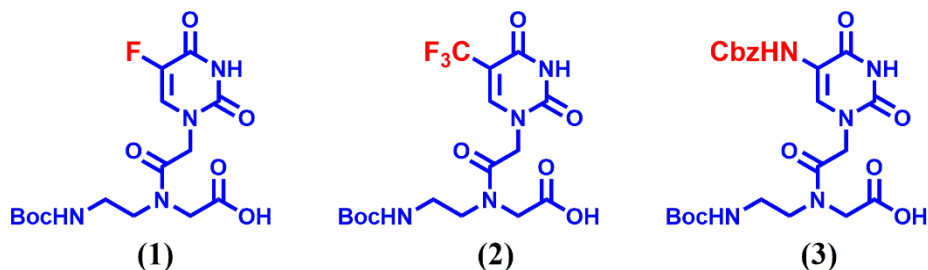


Figure 6.5 Target *aeg*-PNA monomers

2. Incorporation of the above by solid phase synthesis into the following PNA sequences

PNA 1	CGACTTTCAG LysNH ₂
PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂
PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂
PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂
PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂
PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂
PNA 7	CTGAAAGTCG LysNH ₂

3. Study of hybridization of modified PNA oligomers with complementary DNA, RNA and PNA to form PNA:DNA, PNA:RNA and PNA:PNA duplexes respectively

6.4 Results and discussion

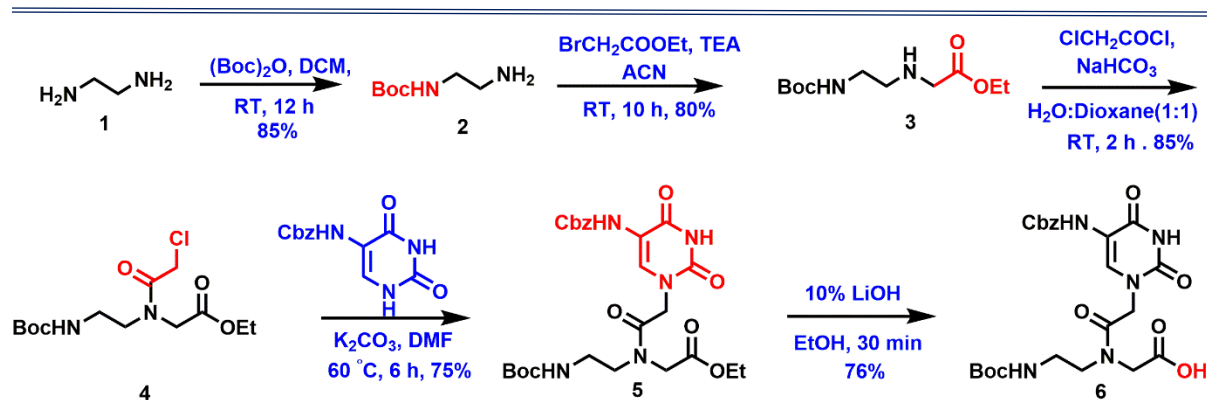
The work in this chapter is presented in two sections: Section A on synthesis of C5-U modified PNA monomers and oligomers and Section B on biophysical studies of the PNA:DNA hybrids.

Section A

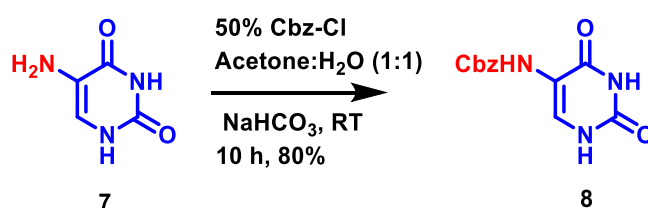
6A.4.1 Synthesis of ethyl *N*1-Boc-aminoethyl-5-NHCbz uracil glycine (5NHCbzU-*aeg*)¹²

Ethylene diamine was treated with t-Boc anhydride in DCM to obtain mono NHBoc protected ethylene diamine (**2**) (Scheme 6.1). This compound was reacted with the ethyl bromoacetate to get N²-alkylated compound **3** which was acylated at N² with chloroacetyl chloride to obtain compound **4**. This compound was coupled with N⁵Cbz protected 5-aminouracil (**8**) which was synthesised from the commercially available 5-aminouracil (**7**) by treatment with 50% benzyl chloroformate (Scheme 6.2). The resulting *aeg* monomer ester **5** was hydrolysed with 10% aq. LiOH gave the desired acid monomer **6**.

Scheme 6.1. Synthesis of protected 5-aminouracil *aeg*-PNA monomer **6**



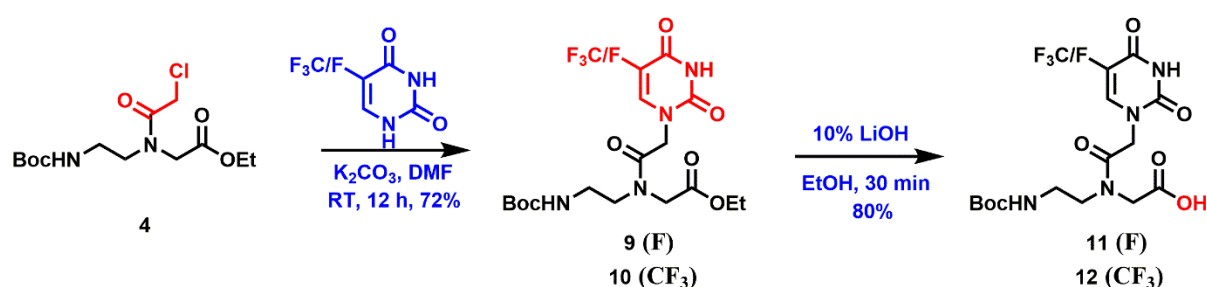
Scheme 6.2. Synthesis of N⁵Cbz protected 5-aminouracil (8)



6A.4.2 Synthesis of N1(Boc)-N2(carbonylmethyl-N¹(5F/5CF₃ uracil) aminoethyl glycine (10, *aeg*-5F/CF₃-U)²⁹

The ethyl [N1(Boc)-N2(chloroacetamide)] aminoethyl glycine **4** was reacted with commercially available 5F/5CF₃ uracil (Scheme 6.3) to give the ester compound **9,10**, which upon hydrolysis with 10% aq. LiOH gave the corresponding *aeg*-PNA acid monomer **11, 12**.

Scheme 6.3. Synthesis of protected 5-fluoro/trifluoromethyluracil *aeg*-PNA monomers (11,12)



All compounds synthesised in Schemes 6.1 to 6.3 were purified by column chromatography and characterised by ¹H, ¹³C, NMR and HRMS analysis.

6A.4.3 Solid phase Synthesis of PNA oligomers

The synthesis of PNA oligomers was carried out using solid phase synthesis protocol using the Boc strategy on MBHA (4-methyl-benzhydryl amine) resin (Figure 6.4). The modified PNA monomers were incorporated at specific positions in *aeg*-PNA sequences. PNA

oligomer synthesis was carried out from the C-terminus to N terminus end using modified monomers with protected amino and carboxylic acid functions maintaining the orthogonality. The monomers were coupled by *in situ* activation with HBTU/HOBt. The synthesis of all oligomers was initiated by first coupling the orthogonally protected NHBoc/NHCbz -L-lysine (S) as the C-terminal spacer amino acid, linked to MBHA resin through an amide bond. The amine loading on the resin was reduced from 0.6 mmol/g to 0.22 mmol/g by partial acylation of amine groups using the calculated amount of acetic anhydride was done to increase the efficiency of coupling by lessening steric crowding with lower loading on the resin.

The deprotection of the resin bound NHBoc protecting group to NH_2 by TFA and the completion of coupling reactions were monitored by Kaiser's test which is the most widely used qualitative test for the presence or absence of free amino group (deprotection/coupling). The *t*-Boc deprotection leads to a positive Kaiser's test in which the resin beads show blue color (Rheumann's purple). After completion of the coupling reaction, the resin beads will become colourless leading to a negative Kaiser's test. Using the standard solid phase synthesis protocol (Figure 6.6), the PNA oligomers of the desired length incorporating PNA monomers were synthesized

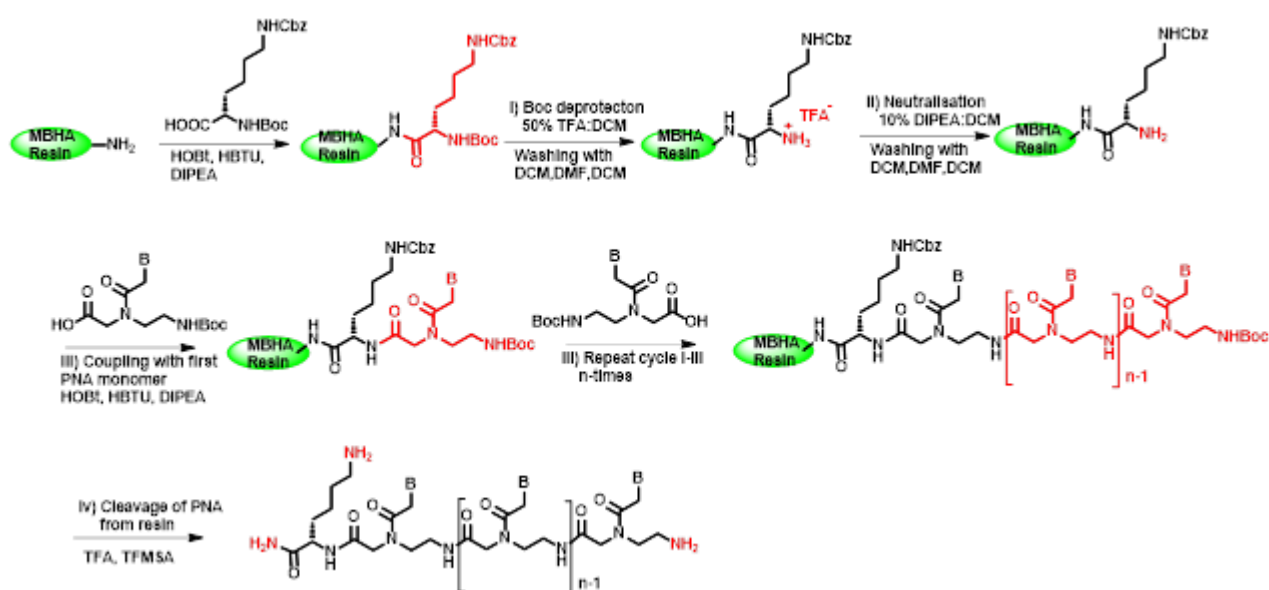


Figure 6.6 Solid phase PNA synthesis protocol by *Boc* strategy; B = Nucleobase such as Adenine(A), Guanine (G), Cytosine (C), Thymine (T), 5(F/CF₃/NH₂) Uracil

6A.4.4 Purification and characterization of PNA oligomers

The synthesized PNA oligomers were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a semi-preparative C18 column using acetonitrile/water gradient elution. The purity of PNA oligomers was checked by reinjecting the sample on

analytical C18 column. The integrity of the synthesized PNA oligomers was confirmed by MALDI-TOF mass spectrometry using 2,5-dihydroxybenzoic acid (DHB) as matrix. The HPLC and MALDI-TOF spectra of the PNA oligomers are shown in Table 6.1. The HPLC-chromatograms and mass spectra are shown in Annexure 1

Table 6.1 Characterisation of synthesised PNA oligomers

Entry	PNA Sequence	RT (min)	Mol. Formula	Calcd. Mass	Obsd. Mass
PNA 1	CGACTTTCAG LysNH ₂	13	C ₁₁₃ H ₁₄₈ N ₅₈ O ₃₂	[M] ⁺ 2830.20	[M] ⁺ 2829.91
PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂	12.6	C ₁₁₂ H ₁₄₇ N ₅₉ O ₃₂	[M] ⁺ 2830.00	[M] ⁺ 2829.94
PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂	11.8	C ₁₁₁ H ₁₄₄ FN ₅₉ O ₃₂	[M-2H] ⁺ 2832.12	[M-2H] ⁺ 2832.78
PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂	12.8	C ₁₁₀ H ₁₄₀ F ₂ N ₅₉ O ₃₂	[M] ⁺ 2838.11	[M] ⁺ 2838.37
PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂	13.3	C ₁₁₂ H ₁₄₄ F ₃ N ₅₉ O ₃₂	[M-H] ⁺ 2883.13	[M-H] ⁺ 2883.10
PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂	13.4	C ₁₁₂ H ₁₄₁ F ₆ N ₅₉ O ₃₂	[M-H] ⁺ 2937.10	[M-H] ⁺ 2837.31
PNA 7	CTGAAAGTCG LysNH ₂	13.1	C ₁₁₄ H ₁₄₆ N ₆₃ O ₃₀	[M-H] ⁺ 2877.18	[M-H] ⁺ 2877.52

Section 6B

Biophysical studies of modified PNA oligomers

In this section temperature dependent UV spectroscopy was used to study the thermal stabilities (T_m) of hybrids derived from modified PNAs towards their complementary antiparallel DNA/RNA/PNA and circular dichroism (CD) studies employed to examine if the modifications alter the secondary duplex structures in the modified PNA:DNA/RNA/PNA hybrids.

6B.4.1 Thermal melting of PNA/DNA, PNA/RNA and PNA/PNA duplexes

The thermal stability of PNA/DNA, PNA/RNA and PNA/PNA duplexes was studied by temperature dependent UV studies⁴⁷ The synthesized PNA decamers (PNA 1-PNA 7 were individually hybridized with complementary antiparallel DNA in 10 mM phosphate buffer (pH 7.0) containing sodium chloride (10 mM). These samples were then annealed by heating at 90 °C for 3 min (in case of PNA:PNA duplexes samples were heated for 10 min) followed by slow cooling to room temperature and then refrigerated at 4 °C for at least 4 to 5 h to constitute duplexes. The thermal stability (T_m) of these duplexes was examined by temperature dependent UV-absorbance changes at 260 nm. The UV-T plots gave sigmoidal transitions and T_m was obtained from the midpoint of the first derivative curve.

6B.4.2 Thermal stability (UV- T_m) of PNA:DNA duplexes

The T_m s derived from the first derivative plots of various PNA:DNA hybrids are shown in Figure 6.7 and compiled in Table 6.2. The unmodified duplex PNA **1**:DNA has T_m value of 47.5 °C. (Figure 6.7)

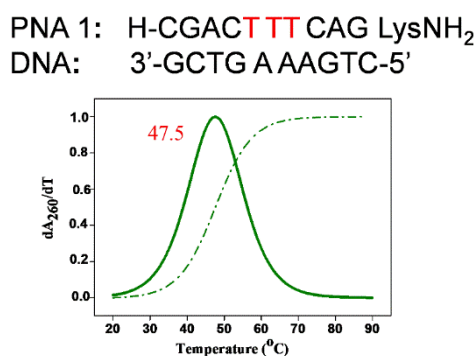


Figure 6.7 UV- T_m profile of unmodified PNA:DNA duplex

When the central thymine (T) is replaced by 5-amino uracil as in PNA 2, the T_m of duplex with DNA increased by 5.6 °C. The PNA 3:DNA duplex has a 5-fluorouracil adjacent to the 5-aminouracil and the thermal stability increased by 6.2 °C compared to the unmodified PNA:DNA duplex. When the central 5-aminouracil is flanked by two 5-F uracil on both sides (PNA 4), the stability of the derived PNA 4:DNA duplex increased drastically i.e. 26.8 °C. 5-CF₃ group adjacent to 5-aminouracil increased stability of corresponding PNA 5:DNA duplex by 17.8 °C compared to the unmodified PNA:DNA duplex. Double modifications 5-CF₃ groups on both sides of 5-aminouracil increased stability only by 5.7 °C.

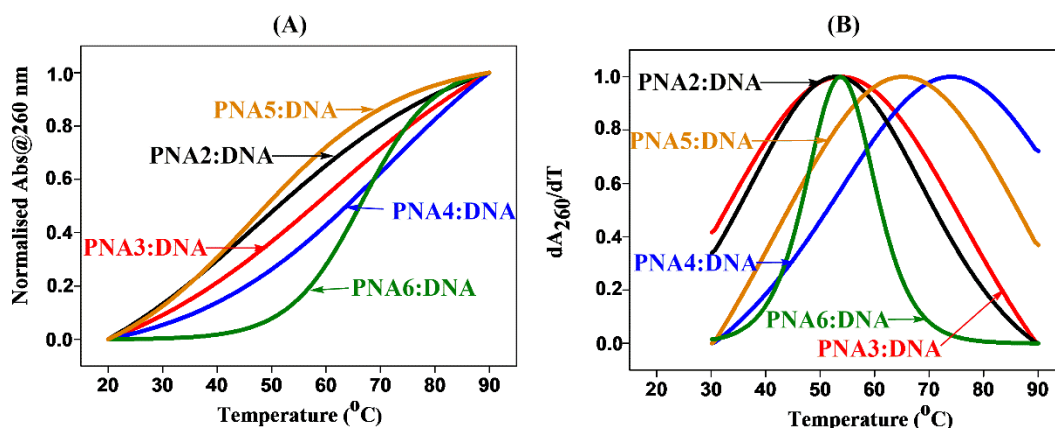


Figure 6.8. Melting curves of PNA:DNA duplexes A) Sigmoidal curve and B) Derivative curve.

Table 6.2 UV- T_m of PNA:DNA duplexes

Sr.No.	PNA code	PNA Sequence	T_m	ΔT_m
01	PNA 1	CGACTTTCAG LysNH ₂	47.5	---
02	PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂	53.1	5.6
03	PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂	53.7	6.2
04	PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂	74.3	26.8
05	PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂	65.3	17.8
06	PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂	53.2	5.7

6B.4.3 Thermal stability (UV- T_m) of PNA:RNA duplexes

The melting temperature curves of PNA:RNA duplexes are shown in Figure 6.10 and T_m values are compiled in in Table 5. The unmodified duplex PNA:RNA has T_m value of 61.1 °C.(Figure 6.9), which is higher by 14.4 oC compared to the corresponding PNA:DNA duplex.

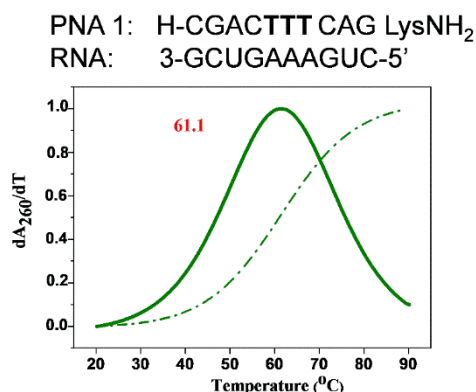


Figure 6.9 UV-T_m profile of unmodified PNA:RNA duplex

The PNA 2:RNA duplex having central T replaced by U^{NH₂} is slightly destabilised by 0.8 °C. The duplex stability of PNA 3:RNA having an adjacent U^F to U^{NH₂} was decreased by 29.1 °C. Double modification of U^F on either side of central U^{NH₂} in the PNA 4:RNA duplex lead to destabilisation than unmodified duplex by 15.1 °C. Single and double U^{CF₃} modifications in PNA 5 and PNA 6 respectively decreased stability of their RNA duplexes by 15.1 °C. and 34 °C respectively (Figure 6.9 and Table 6.3).

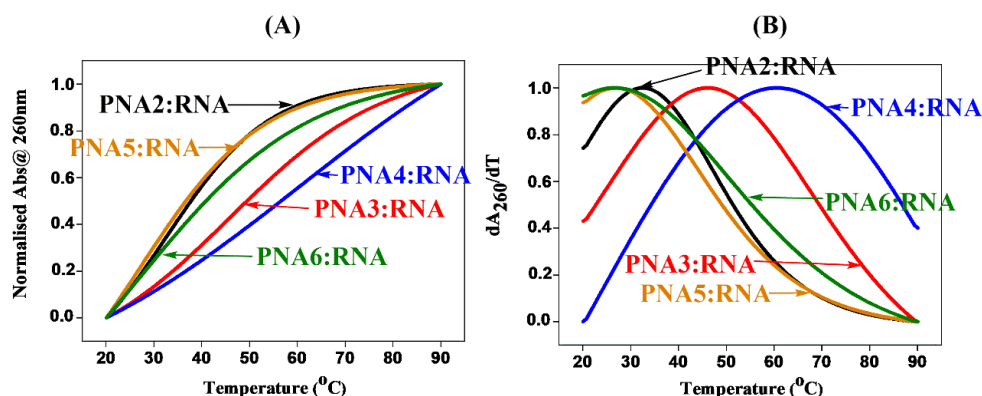


Figure 6.10. Melting curves of PNA:RNA duplexes A) Sigmoidal curve and B) Derivative curve.

Table 6.3 UV-T_m of PNA:RNA duplexes

Sr.No.	PNA code	PNA Sequence	T _m	ΔT _m
01	PNA 1	CGACTTTTCAG LysNH ₂	61.1	---
02	PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂	60.3	-0.8
03	PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂	32	-29.1
04	PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂	46	-15.1
05	PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂	27.1	-34
06	PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂	26.4	-34.7

6B.4.4 Thermal stability (UV- T_m) of PNA:DNA duplexes PNA:PNA duplexes

PNA:PNA duplexes have much higher stability than PNA:DNA and PNA:RNA duplexes. The temperature dependent absorbance curves for the modified PNA duplexes with complementary unmodified PNA duplexes are shown in Figure 6.2. These also showed sigmoidal transition and the T_m derived from the midpoint of first derivative plots given in Table 6.4. The unmodified duplex PNA:PNA has T_m value of 72.9 °C (Figure 6.11) and found to be more stable as compared to PNA:DNA (47.5 °C) and PNA:RNA (61.1 °C) duplexes.

PNA 1: H-CGACTTTCAG LysNH₂
PNA NH₂LysGCTGA AAGTC

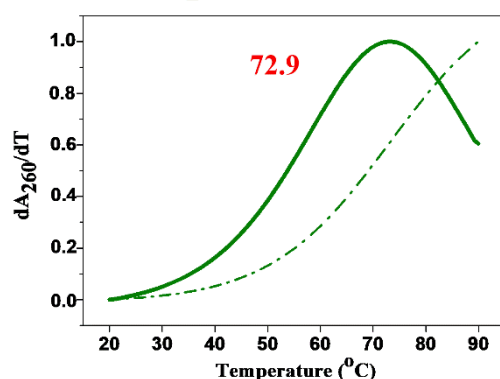


Figure 6.11 UV- T_m profile of unmodified PNA:PNA duplex

The PNA 2:PNA duplex in which one thymine replaced by U^{NH₂} was destabilised by 14 °C and PNA 3:PNA duplex with U^F substitution showed decreased stability by 16.8 °C (Figure 6.11). Double modification of U^F next to U^{NH₂} in PNA 4:PNA duplex exhibited slightly stabilisation by 3.7 °C compared to unmodified duplex. U^{CF₃} modifications in both PNA 5:PNA and PNA 6:PNA duplexes decreased stability by 17.1 and 13.1 °C, respectively.

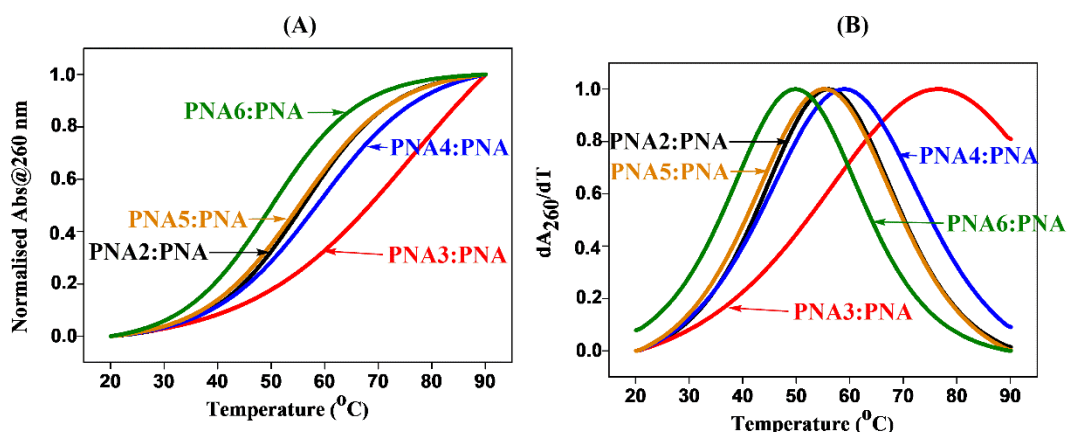


Figure 6.12. Melting curves of PNA:PNA duplexes A) Sigmoidal curve and B) Derivative curve

Table 6.4 UV T_m of PNA:PNA duplexes

Sr.No.	PNA code	PNA Sequence	T_m	ΔT_m
01	PNA 1	CGACTTTCAG LysNH ₂	72.9	---
02	PNA 2	CGACTU ^{NH₂} TCAG LysNH ₂	58.9	-14
03	PNA 3	CGACTU ^{NH₂} U ^F CAG LysNH ₂	56.1	-16.8
04	PNA 4	CGACU ^F U ^{NH₂} U ^F CAG LysNH ₂	76.6	3.7
05	PNA 5	CGACTU ^{NH₂} U ^{CF₃} CAG LysNH ₂	55.8	-17.1
06	PNA 6	CGACU ^{CF₃} U ^{NH₂} U ^{CF₃} CAG LysNH ₂	49.8	-13.1

6B.5 Circular dichroism spectra of C5-U substituted PNA duplexes

In order to examine the effect of incorporation of C5-substituted uracil in place of T in PNA on the conformation of derived PNA duplexes, the CD spectra were recorded. PNA being achiral, do not show any significant CD spectra. However, upon hybridization with chiral DNA / RNA, they exhibit characteristic CD spectra induced by the chiral hybridization component⁴⁸

6B.5.1 Circular dichroism (CD) of PNA:DNA duplexes

The formation of PNA:DNA duplexes results in two positive bands with maxima one in the region of 260 to 280 nm and another band between 210 to 230 nm. PNA:DNA duplexes also showed slightly negative maxima at 240 to 250 nm with cross-over points at approximately 230 and 260 nm. (Figure 6.13). The incorporation of T by C5-substituted uracil (U^F/U^{CF₃}/U^{NH₂}) did not have much effect on the conformation of PNA:DNA duplexes.

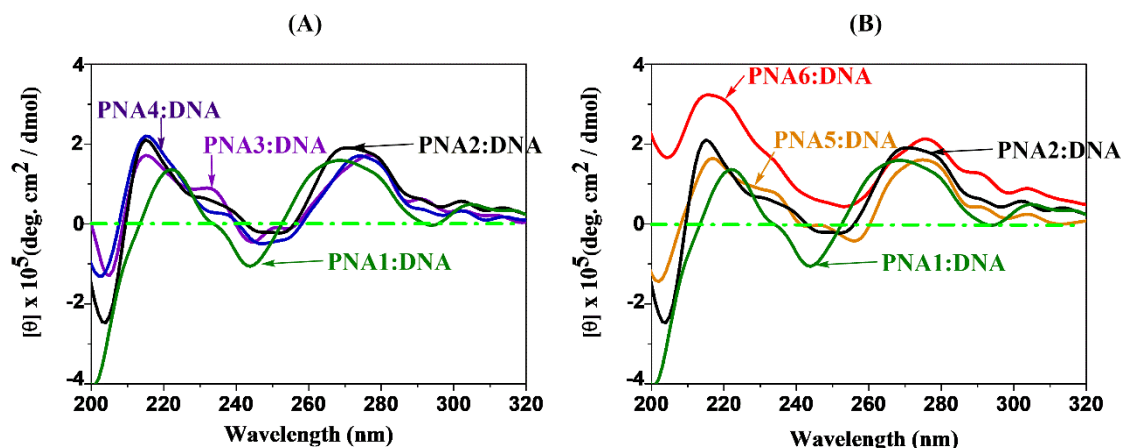


Figure 6.13. CD spectra of PNA:DNA duplexes A) 5-F uracil and B) 5-CF₃ uracil modifications

6B.5.2 Circular dichroism (CD) of PNA:RNA duplexes

The CD profiles of PNA:RNA duplexes showed two positive bands with maxima in the region 255 to 280 nm (with higher intensity) and the second positive band in the region 215 to 230 nm with relatively lesser intensity. PNA:RNA duplexes also exhibited a negative band at 240 nm. (Figure 6.14). Again, incorporation of T by C5-substituted uracil (U^F/U^{CF₃}/U^{NH₂}) did not have much effect on the conformation of PNA:RNA duplexes

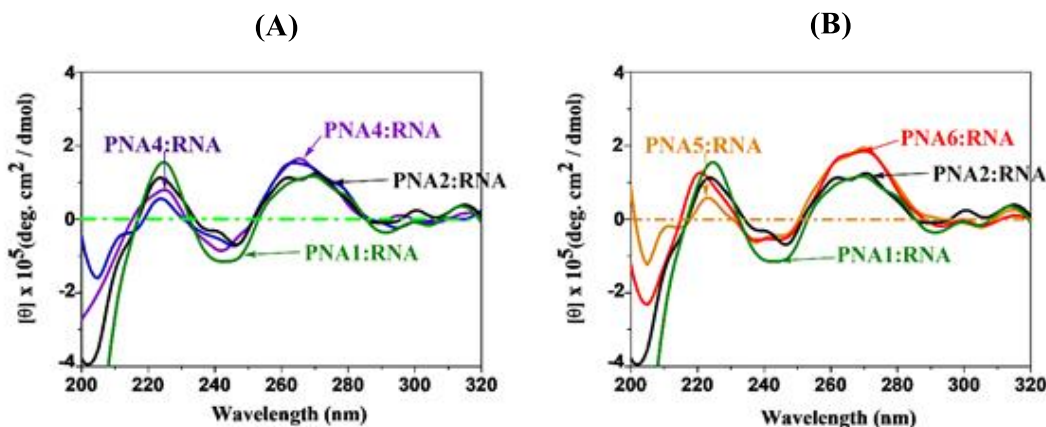


Figure 6.14. CD spectra of PNA:RNA duplexes A) 5-F uracil and B) 5-CF₃ uracil modifications

6B.5.3 Circular dichroism (CD) of PNA:PNA duplexes

CD spectroscopic measurements of PNA:PNA duplexes were done at ambient temperature. The CD bands in the spectra of PNA:PNA duplexes are weak due to absence of chirality in PNA backbone. The unmodified PNA-1:PNA duplexes exhibited the positive band at 242 nm and negative band at 258 nm along with one maxima at 285 nm indicating the formation of double helical structure (Figure 6.15).

From CD spectrums it was observed that 5-F modifications slightly alters the conformation which showed two small bands at 242 nm, 258 nm and at 285 nm. While 5-CF₃ modification did not change the conformation of PNA:PNA duplexes.

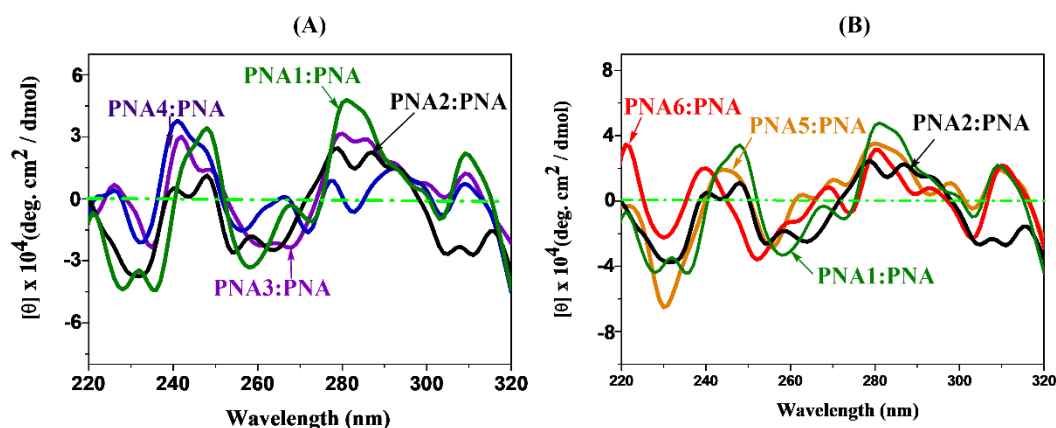


Figure 6.15. CD spectra of PNA:PNA duplexes A) 5-F uracil and B) 5-CF₃ uracil modifications

6B.6 Discussion

6B.6.1 Electronic and local microenvironmental effects of C5 substituents

The substituents at C5 of U effect various electronic effects that influence the strength of the U:A base pair and local effects that influences the microenvironmental polarity effects in the major groove of duplexes. Compared to uracil with H at C5, the substitution by F, CF₃ and NH₂ groups have different inductive effects in terms of electron donation or electron withdrawal which may affect the stability of H-bonding base pairs.(Figure 6.16) F and CF₃ have more electronegative character compared to H, while NH₂ is more electron donating. Generally, electron donation (NH₂) at C5 increases electron density on O⁴ and but slightly decreases the positive character of N3-H. The former increases the H-bond strength, while the latter slightly decreases the H-bond strength,. In case of electronegative, electron withdrawing groups at C5, the electron density on O4 is decreased (lower H-bonding strength), while N3-H assumes more positive character, leading to increase of H-bond strength (Figure 6.16A) However, the co-operative electron delocalization in the cyclic H-bond of base pairs compensates the two effects which determines the overall stabilization / destabilization effects on base pairs (Figure 6.16B) In addition, the C5 substituted groups are projected in to the major groove and influence the polarity of the grooves by their hydrophobic / hydrophilic nature, thereby contributing to the stability of duplexes. The 5-NH₂ group is hydrophilic in nature compared to the 5-CH₃ group of thymine or the 5-H of uracil and thus increase the local dielectric constant in major groove. In contrast, the F in U^F and U^{CF₃} impart polar

hydrophobicity, thus influencing the major groove polarity. Finally, the local environment of the major groove is also different for PNA:DNA, PNA:RNA and PNA:PNA duplexes and hence C5-substituents may impact the stability of various duplexes in different ways.

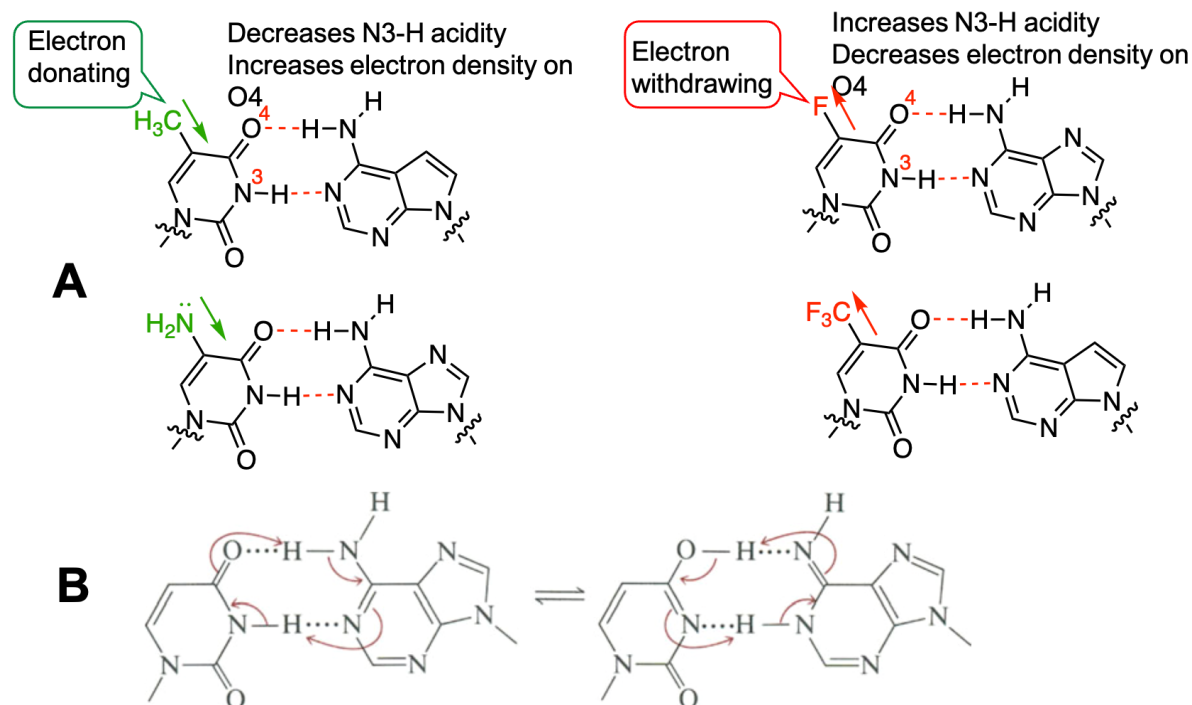


Figure 6.16. (A) Electronic effects of C5 substitutions on strength of H-bonded base pairs (B) Co-operative effects in cyclic H-bonded base pairs⁴⁹

6B.6.2 Comparative effects of C5 substitution on duplex stability

The overall stabilisation order of modified PNA:DNA/RNA/PNA duplexes depended both on the substitution as well as the type of duplexes and the number of substitutions. These are shown in the Figure 6.17 and shown in Table 6.5. Generally, the observed effects of stabilization were not additive in the sense that increasing substitution lead to increasing stabilization or destabilization for any of the duplexes. For PNA:DNA duplexes, U^{NH_2} , U^{F} and U^{CF_3} increased the T_m compared to unsubstituted PNA (entry 1), with maximum stabilization for PNA **4** duplex (entry 4) having $\text{U}^{\text{F}}\text{U}^{\text{NH}_2}\text{U}^{\text{F}}$ substitution, which all stabilise the base pair as expected from electronic effects. The 5- CF_3 substitution stabilised the PNA:DNA duplex compared to corresponding control duplex (entry 2 vs entry 5) but not additive (entry 6). It appears that both the electronic effects of C5-substitutions and groove polarity in conformation adapted by PNA:DNA are favourable for stabilising the duplexes.

In case of PNA:RNA duplexes, all substitutions destabilised the duplexes compared to unsubstituted duplex (entry 1), maximum destabilization being with U^{CF_3} substitutions (entry

5 and entry 6), followed by U^F and U^{NH_2} . Since the electronic effects on base pairing offered by C5 substitutions are similar to that in PNA:DNA duplexes, the destabilization of PNA:RNA duplexes are largely due to incompatibility of substitutions to both the PNA:RNA conformation and the groove microenvironment, which is slightly more polar compared to PNA:DNA duplexes.

In case of PNA:PNA duplexes, all C5-substitutions generally caused destabilization with respect to unsubstituted PNA:PNA duplex (entry 1), except for PNA **4**:PNA duplex with the $U^F U^{NH_2} U^F$ arrangement of substitution (entry 4), which exhibited the maximum T_m . The destabilization decreased in the order $U^{CF_3} > U^{NH_2} \sim U^F$ suggesting that the compatibility of the polarity of groups and the microenvironment of the groove (also determined by the duplex conformation) play a major role in stabilization of duplexes. PNA:PNA duplexes are most hydrophobic and hence F which stabilises the $U^F:A$ base pair by both electronic effects and hydrophobicity seems to be most effective in combination with U^{NH_2} , which is also base pair stabilising in terms of electronic effects.

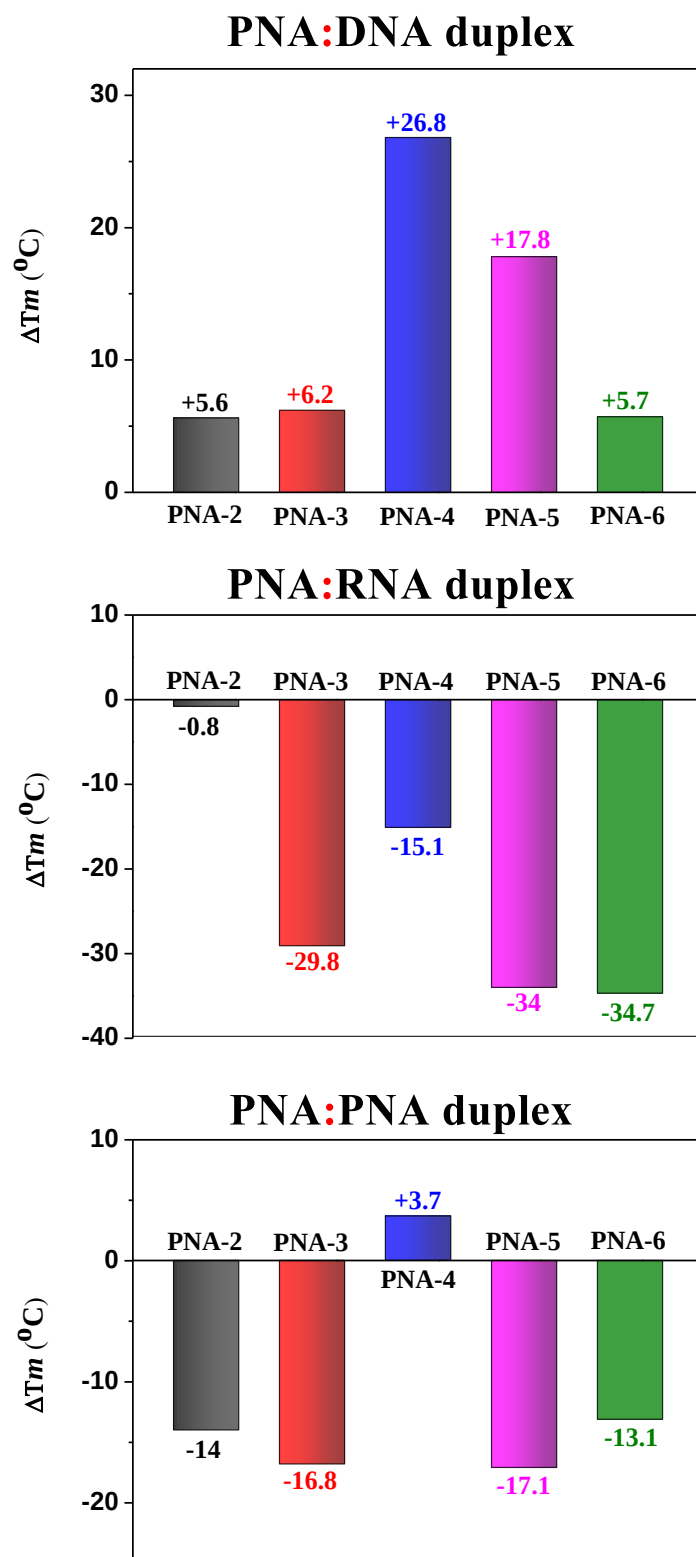


Figure 6.17. Comparison of thermal stabilities of various duplexes.

Table 6.5 Comparative UV T_m of PNA:DNA/RNA/PNA duplexes

Sr.No.	PNA code	C5 substitution	PNA:DNA	PNA:RNA	PNA:PNA
01	PNA 1	TTT	47.5	61.1	72.9
02	PNA 2	TU ^{NH₂} T	53.1	60.3	58.9
03	PNA 3	TU ^{NH₂} U ^F	53.7	32	56.1
04	PNA 4	U ^F U ^{NH₂} U ^F	74.3	46	76.6
05	PNA 5	TU ^{NH₂} U ^{CF₃}	65.3	27.1	55.8
06	PNA 6	U ^{CF₃} U ^{NH₂} U ^{CF₃}	53.2	26.4	49.8

6.7 Conclusions

This chapter presents comparative effects of C5-substitutions in uracil (CH₃, F, NH₂, CF₃) in PNA oligomers on stability of their duplexes with complementary DNA, RNA and PNA. The modified aeg-PNA monomers having C5-substitutions were synthesized and characterized by NMR and mass spectroscopy. They were incorporated into PNA oligomer having central TTT sequence in which T's were systematically replaced by C5-substituted U to get the triplet combinations TU^{NH₂}T, TU^{NH₂}U^F, U^FU^{NH₂}U^F, TU^{NH₂}U^{CF₃} and U^{CF₃}U^{NH₂}U^{CF₃} in the middle of the sequence. This would also impart stability to U:A base pairs based on the electronic inductive effects of the substitutions and change the local microenvironment in major groove depending on their net hydrophobic / hydrophilic character. The duplexes PNA:DNA/PNA:RNA/PNA:PNA incorporating above modified monomers in PNA oligomers were studied to determine their effects on stability of duplexes. It was found that while modified PNA:DNA duplexes were stabilized, while corresponding PNA:RNA and PNA:PNA duplexes were destabilized compared unsubstituted duplexes. The overall results could be explained by a combination of electronic effects of substitutions which is same in all cases and the different microenvironmental features such as local dielectric constant which is different due to different duplex conformations. Future work should focus on the measurement of changes in local dielectric constant due to C5- substitutions as has been done earlier, to get a better molecular insight.

6.8 Experimental section

This section describes the detailed synthetic procedures and spectral characterization of the rationally designed PNA monomers. All compounds synthesised according to reported literature.^{12, 31}

6.8.1 General procedures

All reactions were conducted in oven-dried glassware under argon atmosphere. Low temperature experiments were carried out by cooling down the flasks with ice bath. The flasks were equipped with septum caps. All commercial solvents were distilled prior to use. THF was distilled over sodium/ benzophenone under nitrogen atmosphere, CH₂Cl₂ over CaH₂ and DMF over P₂O₅. Analytical thin layer chromatography (TLC) was performed on precoated 250 µm layer thickness silica gel GF254 (Merck 5554) plates. Visualization was performed by ultraviolet light and staining with a solution of ninhydrin. Column chromatography was performed using 100-200 µm silica gel. ¹H NMR and ¹³C NMR, were recorded using JEOL and Bruker 400 MHz NMR spectrometer at 100 MHz respectively. ¹³C NMR were recorded under broadband decoupling conditions. The delta (δ) values for chemical shifts are reported in ppm and are referred to internal standard TMS or deuterated NMR grade solvents. The optical rotation values were obtained on Rudolph Research Analytical Autopol V polarimeter. Mass spectra for all reaction products were recorded using Synapt G2 high-definition mass spectrometry.

6.8.2 Solid phase synthesis protocol

MBHA resin hydrochloride salt was neutralized in 50% DIPEA/DCM followed by coupling with 3 equivalents of (Boc/Cl-Cbz) L-lysine, coupling agent HBTU, racemization suppressor HOBt and DIPEA as base to get lysine coupled resin. To avoid deletion of sequences, resin was capped by Ac₂O/Pyridine. The resin was tested by ninhydrin solution to check whether any free amine is present. The observation of no color to the resin was the confirmation that no free amine group is bound to the resin. This was followed by washing of resin several times with DCM and then diethyl ether. The resin bound Boc group was knocked out by using 50% TFA/DCM, followed by neutralization with 10% DIPEA/DCM to get the free amine. The free amine bound resin was tested in ninhydrin solution followed by heating, which showed the blue colored resin indicating presence of free amine. The free amine was coupled with *aeg* PNA monomers and heated under microwave condition, which was washed

with DMF and DCM. The resin was again tested in ninhydrin. The polyamide oligomers were cleaved from the resin at final stage using TFA and TFMSA. PNA oligomers purified by RP-HPLC and characterised by MALDI-TOF spectra.

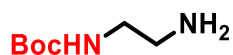
6.8.3 Biophysical studies

6.8.3a UV- T_m measurements. UV-melting experiments were carried out on Varian Cary 300 UVspectrophotometer equipped with a peltier. The samples for T_m measurements were prepared by mixing the calculated amounts of respective oligonucleotides in stoichiometric ratio (1:1, for duplex) in sodium phosphate buffer (10 mM, pH 7.2,) containing NaCl (10 mM), to achieve a final strand concentration of 5 μ M for each strand. samples were annealed by heating at 90 °C for 5 min. followed by slow cooling to room temperature and then storing at 4 °C. The samples (500 μ L) were transferred to quartz cell and equilibrated at the starting temperature for 2 min. The OD at 260 nm was recorded in steps from 20-90 °C with temperature increment of 1 °C/min. 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 of normalized absorbance with respect to temperature and is accurate to ± 0.5 °C. The data was processed using Microcal Origin 8.0/8.5. [The concentration of all oligonucleotides were calculated on the basis of absorbance from the molar extinction coefficients of the corresponding nucleobases i.e. T = 8.8 cm²/μmol; C = 7.3 cm²/μmol; G = 11.7 cm²/μmol and A = 15.4 cm²/μmol].⁵⁰

6.8.3b Circular dichroism. CD spectra were recorded on JASCO J-815 spectropolarimeter. The calculated amounts for PNA oligomers and the complementary DNA/RNA/PNA were mixed together in stoichiometric ratio (1:1, for duplex) in sodium phosphate buffer (10 mM, pH 7.2) and NaCl (10 mM) to achieve a final strand concentration of 5 μ M for each strand. The samples were annealed by heating at 90 °C for 5 min. followed by slow cooling to room temperature and then transferred to refrigerator for at least 4 to 5 h. To record the CD spectra of PNA:DNA/RNA/PNA duplexes and single stranded PNAs, the temperature of circulating water was kept at 25 °C. The CD spectra were recorded as an accumulation of 10 scans from 350 to 190 nm using 2 mm cell, a resolution of 0.1 nm, band-width of 1 nm, sensitivity of 2 m deg, response of 1 sec and a scan speed of 100 nm/min.

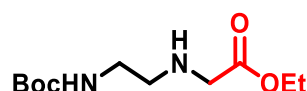
6.8.4. Experimental procedures and spectral data

N1- (Boc)-1,2-diaminoethane (2)



Compound 2 synthesised by standard protocol as mentioned at page no. 94

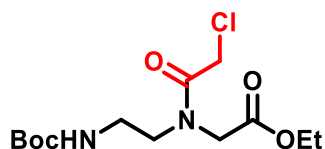
Ethyl N-(2-Boc-aminoethyl) glycinate (3)



Compound 3 synthesised by standard protocol as mentioned at page no. 95

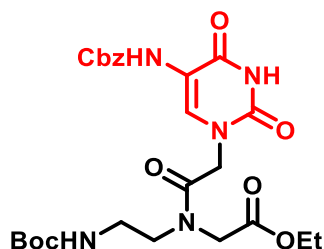
Ethyl N-(2-Boc-aminoethyl) chloroacetyl glycinate (4)

Compound 4 synthesised, purified and characterised according to reported protocol.¹²



Compound 3 (3 g, 11.5 mmol) was dissolved in dioxane (50 mL) at 0 °C and NaHCO₃ (1.9 g, 23 mmol) in water (50 mL) was added to reaction mixture at 0 °C and stirred for 5 min. To this, chloroacetyl chloride (1.0 mL, 12.6 mmol) was added dropwise and reaction was continued for stirring at 0 °C for another 5 min. After 5 min, the reaction mixture was brought to room temperature and stirred for 1.5 h. After completion of reaction (TLC), dioxane was removed under reduced pressure. Reaction mixture was then diluted with water (25 mL) and extracted with ethyl acetate (3 x 75 mL). The combined organic layer was washed with saturated solution of NaHCO₃ followed by washing with brine. Organic layer was dried over anhydrous sodium sulphate, collected by filtration and concentrated on rota evaporator. The residual material was purified on column using silica gel in petroleum ether: ethyl acetate (4:1) (Yield 3.2 g, 85%).

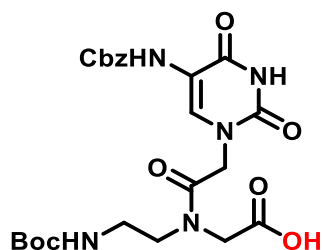
Ethyl 2-(2-(5-(((benzyloxy)carbonyl)amino)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-N-(2-((tert-butoxycarbonyl)amino)ethyl)acetamido)acetate (5)



Compound **4** (3 g, 9.3 mmol) was dissolved in dry DMF under inert atmosphere (25 mL) and stirred at 0 °C temperature for 5 min. After 5 min, dry K₂CO₃ and 5-benzyloxyaminouracil (2 g, 9.3 mmol) was added and stirring continued at same temperature. After 5 min, reaction mixture was moved to oil bath and heated at 60 °C for 2 h. Completion of reaction was monitored by TLC. The hot reaction mixture was allowed to cool at room temperature and then reaction mixture was diluted with water (40 mL) and extracted with ethyl acetate (3 x 75 mL). The combined organic layer was washed with saturated solution of NaHCO₃ (20 mL) and then again washed with water (3 x 40 mL) followed by washing with brine (25 mL). The collected organic layer was dried over anhydrous sodium sulphate, collected by filtration and concentrated on rota evaporator. The crude product was purified on column using silica gel in petroleum ether: ethyl acetate (1.5:3.5) (Yield 3.5 g, 75%). ¹H-NMR (400 MHz, CDCl₃) δ: 9.82 (bs, 1H), 8.03 (bs, 1H), 7.44-7.33 (m, 5H), 7.17 (mi.) 7.13 (ma.) (s, 1H), 5.64(bs, 1H), 5.16 (mi.) 5.15 (ma.), (s, 2H), 4.61 (ma.) 4.47 (mi.) (s, 2H), 4.28 (mi.) 4.20 (ma.) (q, 2H, *J* = 8 Hz), 4.05 (s, 2H), 3.53 (t, 2H, *J* = 6 Hz), 3.33 (t, 2H, *J* = 8 Hz), 1.45 (ma.) 1.43 (mi.) (s, 9H), 1.32 (mi.) 1.27 (ma.) (t, 3H, *J* = 6 Hz) ppm; ¹³C-NMR (100 MHz, CDCl₃) δ: 169.4, 169.0, 167.03, 159.7, 156.1, 152.9, 149.1, 135.7, 129.6, 128.5, 128.1, 114.8, 80.0, 67.3, 62.3, 61.6, 48.8, 38.8, 28.3, 14.0 ppm; MS (HRMS-ESI): *m/z* calcd for C₂₅H₃₃N₅O₉Na 547.2278, found 570.2175 (M+Na), 448.1895 (M-Boc).

Ethyl 2-(2-(5-(((benzyloxy)carbonyl)amino)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-N-(2-((tert-butoxycarbonyl)amino)ethyl)acetamido)acetic acid (6)

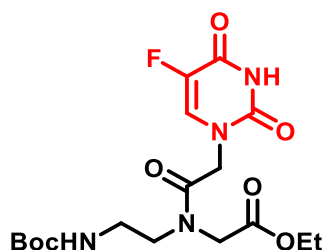
Compound **6** was synthesised, purified and characterised according to reported protocol.¹²



Compound **5** (1 g, 2 mmol) was dissolved in ethanol and stirred at 0 °C. To this, 10% LiOH (1.5 mL) was dropwise added at the same temperature for 10 min. The completion of reaction was monitored by TLC. After completion of reaction, solvent was removed on rotary evaporator. Ethyl acetate was added to the residue and it was acidified by addition of saturated aqueous solution of potassium bisulfate till the pH comes down to 3-4. Organic layer was collected and washed with brine solution. The organic layer was concentrated on rota evaporator to get solid product which was further dried on high vacuum and stored in vacuum dessicator (Yield 0.85 g, 90%).

Ethyl 2-(N-(2-((tert-butoxycarbonyl) amino) ethyl)-2-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl) acetamido) acetate (9**)**

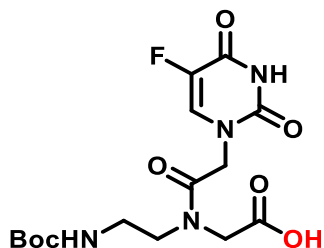
Compound **9** was synthesised, purified and characterised according to reported protocol.³¹



To a solution of compound **4** (2 g, 6.21 mmol) in DMF (5.0 mL), 5-fluorouracil (0.83 g, 6.21 mmol) and K₂CO₃ (0.85 g, 6.21 mmol) were added at RT and stirring was continued at same temperature for 12 h, monitoring the completion of reaction by TLC, after completion of reaction, water was added and extracted with ethyl acetate (3x25 mL). The organic layer was dried over anhydrous Na₂SO₄. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (1:1) to obtain the compound **9**. (Yield 2.1 g, 77%).

2-(N-2((tert-butoxycarbonyl)amino)ethyl)-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamido)aceticacid (11**)**

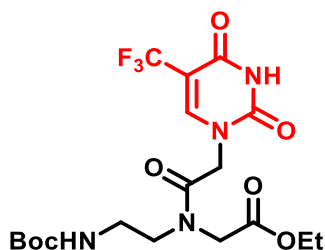
Compound **11** was synthesised, purified and characterised according to reported protocol.³¹



To the ester compound **9** (2.1 g, 5.05 mmol) in THF (5 ml), 10% LiOH was added at 0 °C and stirred for 3 h. After completion of reaction, the mixture was acidified with *sat. aq.* KHSO₄ solution extracted with ethyl acetate and dried over Na₂SO₄. The solvent was evaporated under reduced pressure to obtain the desired monomer **11** in good yields.

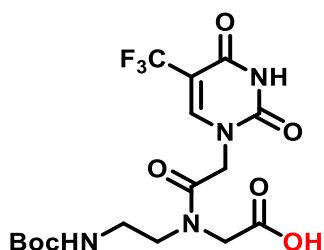
Ethyl-2-(N-(2-((tert-butoxycarbonyl)amino)ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetate (10**)**

Compound **10** was synthesised, purified and characterised according to reported protocol.³¹



To a solution of the compound **4** (1.0 g, 3.10 mmol) in DMF (5 ml), K₂CO₃ (428 mg, 3.10 mmol) and 5-trifluoromethyluracil (560 mg, 3.10 mmol) were added at RT and stirring was continued at same temperature for 12 h, monitoring the completion of reaction by TLC, after completion of reaction, water and *aq.* KHSO₄ were added and extracted with ethyl acetate (3x25 mL). The organic layer was dried over anhydrous Na₂SO₄. The solvent was removed in vacuo and product was purified by column chromatography, eluting with petroleum ether/ EtOAc (1:1) to obtain the compound **10**. (Yield 1.10 g, 76%). HRMS (ESI-TOF) *m/z* Calcd for C₁₈H₂₅F₃N₄NaO₇ [M+Na]⁺ 489.1573, found 489.1571.

Synthesis of 2-(N-(2-((tert-butoxycarbonyl)amino)ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2H)-yl)acetamido)acetic acid (12**)**



The ester compound **10** (1.0 g, 2.14 mmol) in THF was saponified with 10% LiOH at 0 °C for 3 h. After completion of reaction, the mixture was acidified with *sat. aq.* KHSO₄ solution and then the acid compound was extracted with ethyl acetate, dried over Na₂SO₄ and evaporated under reduced pressure to obtain the desired monomer **12** in good yields. (Yield 750 mg, 80%). ¹H-NMR (400 MHz, DMSO-d₆) δ: 1.31(min) 1.33 (maj) (s, 9H), 2.96-3.15 (m, 2H), 3.25-3.35 (m, 2H), 3.93 (maj) 4.04 (min) (s, 2H), 4.58 (min) 4.76 (maj) (s, 2H), 6.73 (min) 6.89 (maj) (br, 1H), 8.18 (min) 8.24 (maj) (s, 1H), 11.81 (bs, 1H); ¹³C-NMR (100 MHz, DMSO-d₆) δ: 28.6, 38.5, 47.3, 48.3, 48.8, 78.2, 78.5, 99.9, 121.5, 121.8, 124.5, 148.5, 150.6, 156.1, 156.2, 159.8, 167.1, 167.6, 170.9; HRMS (ESI-TOF) *m/z* Calcd for C₁₆H₂₁F₃N₄NaO₇ [M + Na]⁺ 461.1260, found 461.1264.

6.9. References

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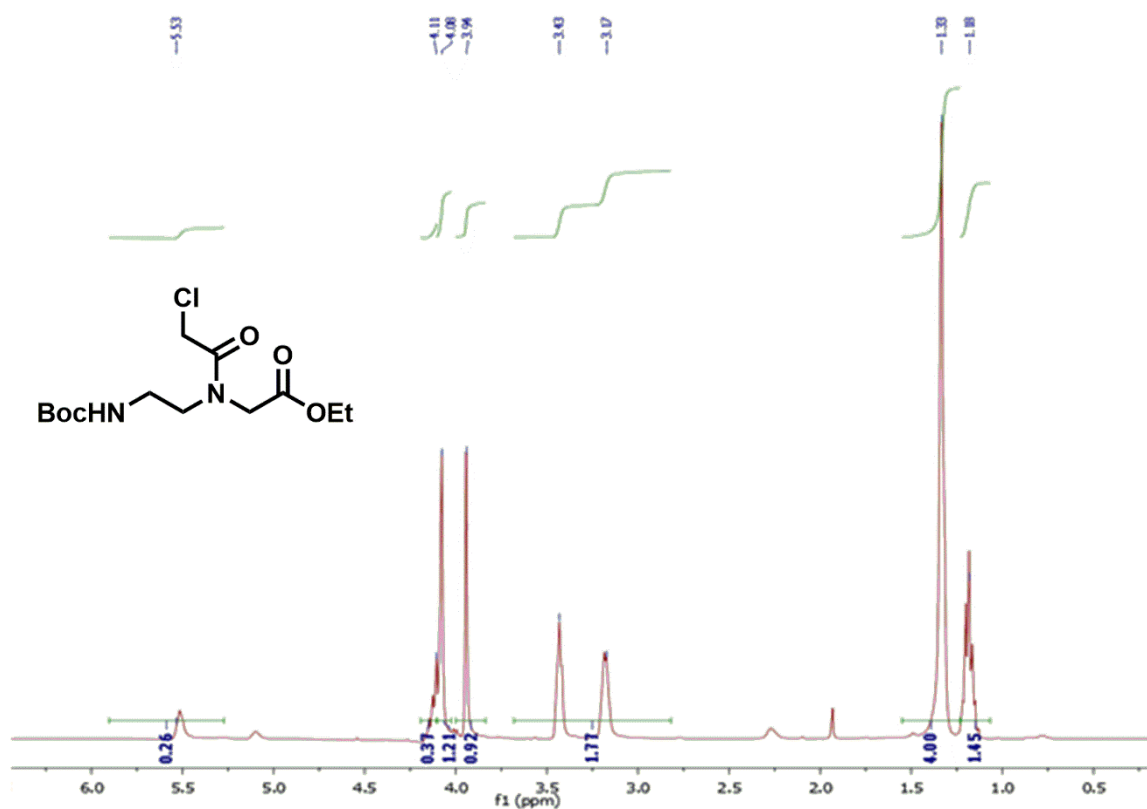
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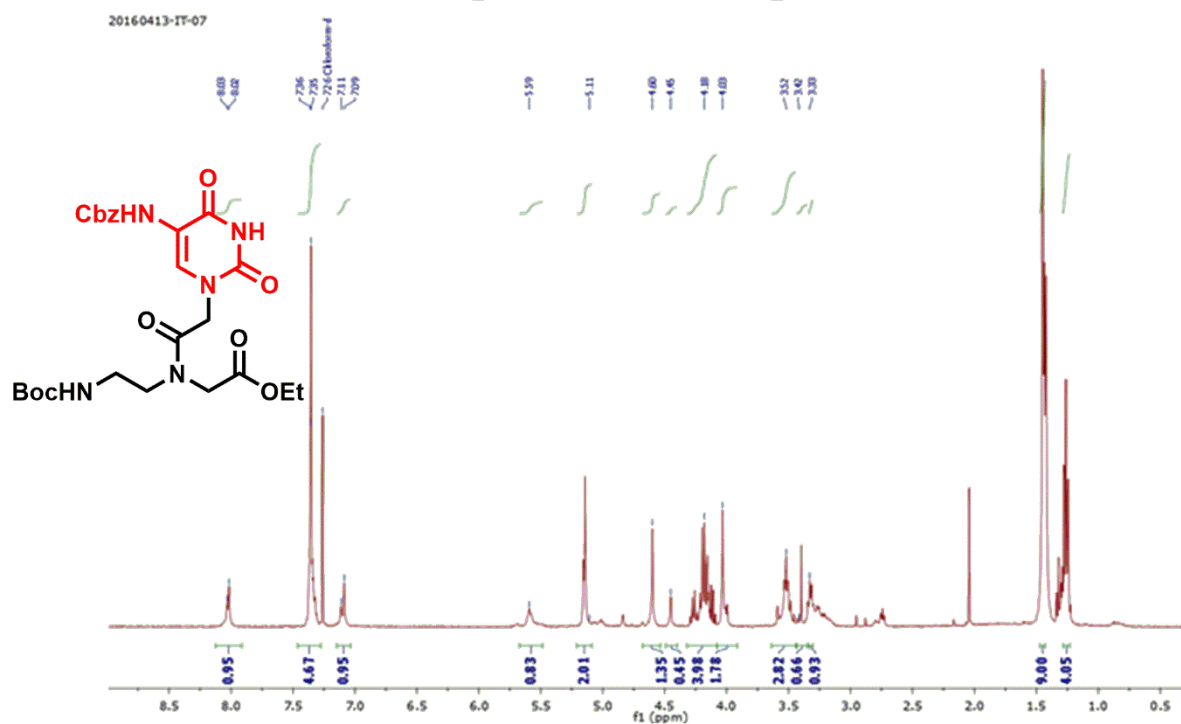
6.5 Appendix-I: Characterization data of synthesized compounds/PNAs

Entry	Table of contents	Page No.
01	^1H , ^{13}C , HRMS of compounds.	28-31
02	HPLC and MALDI profiles of all synthesized PNA oligomers	32-38

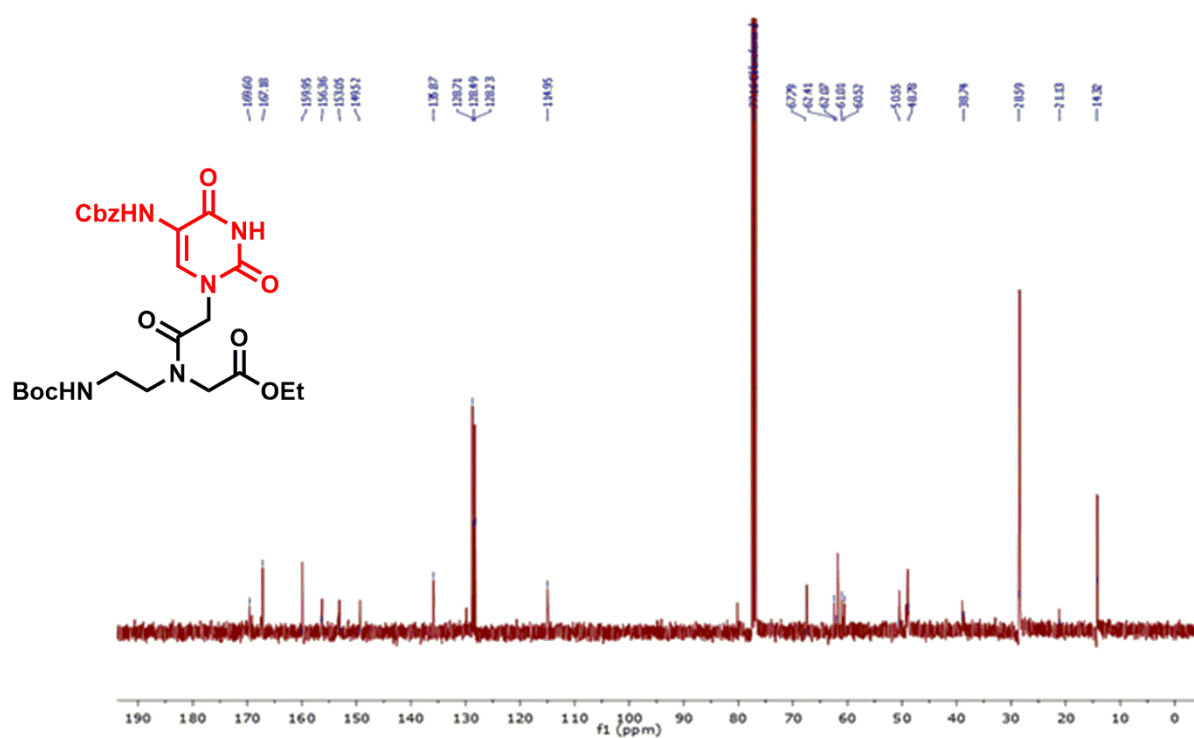
^1H spectrum of compound 4

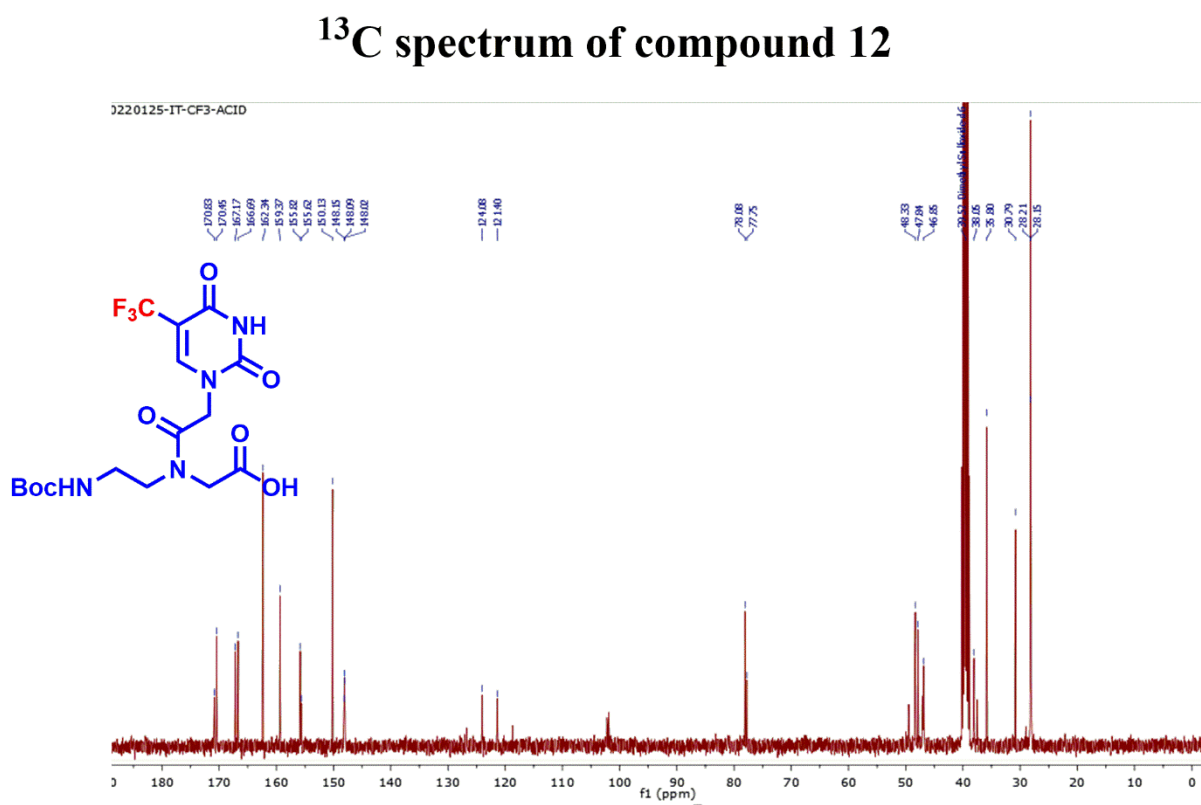
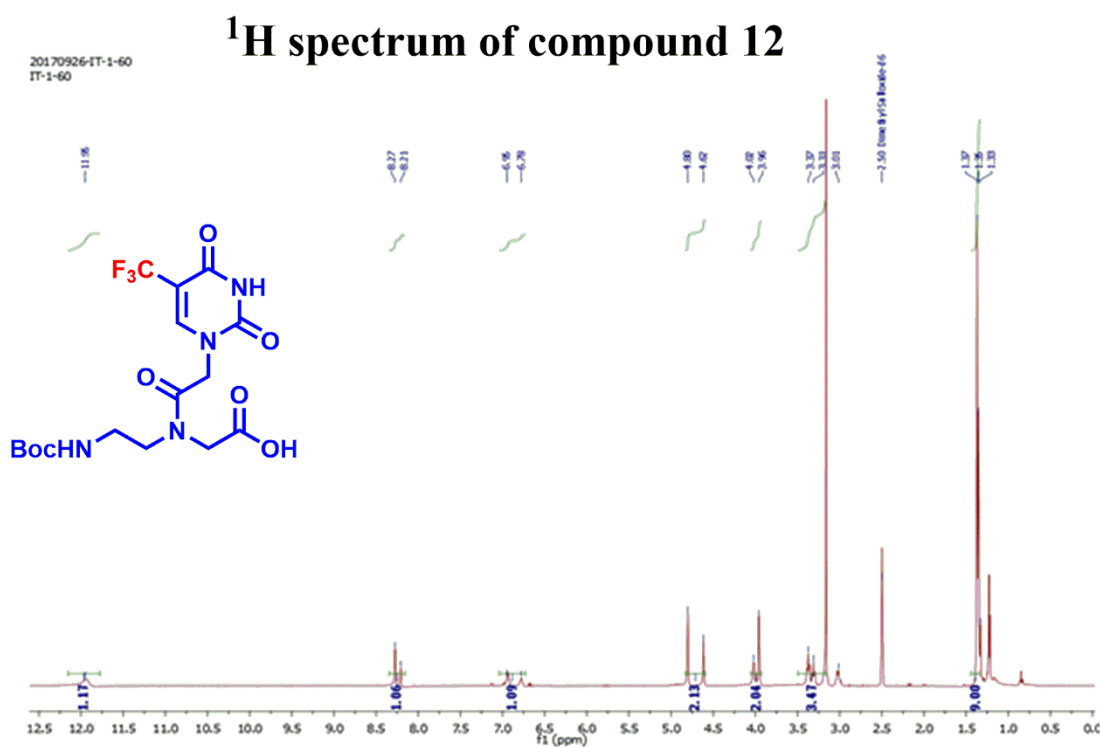


¹H spectrum of compound 5



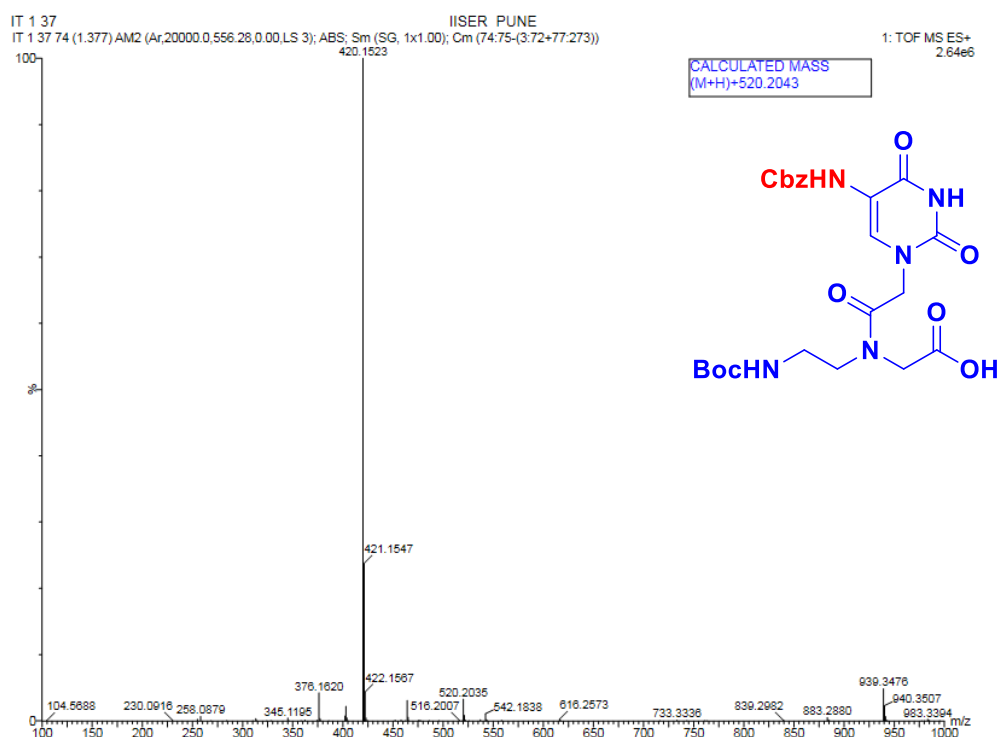
¹³C spectrum of compound 5



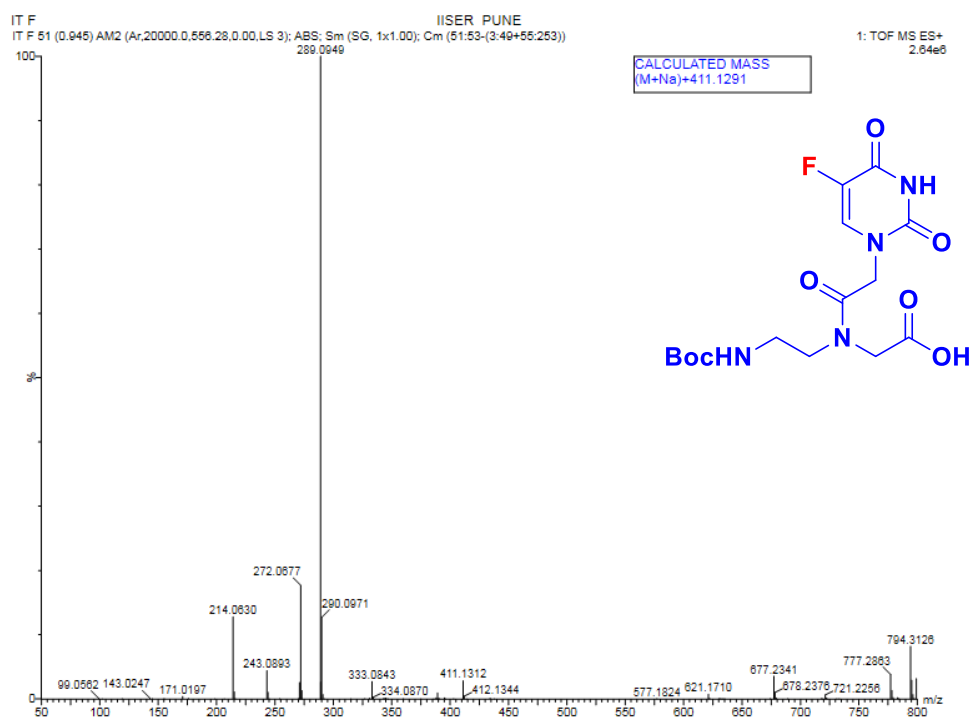


HRMS of all monomers observed [M-Boc] as high intense peak

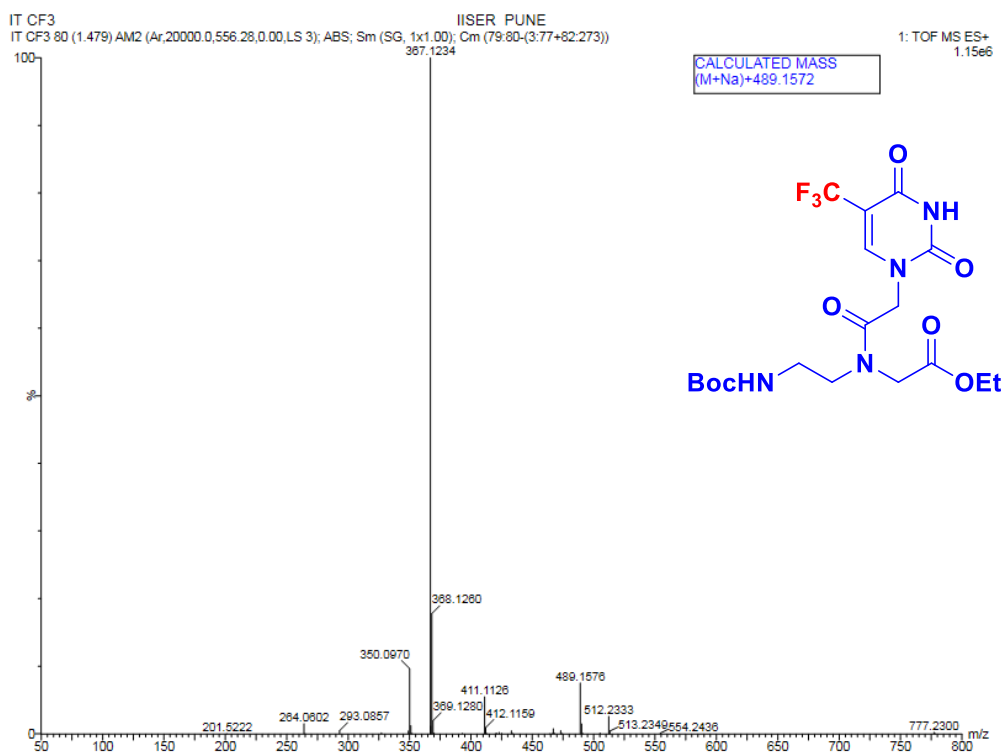
HRMS of compound 6



HRMS of compound 11

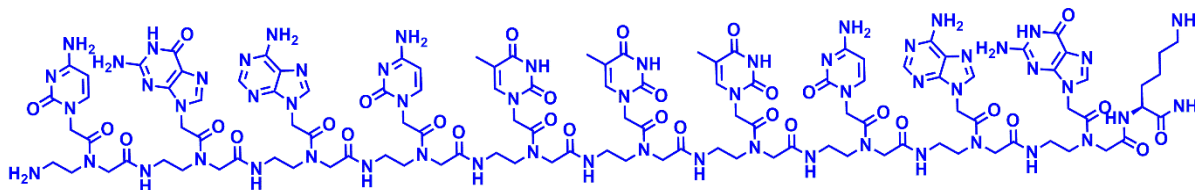


HRMS of compound 10

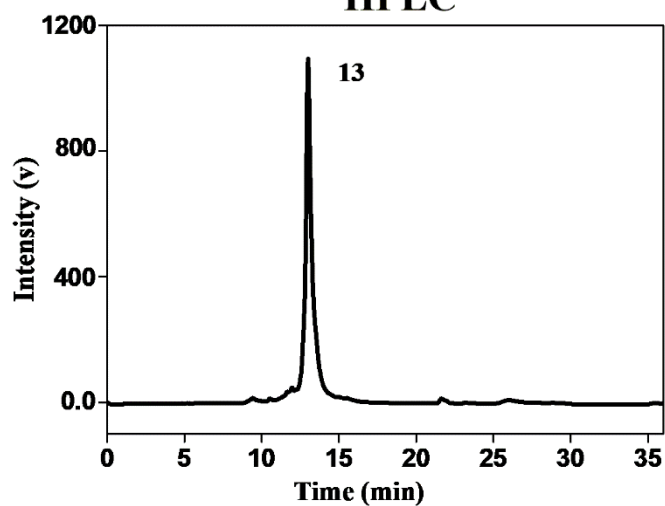


HPLC and MALDI-TOF profiles:

PNA-1



HPLC



MALDI-TOF

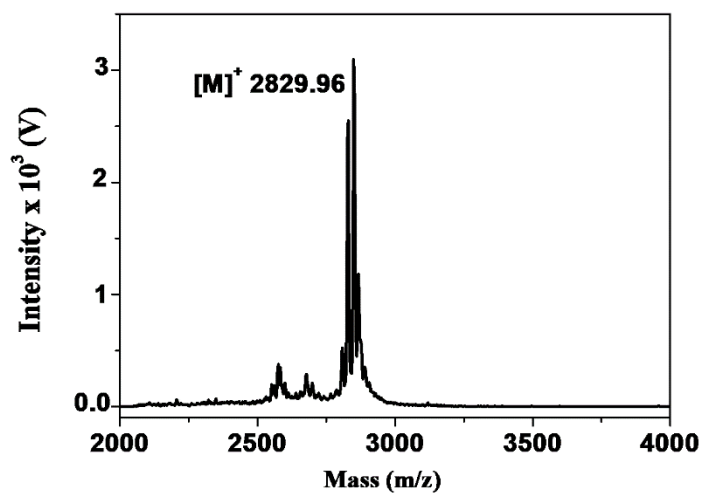


Figure 6.18 RP-HPLC and MALDI-TOF of PNA 1

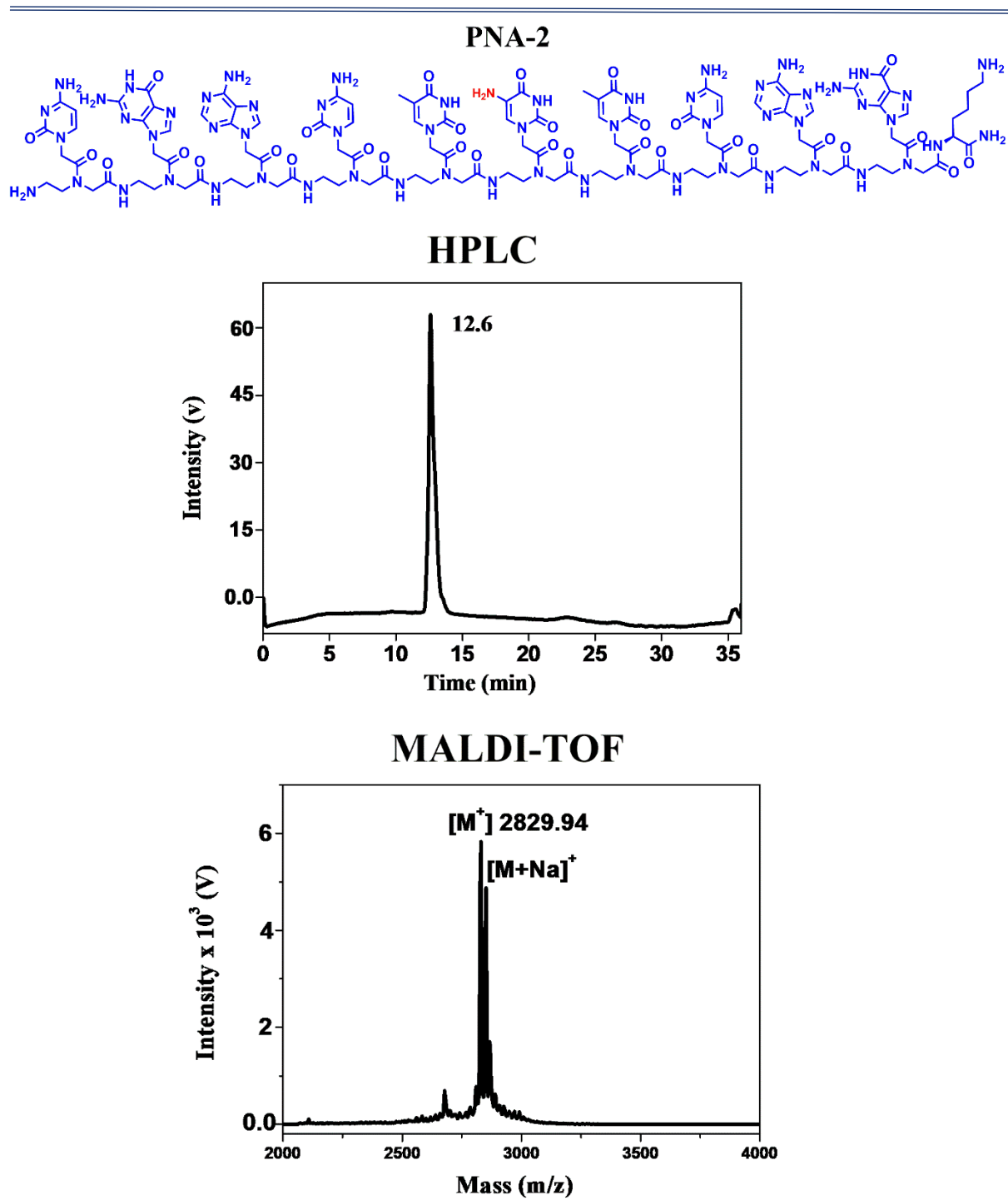


Figure 6.19 RP-HPLC and MALDI-TOF of PNA 2

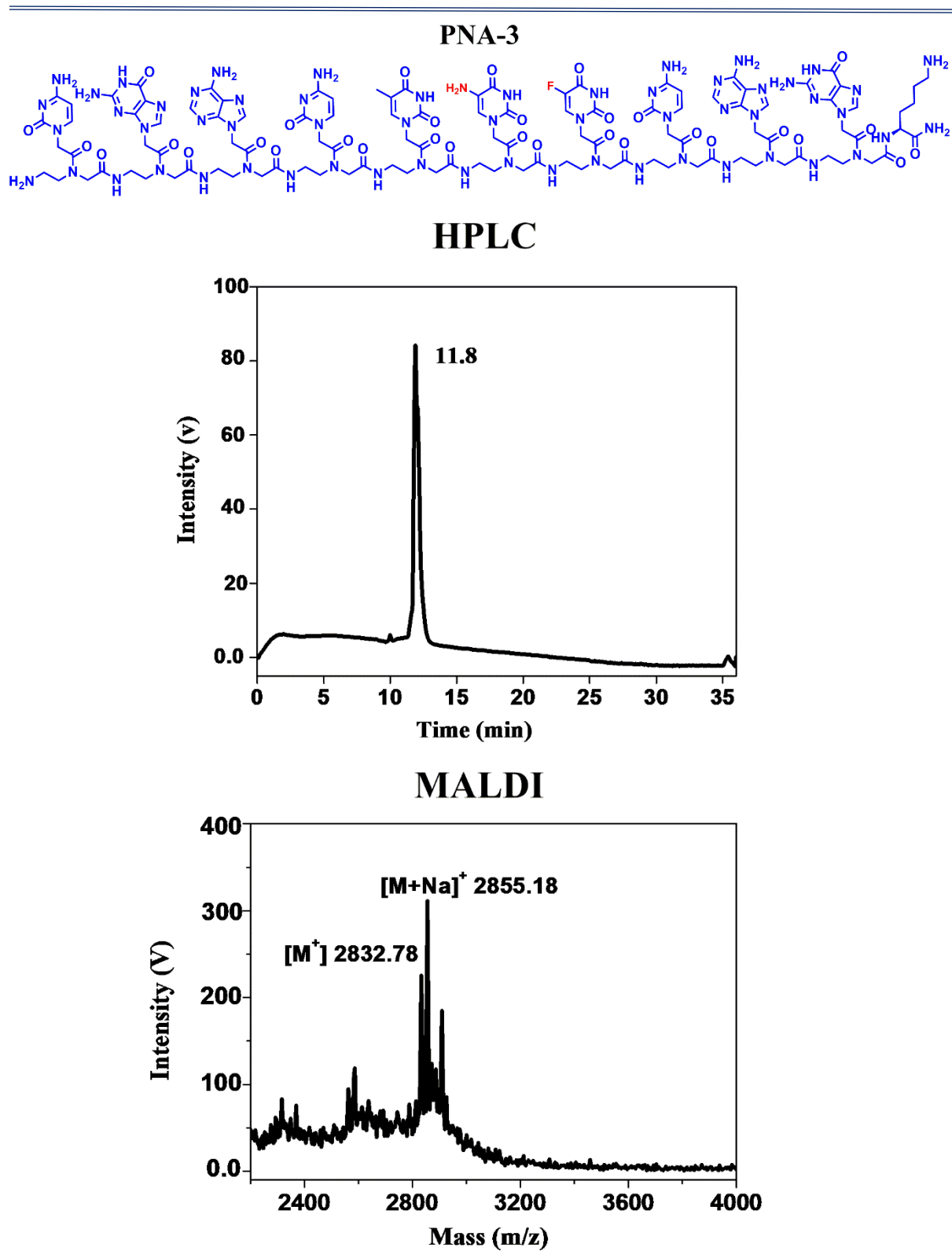
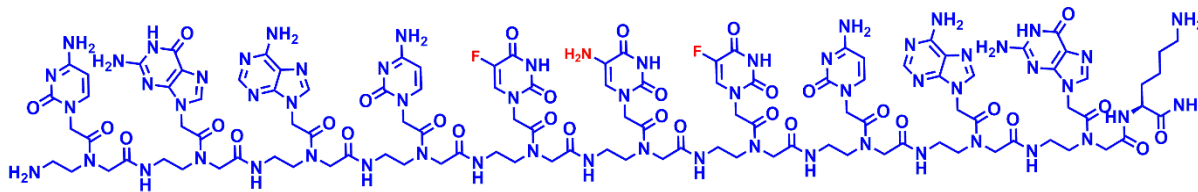
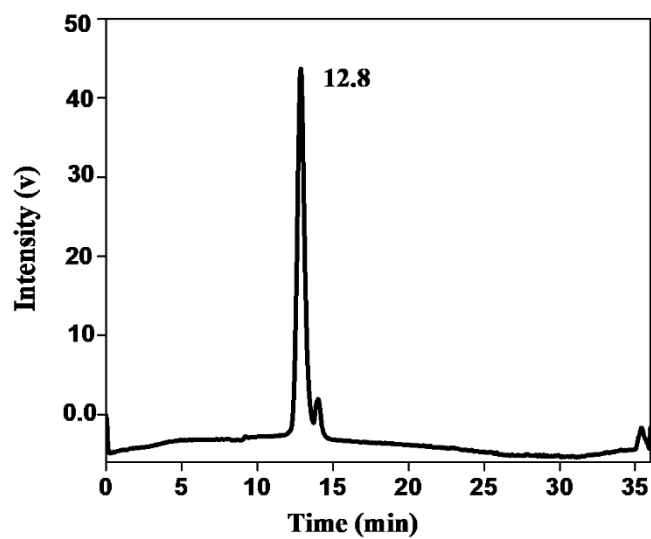


Figure 6.20 RP-HPLC and MALDI-TOF of PNA 3

PNA-4



HPLC



MALDI-TOF

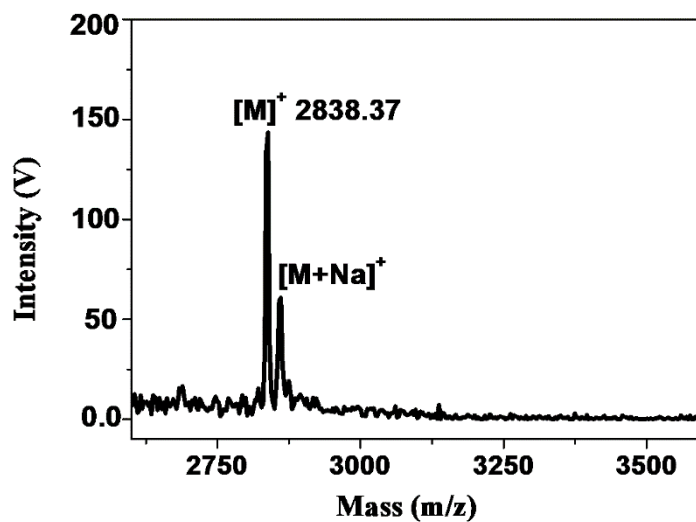


Figure 6.20 RP-HPLC and MALDI-TOF of PNA 4

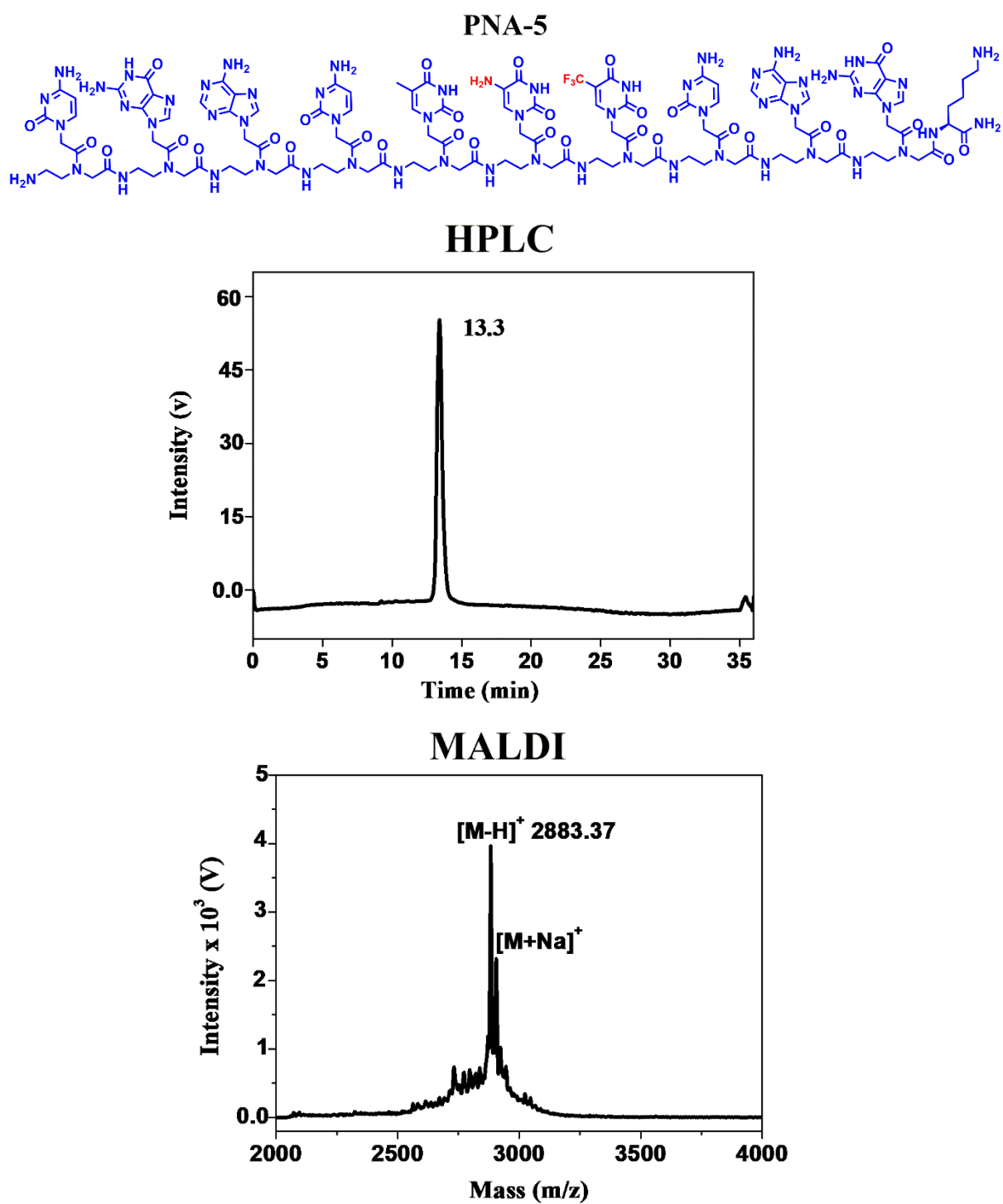


Figure 6.22 RP-HPLC and MALDI-TOF of PNA 5

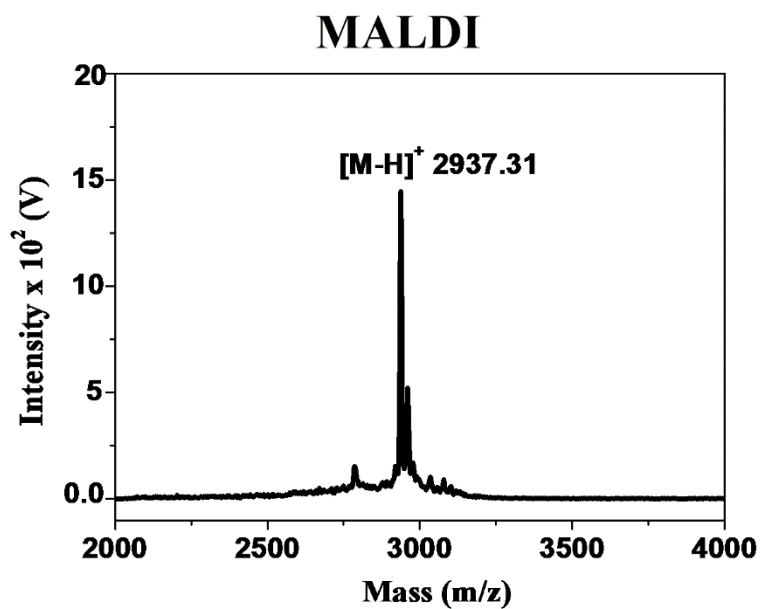
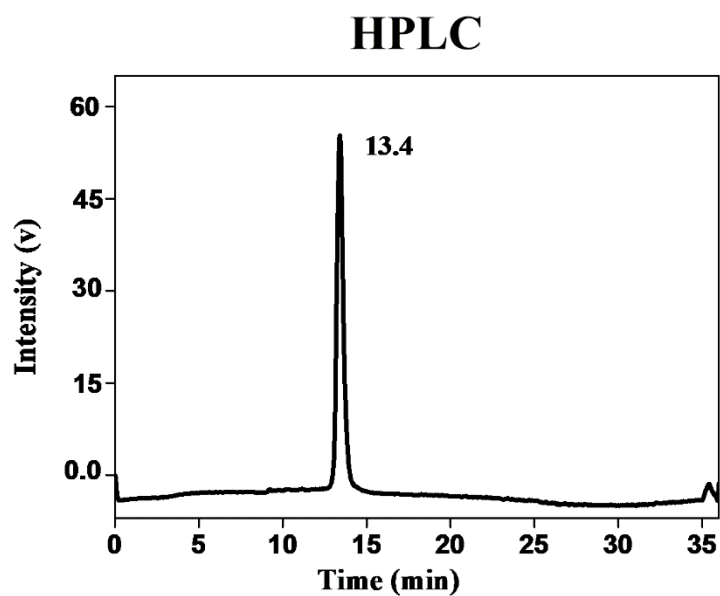
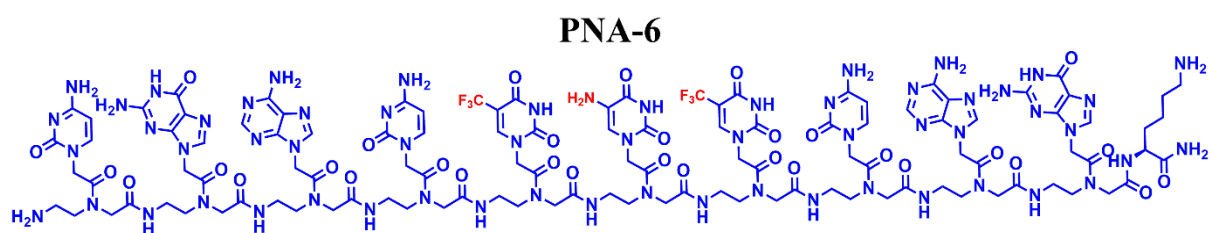
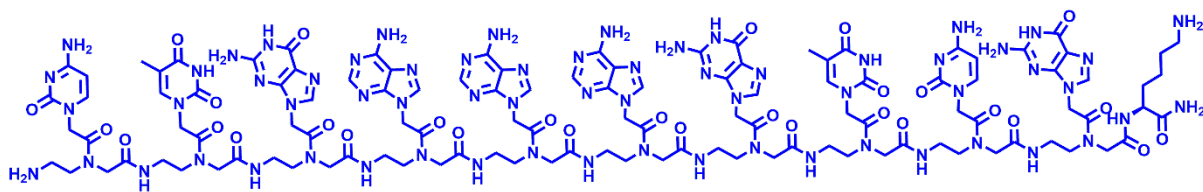
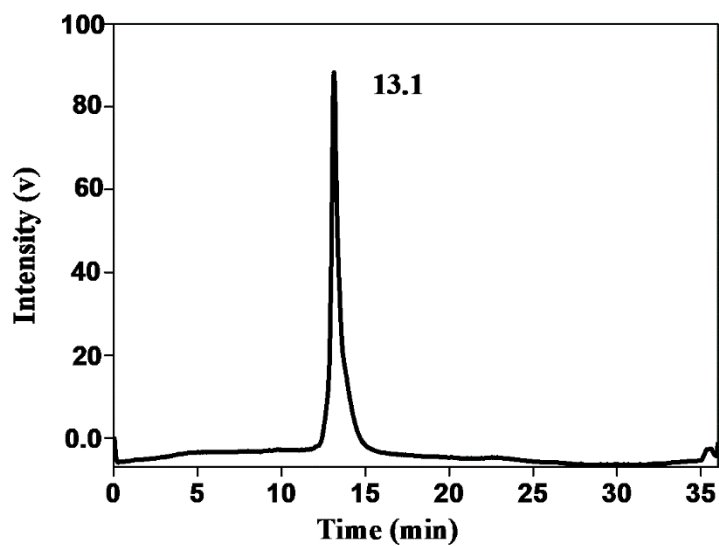


Figure 6.23 RP-HPLC and MALDI-TOF of PNA 6

PNA 7



HPLC



MALDI

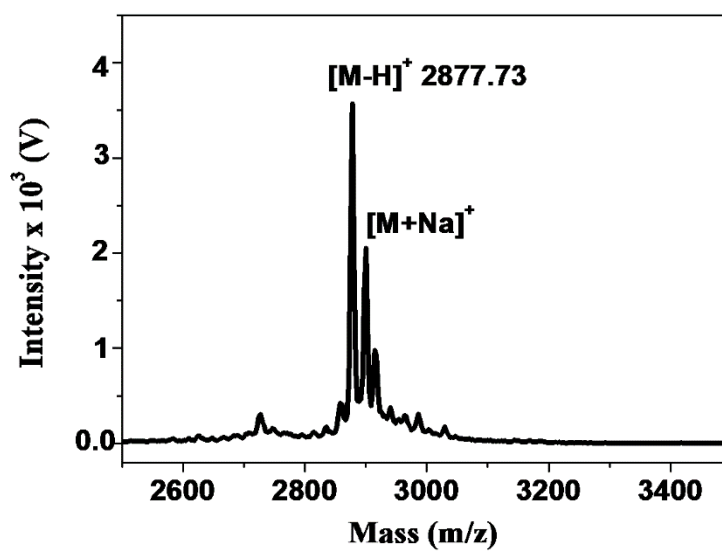


Figure 6.24 RP-HPLC and MALDI-TOF of PNA 7