Pyrrolidine and Piperidine Nucleic Acids: Novel Class of PNA Analogues

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CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Pyrrolidine and Piperidine Nucleic Acids: A novel Class of PNA Analogues"** submitted by Pallavi S. Lonkar, was carried out by the candidate at the National Chemical Laboratory Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

Vaijayanti A. Kumar **October 2004**
Research guide
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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled **"Pyrrolidine and Piperidine Nucleic Acids: A novel Class of PNA Analogues"** submitted for the degree of Doctor of Philosophy in Chemistry to the University of Pune, has not been submitted by me to any other university or institution. This work was carried out at the National Chemical Laboratory, Pune, India.

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pallavi

CONTENTS

PUBLICATIONS

1. *Trans* 5-aminopipecolyl-*aeg*PNA chimera: Design, synthesis and study of binding preferences with DNA/RNA in duplex/triplex modes **Lonkar, Pallavi S**.; Kumar V. A. **2004** (Manuscript submitted to *J. Org. Chem.*).
2. Chimeric Pyrrolidine PNA:Synthesis and stereodiscriminative duplex binding with DNA/RNA. **Lonkar, Pallavi S**.; Kumar V. A.; Ganesh K. N. *Organic & Bio. Chem*. **2004**, *2*, 2604-2611.
3. Design and Synthesis of Conformationally Frozen Peptide Nucleic Acid Backbone: Chiral Piperidine PNA as a Hexitol Nucleic Acid Surrogate. **Lonkar, Pallavi S**.; Kumar V. A. *Bioorg. Med. Chem. Lett.* **2004,** *14*, 2147-2149.
4. Conformationally restricted cationic polyamide analogs of nucleic acids: Design, synthesis and DNA/RNA binding studies. Kumar, V. A.; D'Costa, M.;**Lonkar, Pallavi S**.; Meena.; Pallan, P. S.; and Ganesh, K. N. *Pure Appl. chemistry* **2004** *76*, 1599-1603.
5. Piperidinyl Peptide Nucleic Acid: Synthesis and DNA complementation Studies. **Lonkar, Pallavi S**.; Kumar, V. A. *Nucleosides, Nucleotides and Nucleic Acids* **2003,** *22*, 1105-1108.
6. Constrained flexibility in PNA:DNA binding studies with bridged aminopropylglycyl PNA**. Lonkar, Pallavi S**.; Kumar, V. A.; Ganesh, K. N. *Nucleosides, Nucleotides and Nucleic Acids* **2001**, *20*, 1197-1200.

ABSTRACT

The thesis entitled "**Pyrrolidine and Piperidine Nucleic Acids: A Novel Class of PNA Analogues"**

The thesis comprises the studies towards design, synthesis and DNA/RNA recognition properties of nucleic acid analogs having chiral polyamide backbone (Peptide Nucleic Acids, PNA) with five- and six-membered nitrogen heterocycles. The polyamide backbone is stable towards intracellular enzymes and has potential applications in therapeutics. The thesis is divided into four chapters.

Chapter 1 introduces the background literature for undertaking the research work.

Chapter 2 details the synthesis and DNA/RNA hybridization studies of diastereomeric pyrrolidinyl-aminoethylglycyl chimeric PNA.

Chapter 3 describes the synthesis and DNA/RNA hybridization studies of 4-amino and 5-amino pipecolyl-aminoethylglycyl chimeric PNA.

Chapter 4 introduces the piperidinyl-aminoethylglycyl PNA chimera and their DNA recognition properties.

Chapter 1: Introduction

The genetic information encoded in double–stranded DNA (dsDNA) and transcribed into single stranded mRNA, is an attractive target for potential therapeutic interventions. A variety of chemical reagents, both natural and synthetic, are capable of interacting with DNA or RNA. Such interactions can lead to inactivation or destruction of these molecules. Selective inhibition of disease causing genes is theoretically possible by taking advantage of the known specific hydrogen bonding interactions between complementary base pairs of nucleic acids. Based on these interactions, designed short nucleic acid sequences or oligonucleotides, could selectively bind to the target DNA/RNA. Oligonucleotides that interact with single stranded RNA are termed as 'antisense' oligonucleotides whereas those interacting with double stranded DNA are called 'antigene' oligonucleotides. However, intracellular enzymes such as nucleases, rapidly cleave the unmodified ODNs with sugar-phosphate backbone. The development of several

new classes of modified oligonucleotides has been triggered to allow the fruitful application of antisense/antigene principle.

Peptide nucleic acids, the most prominent outcome of this search is a class of compounds in which the entire anionic sugar phosphate backbone of DNA is replaced by a neutral and achiral polyamide backbone consisting of *N*-2-(aminoethylglycyl) units (Figure 1). The nucleobases are attached to the backbone through an acetamide linker. The internucleobase distance in PNA is conserved, allowing its binding to the target DNA/RNA sequences with high sequence specificity and affinity. PNA is also stable to intracellular enzymes. The binding of homopyrimidine PNAs to homopurine targets in dsDNA usually does not result in a conventional PNA:DNA_2 triplex but rather results in a strand displacement complex leading to a PNA_2:DNA triplex and a single stranded DNA loop. Due to its

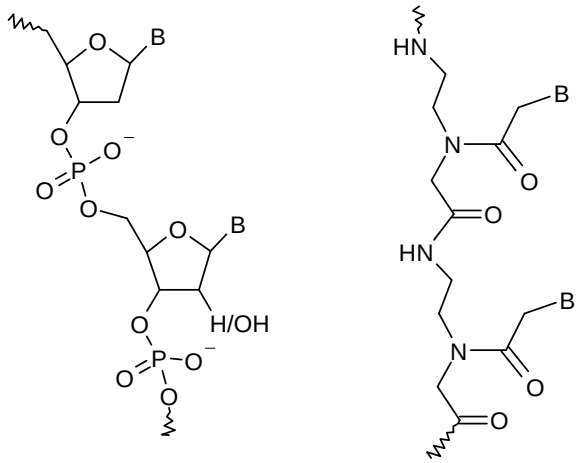


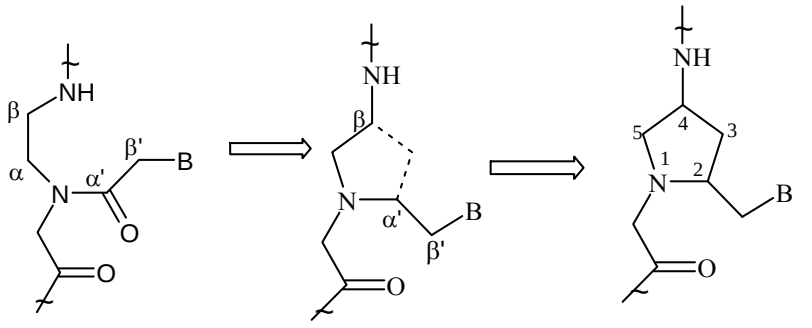
Figure 1 DNA/RNA and PNA

favorable properties, PNA is regarded as a very promising lead for developing into efficient thereapeutic agents. However PNAs have some drawbacks like low water solubility, binding to target DNA/RNA in parallel or antiparallel orientations, low membrane penetration and inefficient cellular uptake. Various attempts have been made to address these shortcomings of PNA. Introduction of various modifications/substitutions in the PNA backbone that resulted in chiral PNA, are aimed to achieve directional selective binding to target DNA/RNA. PNAs suffixed with negatively charged DNA or modified PNAs with positively charged

polypeptide sequence at either '*C*' or '*N*' terminus, resulted in PNA-DNA/PNA-peptide chimera with favorable aqueous solubility, cellular uptake and DNA binding/recognition properties. This chapter gives an overview of the oligonucleotide analogues and various modifications of PNAs carried out to improve their applications as therapeutic agents with special emphasis on PNAs containing 5- and 6- membered rings. Rationale for the design of chiral, pyrrolidine/piperidine based new PNAs is presented that forms the basis for the present work. The five-membered pyrrolidinyl PNAs and six-membered piperidinyl as well as pipecolyl PNAs were designed to impart controlled flexibility to the backbone, and at the same time introduce positive charges and chirality.

Chapter 2: Synthesis of (4*R*/*S*)-4-(t-butyloxycarbonylamino)-2-(*R*/*S*) (thymin/guanin/cytosin/adenin-yl-methyl)-pyrrolidin-*N*-1-acetic acid: Synthesis and DNA binding studies.

Our efforts towards generating positively charged PNA analogues resulted in the synthesis of (2*S/R*, 4*R/S)*-4-(t-butyloxycarbonylamino*)*-2-(thymin-1-yl-methyl*)*-pyrrolidin-*N*-1-acetic acid. This modification allows the introduction of rigidity into the PNA backbone in the form of pyrrolidinyl ring, while simultaneously keeping a certain degree of flexibility in the linker to the nucleobase. The β carbon atom of ethylenediamine segment and β' carbon atom of linker to the nucleobase of *aeg*PNA



*aeg*PNA **I** pyrrolidine PNA **II**

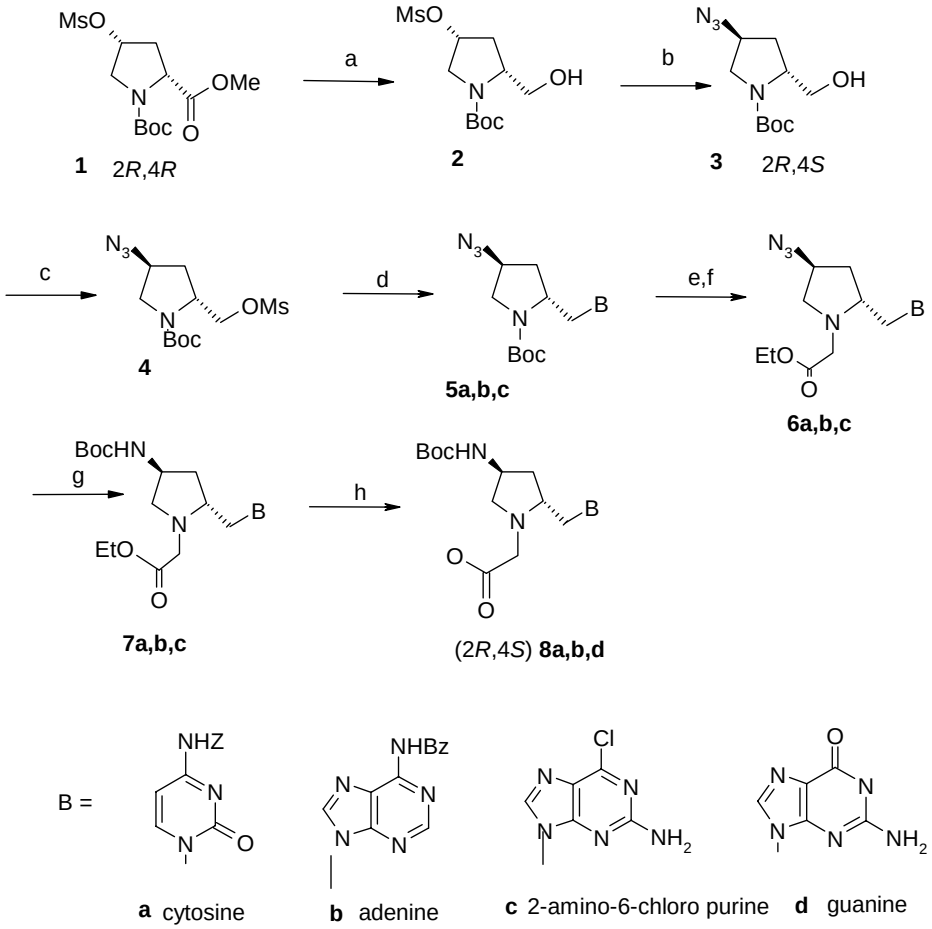
Figure 2 Pyrrolidine PNA

I are bridged by a methylene group (Figure 2). The nucleobase is attached to C-2 position of the ring through methylene linker. The pyrrolidine ring nitrogen is a tertiary amine and bears a positive charge at physiological pH and helps to improve the solubility of PNA oligomers. The synthesis of all four stereoisomers with

thymine as a nucleobase is described. Also, the monomers incorporating other naturally occurring nucleobases adenine, guanine and cytosine with (2*R*,4*S*) stereochemistry is described.

Synthesis of pyrrolidine PNA monomers and oligomers:

The synthesis of the (2*R*,4*S*)-4-*(t-butoxycarbonylamino)-2-*(*R*) (*thymin*/*guanin*/*cytosine/adenin-yl-methyl*)-*pyrrolidin-N-1-acetic* acid was achieved



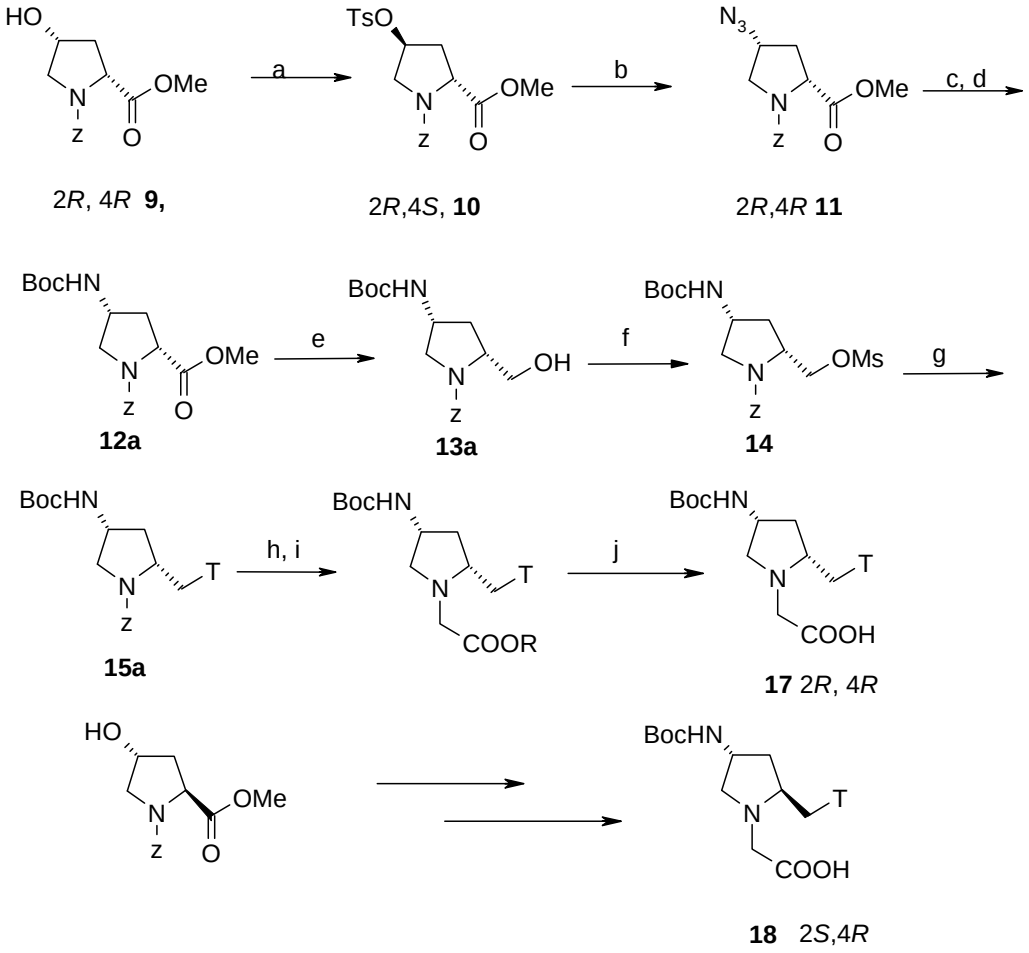
1 2*R*,4*R* 2 3 2*R*,4*S* 4 **5a,b,c** **6a,b,c** **7a,b,c** (2*R*,4*S*) **8a,b,d**

Scheme 1: a) LiCl:$NaBH_4$, 90% b) NaN_3, DMF 80% c) MsCl, 89% d) B, K_2CO_3, DMF e) 50%TFA, DCM f) $BrCH_2COOEt$, DIPEA, THF, 89% g) H_2, Pd-C, Boc-N_3 h) 1N NaOH, MeOH, 98%

from naturally occurring 4(*R*)-hydroxy-2 (*S*)-proline as shown in Scheme 1. The 4 (*R*)–hydroxy group was mesylated after the carboxyl and amino functions were protected as methyl ester and *N*-Boc derivatives respectively. The reduction of the methyl ester was followed by conversion to the corresponding azide **3**, with

inversion of the C-4 stereocenter. The nucleobase was attached to C-2 methylene position via mesyl intermediate **4**. The pyrrolidine ring nitrogen was alkylated after Boc deprotection to get 6. Hydrogenation and Boc-protection furnished 4(*S*)-(*N*-Boc amino)-2-(*S*)-thymine/guanine/cytosine-1ylmethyl)-pyrrolidin-*N*1-ethyl acetate **7**. Subsequent hydrolysis of ester function gave the desired monomers with free carboxylic acid (**8a,b,d**).

The synthesis of the other diastereomers with (2*S*,4*R*) and (2*R*,4*R*) stereochemistry having thymine as a nucleobase requires an additional Mitsunobu



Scheme 2 a) PPH_3,DIAD,Methyl Tosylate b) NaN_3, DMF,80% c) Ra/Ni, H_2 d)Boc unhydride, 85% e) LiCl:$NaBH_4$ 80% f)MsCl, Py. 90% g)Thymine, K_2C0_3, DMF h)H_2,Pd-C I)$BrCH_2COOEt$, THF 90% j)1N NaOH/MeOH 96%

reaction to invert the stereochemistry at C-4 center (Scheme 2). The 4*R* hydroxy group was converted to its 4*S* tosylate **10**, which was then converted to its azide

derivative **11** *via* SN2 displacement reaction to give 4*R* derivative **11**. The azide function in **11** was reduced to amine and protected as its Boc-derivative **12**. The reduction of methyl ester was followed by conversion of the alcohol to its mesyl intermediate **14.** *N*-1 alkylation of thymine was effected by reaction with mesylate **14** to get **15**. The pyrrolidine ring nitrogen in **15** was then deprotected by hydrogenation and immediately alkylated with ethyl bromoacetate to afford 4(*S*)-(*N*-Boc-amino)-2-(*S*/*R*)-thymine-1-ylmethyl)-pyrrolidine-*N*1-ethyl acetate **16** which upon hydrolysis yielded the free acid **17.** Synthesis of (2*R*,4*S*) thyminyl monomer **18** was accomplished using the same steps starting from (2*R*,4*S*) proline methyl ester **9b**. The monomers were unambiguously characterized by optical rotation, NMR and mass spectroscopy.

The monomers with different stereochemistries were incorporated into oligomers at different, pre-determined positions using solid phase peptide synthesis employing the Boc-protection strategy. The peptide oligomers were cleaved from the Merrifield resin using TFA/TFMSA, which yielded oligomers bearing a C-terminal β- alanine residue with a free carboxylic acid. These were suitably purified and characterized

19	H-T T T T T T T T -NH$(CH_2)_2$COOH
20	H-T TT T T T T **t**-NH$(CH_2)_2$COOH
21	H-T T T **t** T T T **t**-NH$(CH_2)_2$COOH
22	H-T T T **t** T T T T-NH$(CH_2)_2$COOH
23	H-**t** T T T T T T T -NH$(CH_2)_2$COOH
24	H-GTAGATCACT-NH$(CH_2)_2$COOH
25	H-GTAGA **t** (2*R*,4*S*) CACT-NH$(CH_2)_2$COOH
26	H-GTA **g** (2*R*,4*S*)ATCACT-NH$(CH_2)_2$COOH
27	H-GT **a** (2*R*,4*S*) GATCACT-NH$(CH_2)_2$COOH
28	H-GTAGAT **c** (2*R*, 4*S*) ACT-NH$(CH_2)_2$COOH
	DNA/RNA sequences
29	5'- AGT GAT CTA C-3' DNA (*ap*)
30	5'-CAT CTA GTG A-3' DNA (*p*)
31	5'- AGU GAU CUA C-3' RNA (*ap*)
32	5'- AGT G T T CTA C-3' DNA (*ap*)

Table -1 PNA and DNA/RNA sequences T, C, G, A represent *aeg*PNA unit and **t, c, g, a** represent pyrrolidine PNA unit, **T** is a mismatched base.

by MALDI-TOF mass spectrometry prior to studying their binding properties.

Mixed sequence oligomers were also synthesized as shown in the table (Table 1). These were subjected to biophysical studies and examined for complementary DNA/RNA binding affinity.

UV melting experiments :

Triplexes derived from PNAs with four thyminyl distereomers of pyrrolidinyl unit (**19-22**) showed stability that was dependent on stereochemistry as well as position of the modified unit in the sequence. The complex formation was found to be stringent as evident from the melting studies with a single T-T mismatch DNA sequence **32** introduced at a central position. In the case of PNA:DNA/RNA duplexes with (2*R*,4*S*) stereochemistry having A,G,C and T nucleobases, at their respective positions (**25-28**) destabilized the complexes. It was found that (1) (2*S*,4*R*) and (2*R*,4*R*) stereoisomers were effective in enhancing DNA duplex stability (2) *cis* (2*S*,4*S*) and (2*R*,4*R*) remarkably enhance PNA:RNA duplex stability (3) all pyrrolidinyl modifications preferred antiparallel DNA/RNA binding and (4) PNA:RNA hybrids were more stable than DNA hybrids.

Chapter 3: Synthesis and hybridization studies of (2*S*, 4/5*S*)-*N*(t-butoxycarbonyl)-*N*- (thyminylacetyl) pipecolic acid

This chapter describes the modification of neutral, achiral PNA with an aim to

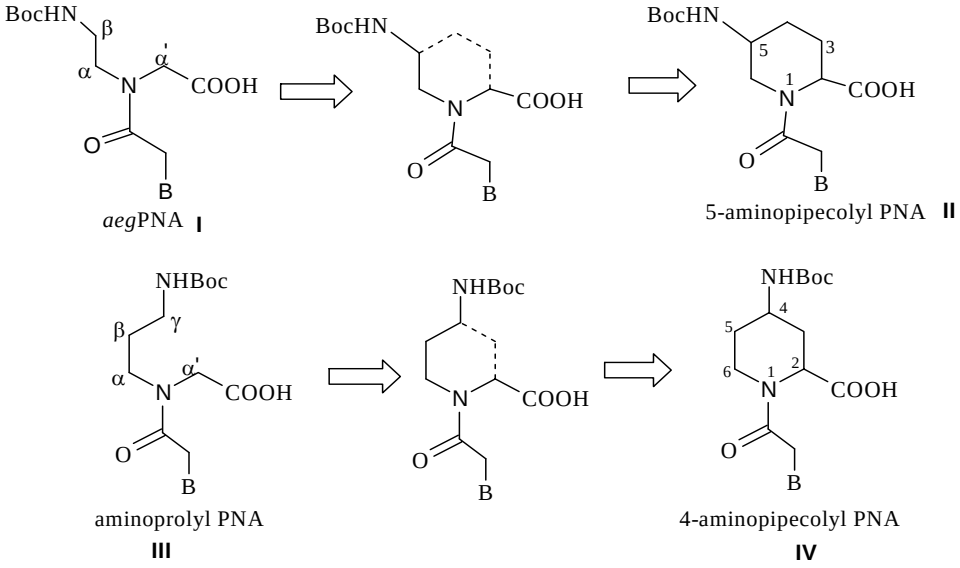
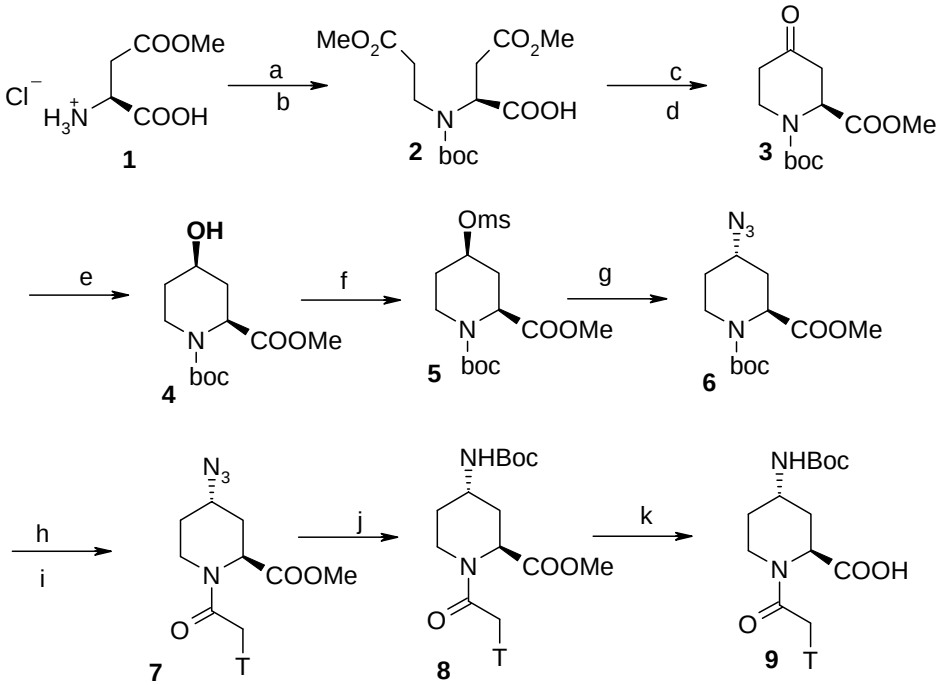


Figure 3: 5-amino- (II) and 4-amino- (IV) Pipecolyl PNA

improve its hybridization properties to complementary DNA/RNA sequences. New chiral pipecolyl backbone is introduced in place of the *aeg* backbone of PNA (Figure 3, I). Ethylene bridge was introduced between β carbon atom of ethylene diamine and α'carbon atom of the glycine unit to get 5-Aminopipecolyl PNA monomer (Figure 3, II). In the case of 4-amino backbone (Figure 3, IV) methylene bridge was introduced between the γ carbon atom of the aminopropyl segment (Figure 3, III) and α' carbon atom of the glycyl segment.

Synthesis of 4-aminopipecolyl PNA monomer:

4-amino-pipecolylPNA monomer (IV) was synthesised from 4-hydroxy L-pipecolic acid (Scheme 3), which in turn was synthesised from β methyl-L-aspartate hydrochloride according to the reported procedure. Thus β methyl-L-aspartate hydrochloride was treated with excess of triethyl amine and methyl acrylate followed by N-protection as its Boc derivative. Formation of piperidine ring was accomplished by treating diester **2** with sodium methoxide. The β keto ester

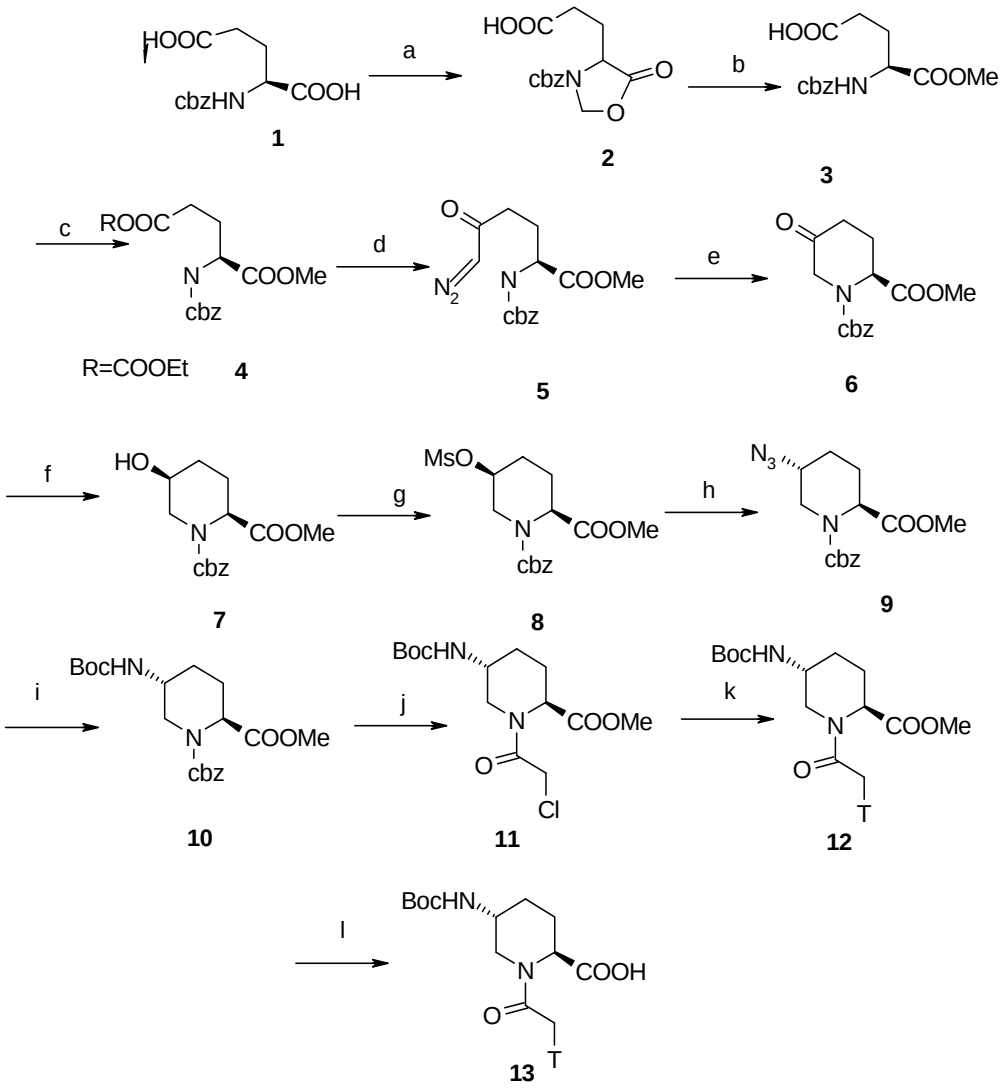


Scheme 3 a) Et_3N, Methyl acrylate b) $(Boc)_2O$, 79% c) NaOMe, MeOH d) H_2O e)$NaBH_4$, MeOH, 80% f) MsCl, Py, 80% g) NaN_3, DMF, 75% h) TFA,DCM I) Thyminyl acetic acid, HOBt, DIPCDI, DMF j) H_2, Pd-C, $(Boc)_2O$ k) 1N NaOH, MeOH

obtained was decarboxylated *insitu* by addition of H_2O and heating to get compound **3**. The keto group in **3** was reduced to alcohol by sodium borohydride. The 4-hydroxy group was converted to azide derivative **6** *via* mesylate **5**. The Boc group was deprotected with TFA/DCM and resulting free amine was coupled with thyminyl acetic acid to get compound **7.** The azide group in **7** was was hydrolyzed to get free acid **9**, which was used to build the PNA oligomers (**14-17** Table 2).

Synthesis of 5-Aminopipecolyl PNA monomer

The chiral pipecolyl PNA thymine monomer *trans* 5*R*-(*N*-Boc–amino)-*N*-



Scheme 4 a) PTSA, $(CH_2O)_n$, 89%. B) NaOMe, MeOH, 90%, c) ClOOEt d) CH_2N_2, 60% e) $Rh(OAC)_2$, 40% f) $NaBH_4$,MeOH, 85%g) MsCl,Py, 60% h) NaN_3, DMF, 70%, i)H_2,/ Pd-C, Boc unhydride, 90% j) $ClCH_2COCl$ 60% k) Thymine, K_2CO_3, DMF, 73% l) 1N NaOH,MeOH, 90%

(thymin-1-ylacetyl)-2*S*-pipecolic acid **13** was synthesized from *cis*-*N*-benzyloxycarbonyl-5*S*-hydroxy–2*S*-pipecolic acid methyl ester **7** as shown in Scheme 4. Compound **7** was synthesized from L-glutamic acid **1** by known procedure. The 5-hydroxy group was converted to azide derivative **9** *via* mesylate **8** with inversion of configuration. The azide group in **9** was selectively reduced to amine, which was then protected as its Boc derivative **10**. The ring nitrogen in **10** was deprotected by hydrogenation and then subjected to acylation with chloroacetyl chloride to get **11**. *N*-1 alkylation of thymine was effected by treatment with chloro derivative **11** in presence of K_2CO_3 to yield **12**. The methyl ester in **12** was hydrolyzed to free acid **13**, which was used to build the PNA oligomers **14-17** listed in Table 2. The binding properties of these modified PNA oligomers with complementary DNA sequences using CD and UV is also described.

Hybridization studies:

The binding stoichiometry of PNA_2:DNA complexes was ascertained by CD-mixing experiments and was found to be 2:1. Complex formation was evaluated from UV-Tm measurements and substantiated by gel electrophoresis data. In general, the hybridization affinities of oligomers containing 5-aminopipecolyl (2*S*,5*R*) units for complementary DNA were greater than those containing 4-aminopipecolyl (2*S*,4*R*) units, with the exception of a single C-terminal (2*S*,5*R*) unit (Table 3) .The (2*S*,5*R*) unit, thus seems to be more efficient in inducing a conformation that is preorganised to bind complementary DNA better than the (2*S*,4*R*) unit in the case of PNA_2:DNA triplexes.

Sequences	PNA	2*S*, 4*S*	2*S*, 5*S*
PNA-14	H- T T T T T T T T T t –β-ala-OH	67	61
PNA-15	H -t T T T T T T T T T –β-ala-OH	56	68
PNA-16	H- T T T t T T T T T t –β-ala-OH	34	78
PNA-17	H- T T T t T T T T T T –β-ala-OH	47	86
PNA-18	H- T T T T T T T T T T –β-ala-OH	67	
DNA–19	5’- G C A A A A A A A A C G – 3’		
DNA-20	5’- G C A A A T A A A A C G – 3’	-	57(14:20)

Table-2 t= 4/5-*aminopipecolyl*PNA**,** T = *aeg*PNA **T** =mismatched base in DNA

The introduction of a single mismatch in the complementary DNA strand (DNA **20**) led to the anticipated decrease in the Tm value of the corresponding triplex

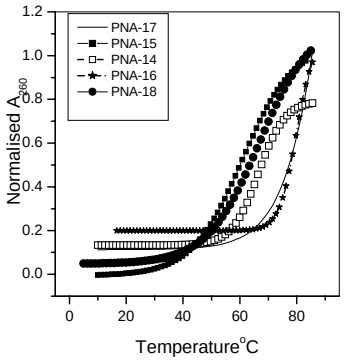


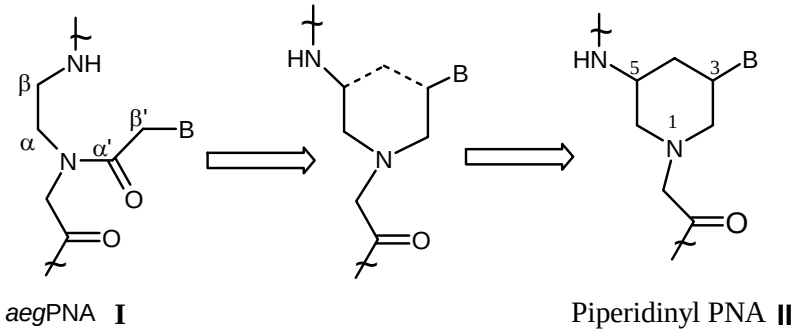
Figure-4 UV-Tm plots of 5-aminopipecolyl PNA:DNA

(**14:20**) as compared to the fully complementary triplex (14:19). This result supported that the 5-aminopipecolyl monomer participated in Watson-Crick hydrogen bonding interactions and contribute to over all sequence-specific recognition and binding of octamers to complementary DNA.

Mixed sequence oligomers were also synthesized. In all the cases with DNA, antiparallel duplex is more stable than the parallel one. Destabilization was observed upon introduction of a single 4/5 aminopipecolyl unit in the middle of an antiparallel PNA sequence. The observed results are discussed with respect to PNA structure.

Chapter 4: Synthesis and biophysical studies of piperidinyl PNA

The efforts directed towards refining *aeg* properties were extended by the

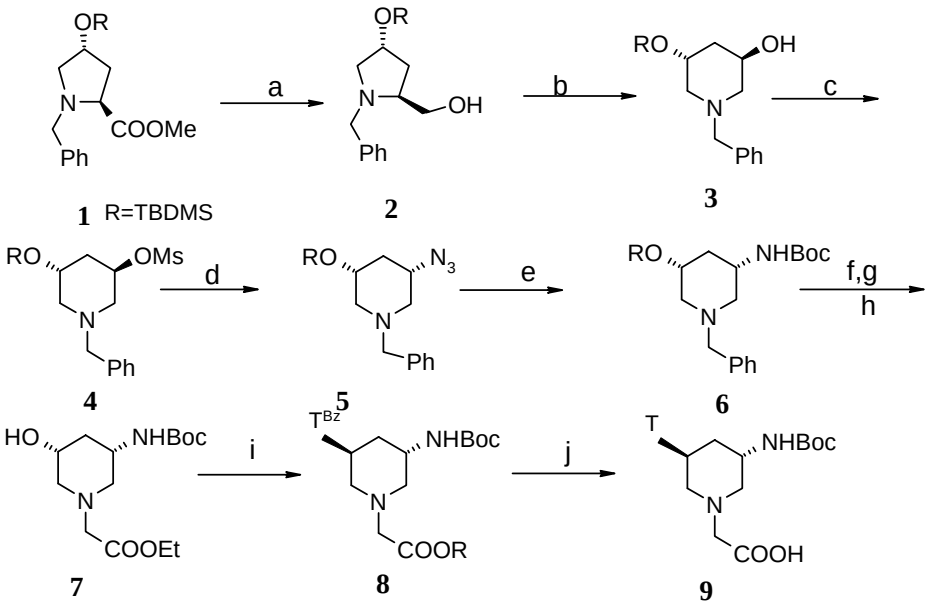


*aeg*PNA **I** Piperidinyl PNA **II**

Figure 5 Piperidinyl PNA

construction of five-membered pyrrolidine based conformationally restricted chiral PNA. Our efforts directed towards generating positively charged chiral six-membered PNA resulted in the synthesis of piperidinyl PNA. (Figure 5, II) The introduction of rigid conformation of six-membered rings that determines the orientation of the ring substitution with respect to each other will further add to the structural diversity of PNA. The new six-membered piperidine PNA analogue, which is a hexitol nucleic acid surrogate has been synthesized by introducing a methylene bridge between the β carbon atom of the ethylene diamine and β' carbon atom of the linker to the nucleobase (Figure 5).

The design of the monomer is configured in such a way that, to maintain the preferred *N*1- equitorial alkyl substitution on piperidine ring and 1,3 *cis* diequitorial



Scheme 5 a)$LiBH_4$/THF; b) TFAA,Et_3N c) MsCl/Pyridine; d) NaN_3/DMF; e) Ra-Ni/H_2, Boc anhydride; f)H_2/Pd-C; g) $BrCH_2COOEt$/DIPEA; h) TBAF/THF; i) N3-benzoylthymine, PPh_3/DIAD j)NaOH/water:MeOH

disposition of the backbone, the orientation of the base needs to be axial. This is a unique example of a rationally designed PNA analog that takes advantage of the conformationally frozen six-membered ring having substituents in definite preferred orientations with respect to each other. The synthesis of *trans*- (3*S*,5*S*)-3-(thymin-1-yl)-5 (t-butyloxycarbonylamino)-piperidine-1ylacetic acid was achieved from *trans*-

4 hydroxy proline. The suitably protected derivative of *trans*-(2*S*,4*R*)-4-hydroxy-L-proline (Scheme 5) was converted to the *trans*- (2*S*,4*R*) pyrrolidine-2 methanol 2 by reduction of the ester function. Treatment with trifluroacetic anhydride followed by diisopropylethyl amine gave six membered rearranged product **3** (3*R*,5*R*) with retention of configuration. Mesylation of resulting unprotected hydroxyl group and reaction with excess sodium azide gave *cis* azide **5** (3*S*,5*R*) with inversion of configuration at C3. Compound 5 was selectively hydrogenated with Ra-Ni and the resulting free amine was then protected as its Boc derivative **6**. The ring nitrogen protection was removed by hydrogenation and was set for alkylation with ethylbromoacetate followed by removal of silyl protection to give **7**. *Trans*-5*S*-(*N*3-benzoyl-thymin-1-yl-3*S*-t-butoxycarbonylamino)methyl piperidine derivative **8** was synthesized under Mitsunobu condition. The ester function and *N*-3 - benzoyl group of thymine were hydrolyzed with methanolic sodium hydroxide to get the free acid **9**.The piperidinyl PNA monomer **9** was used to build the PNA oligomers listed in Table 3. The biophysical studies of the complexes of modified oligomers with complementary DNA sequences in order to evaluate their binding affinity and specificity are described. The binding stoichiometry of PNA:DNA complexes were ascertained by CD- mixingexperiments. Mixed sequence oligomers were also synthesized and were subjected to biophysical studies and examined for complementary DNA binding affinity. DNA complementation studies of the piperidinyl PNAs by UV-Tm measurements indicates that these PNAs form stable PNA_2:DNA complexes(Table 3).

	Sequences[b]	UV-T_m °C
PNA 10	H-TTTTTTTT-β-ala-OH	43 (10)[c]
PNA 11	H-TTTTTTTt-β-ala-OH	50.7 (15)
PNA 12	H-TTTtTTTt-β-ala-OH	54.4 (9)
PNA 13	H-TTTtTTTT-β-ala-OH	41.4 (6)
PNA 14	H-tTTTTTTT-β-ala-OH	35 (15)
DNA 15	5'-GC$(A)_8$ CG-3'	

Table 3 [b]T represents *aeg*PNA unit and **t** represents piperidine PNA unit.[c]c in the parenthesis indicate % hyperchromicity.

ABBREVATIONS

β-ala	β-alanine
A	Adenine
aeg	Aminothylglycine
Aep	Aminoethylprolyl
ala	Alanine
ap	Antiparallel
Boc	Tert. butyloxycarbonyl
C	Cytosine
Cbz	benzyloxy carbonyl
CD	Circular Dichroism
dA	Deoxy adenine
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexyl urea
dG	2’-deoxyguanine
DIAD	Diisopropylcarbodiimide
DIPCDI	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMF	N,N-Dimethylformamide
DNA	2’-deoxynucleic acid
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
Fmoc	9-Fluorenylmethoxycarbonyl
FPLC	Fast Protein Liquid Chromatography
g	Gram
G	Guanine
gly	Glycine
h	Hours

HBTU	O-Benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate.
HOBt	1-Hydroxybenztriazole
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IR	Infra red
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time Of Flight
MF	Merrifield Resin
mg	Milligram
MHz	Megahertz
μM	Micromolar
ml	Milliliter
mM	Millimolar
mmol	Millimoles
N	Normal
nm	Nanometer
NMP	N-methyl pyrrolidine
NMR	Nuclear Magnetic Resonance
p	Parallel
PCR	Polymerase Chain Reaction
PPh3	Triphenyl phosphine
PNA	Peptide Nucleic Acid
Pro	Proline
Pyr	pyrrolidinone
RNA	Ribonucleic acid
r.t	Room temperature
ss	Single strand/ Single stranded
T	Thymine
t-Boc	Tertiarybutyloxy carbonyl

TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TEA	Triethylamine
TFA	Trifluroacetic acid
TFAA	Trifluroacetic unhydride
TFMSA	Trifluromethanesulphonic acid
THF	Tetrahydrofuran
UV-Vis	Ultraviolet- Visible

Chapter 1

Introduction

1.1. INTRODUCTION

All organisms function through transfer of genetic information from DNA to RNA (Figure 1) to proteins, except in the case of retroviruses, where the information

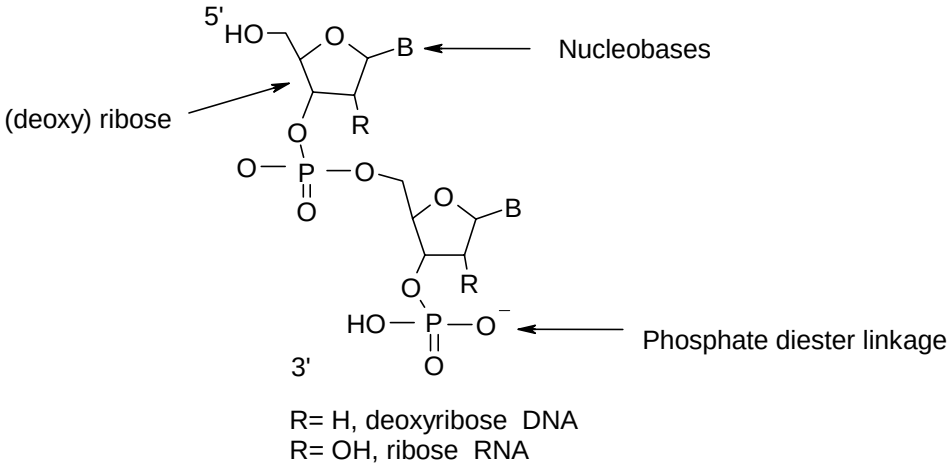


Figure 1 Chemical structure of DNA/RNA

is stored in viral RNA. In the past few years novel approaches to therapeutics, which involve intervention in these processes using synthetic oligonucleotides, have been developed. These have the capability of emerging as effective pharmaceutical agents. In effect, this approach represents a completely novel strategy that is in some ways intermediate between the classical drug paradigm of small molecule (lock and

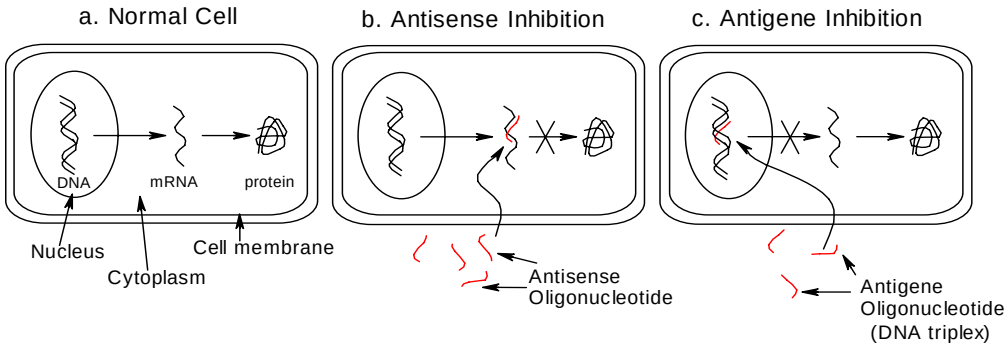


Figure 2 Principle of action of antisense and antigene oligonucleotides.

key) inhibitors, and gene therapy that involves the introduction of whole genes into affected individuals. In 1978 Zamecnik and Stephenson[1] proposed synthetic oligodeoxynucleotide analogs as a new class of potential therapeutic agents that could be rationally designed and interact selectively with target mRNA (Figure 2b). The principle mode of action of these modified oligonucleotides (ODNs) is through the binding *via* Watson-Crick base pairing (Figure 3),[2] to a specific mRNA sequence

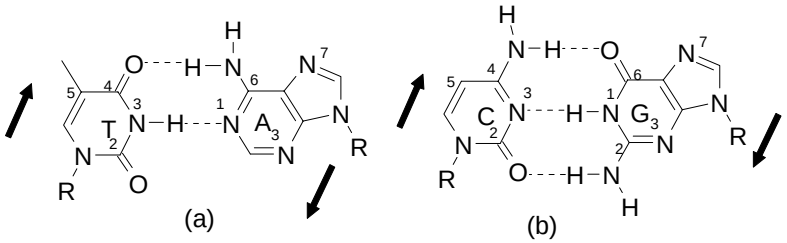


Figure 3 Watson-Crick hydrogen-bonding scheme for (a) T:A and (b) C:G base pairs.

associated with a diseased state and the subsequent inhibition of the translational event leading to a disease causing protein (antisense strategy).[3] Watson-Crick base pairing of complementary bases is a key feature of this strategy, as it ensures selective binding of antisense ODNs to the corresponding mRNA strand. If this mRNA:antisense-DNA duplex is stable and hence blocks the translational machinery of cell, then protein expression from this mRNA is prevented. Nature itself utilizes the antisense principle for the regulation of a variety of gene products and antisense RNA molecules have been found to be expressed intra-cellularlly. In addition, transcription can also be inhibited by the binding of an ODN to a duplex DNA *via* formation of triple helix (antigene strategy)[4] (Figure 2). In this 'antigene' concept, similar to antisense approach, the therapeutic oligonucleotide is targeted to the complementary duplex DNA principally through Hoogsteen base pairing[5] (Figure 4) to tackle the problem at its origin.

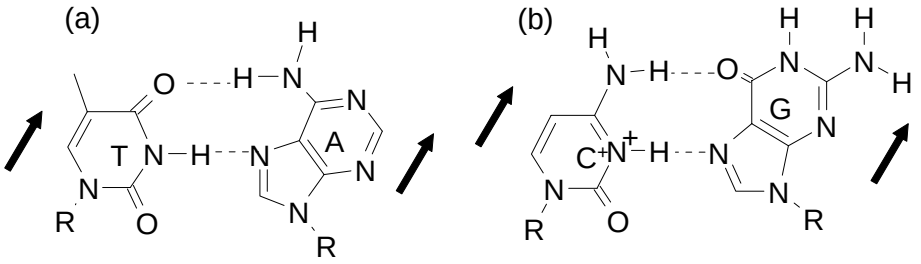


Figure 4 Hoogsteen base pairing scheme for (a) T:A (b) C:G

While the antisense approach is a convincing strategic model for the inhibition of gene expression, several challenges have to be met in order for synthetic ODNs to become therapeutic agents in biological system. Unmodified natural ODNs are rapidly degraded in the cellular environment due to the action of nucleases that hydrolyze the phosphodiester (PO) bond. As a consequence, considerable efforts have been focused on the synthesis of ODNs having modifications that render these compounds nuclease-resistant but still allow the formation of stable duplexes/triplexes with target nucleic acids and suppress translation. Additionally, these ODNs should be water-soluble, penetrate cell membranes and reach the cytoplasm or nucleus for their biological effect.

1.2. Mechanism of action of Antisense oligonucleotides

The apparent paradox to be understood is the mechanism through which most antisense oligonucleotides act. Following hybridization of an oligonucleotide to a complementary target sequence, the fate of the resulting DNA:RNA heteroduplex depends on the nature of the component oligonucleotide. Oligonucleotides possessing anionic internucleoside linkages, form heteroduplexes that are substrates for the ubiquitous enzyme ribonuclease H (RNaseH).[6,7] RNaseH cleaves the RNA

component of the heteroduplex, rendering the message permanently unavailable for translation.[8] In fact, the two RNA cleavage fragments produced from this reaction are generally unstable in the cell and are degraded rapidly. The oligonucleotide however, is unaffected by this reaction and may then dissociate from the cleaved RNA and bind to another target message. Therefore, the potential exists for a few oligonucleotide molecules to completely eliminate an entire pool of target message.

A second possible mode of action operates when the DNA:RNA heteroduplex is not a substrate for intracellular RNaseH. This occurs when the heteroduplex contains a chemically modified oligonucleotide that is incapable of activating RNaseH. Such oligonucleotides have been shown to be effective antisense agents[9,10] and are thought to act by a steric blocking mechanism that inhibits the ribosome, initiation factors or other proteins from binding to specific sites on the mRNA transcript. It seems likely that strong binding to the target mRNA is essential for steric blocking ODNs, and that these agents are more effective when targeted to non-coding regions.

1.3 Antisense oligonucleotide modifications

Since antisense ODNs are 18-20 mer negatively charged molecules, their cellular uptake poses a major challenge. In addition to the requirements outlined above, antisense ODNs must display good pharmacokinetic and pharmacodynamic properties.

Overall bioavailability should be high with slow degradation and clearance from the body. To address these requirements of a successful drug candidate, various structural modifications of ribo/deoxyribonucleotides are studied. These can be broadly distinguished as, i) analogs with unnatural bases[11] ii) modified sugars

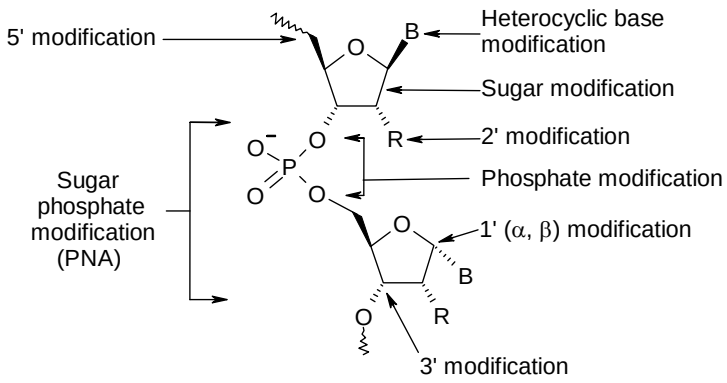


Figure 5. Structurally possible DNA modification sites.

(especially at the 2' position of the ribose) iii) altered sugar-phosphate backbone[12]. A significant knowledge is made available over the last two decades regarding the uptake,[13] degradation,[14] specificity,[15] toxicity[16] and efficacy of antisense oligonucleotides. The information derived from asking the complex mechanistic questions has driven the development of several new classes of modified oligonucleotides. Modifications involving the phosphate bond, base, and sugar moiety of oligonucleotides have created numerous compounds with varying physical and biological properties. The major representatives of first and second generation DNA analogs that are the best known and most widely used AS-ODNs todate are phosphate modifications such as the phosphorothioates,[17,18] phosphorodithioates, 2'-O-methyl and 2'-O-methoxyethyl RNA,[19] phosphoramidates[20] and phosphotriesters and have already shown promising results.[21] Vitravene, a drug, based on the phosphorothioate ODNs is already approved.[22] The replacement of the ribose sugar by hexose or carbocycles[23] has not been very successful in terms of specificity of binding/hybridization. However, morpholino oligomers,[24] where the monomers are linked through neutral carbamate linkages[25] or through phosphoramidate linkages[16]

(Figure 6), have shown promising antisense activity as they have superior permeability properties.

The locked nucleic acids (LNAs)[26] invented by Wengel *et al.* were found to exhibit unprecedented stability of their complexes with complementary DNA and RNA. They are also stable to 3'-exonucleolytic degradation and possess good water solubility. LNAs are oligonucleotides containing one or more 2'-O, 4'-C-methylene-β-D-ribofuranosyl nucleotides (Figure 6). The conformational preorganization of LNA could be instrumental in imparting the enhanced binding affinity to DNA.

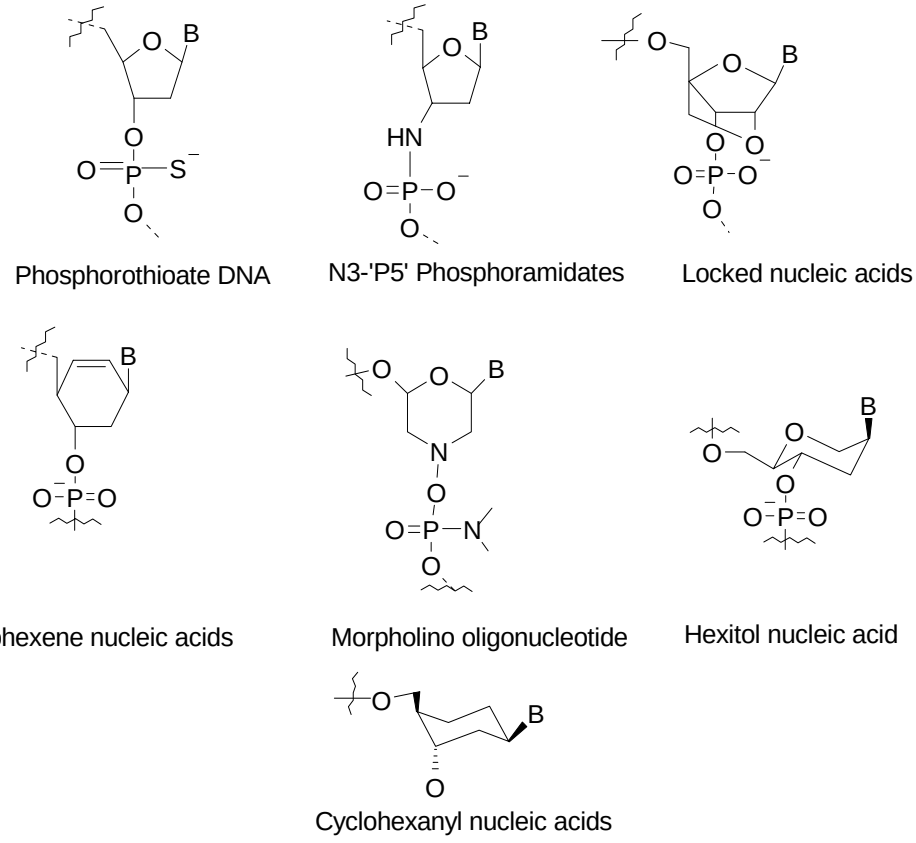


Figure 6 Modified antisense oligonucleotides.

However, more biochemical investigations are required to evaluate their success in the antisense/antigene context.

Replacement of the five membered ring by a six membered ring is the basis for cyclohexene nucleic acids, which are characterized by a high degree of conformational rigidity of the oligomers. Hexitol nucleic acids (Figure 6), composed of 2,3-dideoxy-1,5-anhydro-D-arabinohexitol forms very stable complexes with DNA and RNA and are enzymatically stable. Cyclohexanyl nucleic acid, which adopt conformation with an equatorial oriented base, found to be unable to hybridize with natural nucleic acids. [27]

1.4 Peptide Nucleic Acids (PNA)

Peptide Nucleic Acids (PNAs) developed by Nielsen and co-workers[28] are a remarkable example of a simple, neutral, and achiral whole backbone replacement, which in many ways have surpassed other attempts to mimic the native nucleic acid

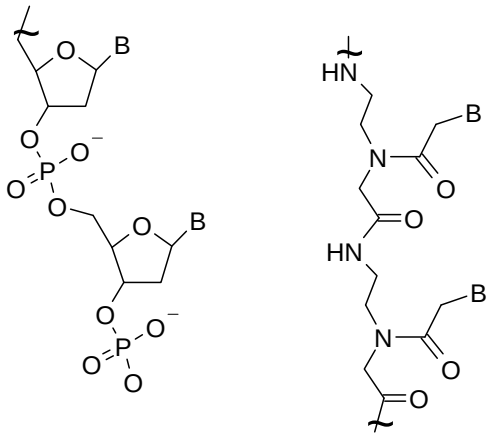


Figure 7 DNA and PNA structures

structures in terms of molecular recognition properties.[29] It was designed by computer assisted modeling of triple helices. The deoxyribose backbone of the third

strand (also known as the Hoogsteen strand) was replaced by a pseudopeptide backbone by atom to atom replacement, thus obtaining a DNA mimic that is uncharged and achiral (Figure 7).[30] The nucleobases are attached to the backbone *via* methylene carbonyl linkages. The PNA backbone maintains the same approximate length per repeating unit as in DNA or RNA and the appended nucleobases project from the backbone to form stable double or triple helical complexes with target nucleic acids.

Because PNAs have a neutral backbone, hybridization with target nucleic acids is not affected by the interstrand negative charge electrostatic repulsions.[31] PNAs are not the substrates for nucleases or proteases[32] and absence of repetitive charged backbone also prevents PNAs from binding to proteins that normally recognize polyanions, avoiding a major source of nonspecific interactions.[33]

Being achiral, PNAs bind DNA/RNA in either parallel or antiparallel modes, the antiparallel mode being slightly preferred over the parallel one. The antiparallel mode refers to the instance when the PNA 'N' terminus lies towards the 3'- end and

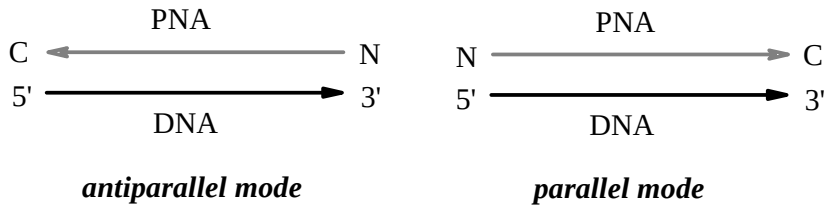


Figure 8 Parallel and antiparallel modes of PNA-DNA binding

the 'C' terminus, towards the 5'- end of the complementary DNA/RNA oligonucleotide. Likewise, the parallel mode of binding is said to occur when the PNA 'N' terminus lies towards the 5'- end with the 'C' terminus towards the 3'- end of the complementary DNA/ RNA oligonucleotide (Figure 8).

1.4.1. Peptide Nucleic Acid: Targeting double stranded DNA

PNA was originally conceived as a mimic of a triplex forming oligonucleotide (Figure 9a). It was observed that the homopyrimidine PNAs bind to a target homopurine nucleic acids strongly and sequence specifically by the formation of 2:1 PNA_2:DNA complex.

Further, these homopyrimidine PNAs are capable of forming unprecedented strand invasion complexes by displacing one of the DNA strands, with one PNA strand binding by Watson and Crick base pairing in an antiparallel orientation and the other binding in a parallel Hoogsteen fashion (Figure 9b). These strand invasion complexes, once formed, are extremely stable and can effectively interfere with DNA-protein interactions involving restriction enzymes.[34] The efficiency of this strand invasion can be further increased using *bis*-PNAs, consisting of two homopyrimidine PNA oligos connected *via* a flexible linker because attachment of

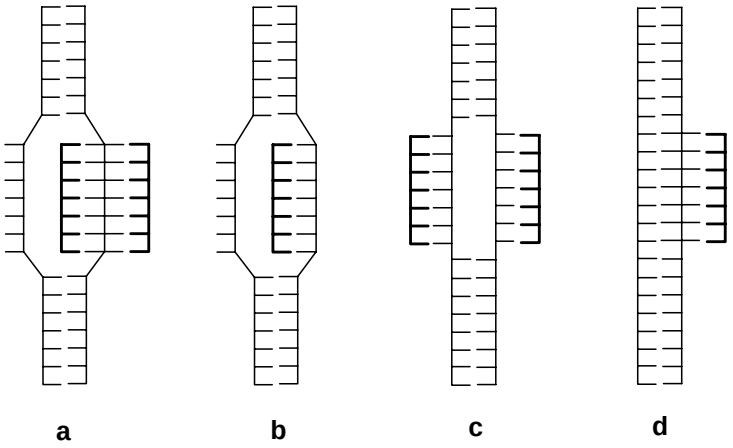


Figure 9 Schematic drawing of the mode by which PNA has been shown to bind to duplex DNA targets. PNA oligomers are in bold.

the two PNAs reduces the entropic penalty of binding. Furthermore, *bis*-PNAs with pseudoisocytosine[35] or *N*-7 guanine[36] bases, instead of cytosine bases on the Hoogsteen half, bind more tightly to dsDNA independent of pH. Some evidence also

suggests that strand invasion by *bis*-PNAs is likely to occur by a two step process where the Hoogsteen H-bonds form first to give a PNA$(DNA)_2$ intermediate. This is then followed by Watson and Crick base pairing and strand invasion, resulting in the bimolecular P-loop. This property has earmarked PNA as a potential antigene agent.[37] The strand invasion complex formation by mixed purine-pyrimidine sequences *via* duplex formation (Figure 9c) is reported as a rare example[38]. Using alternative pseudocomplementary nucleobases, such as the 2,6 diaminopurine, 2-thiouracil base pair, very stable, double duplex invasion complexes can be formed (Figure 9d).[39]

1.4.2. PNA complex stability

Duplexes between PNA and DNA or RNA are in general thermally more stable than the corresponding DNA-DNA or DNA-RNA duplexes.[33,40] PNA-DNA duplexes show increased stability when the purines are in the PNA strand. It is also observed that the thermal stability of PNA-PNA duplexes exceeds that of PNA-RNA duplexes, which again are more stable than PNA-DNA duplexes. Very importantly, the stability of PNA-DNA duplexes are almost unaffected by the ionic strength of the medium (actually the stability decreases slightly with increasing Na^+ concentration due to counterion release upon duplex formation). This is in sharp contrast to the behavior of DNA-DNA (or RNA) duplexes, the stability of which decreases dramatically at low ionic strength because of the requirement of counterion shielding of the phosphate backbone.[41]

PNA-DNA-PNA triplexes exhibit extraordinarily high stability (the thermal stability for a ten-mer is typically upto 70^oC). However, the rate of formation of

such ternary triplexes is slow giving rise to significant hysteresis of the thermal transition.

1.4.3. Structure of PNA Complexes

So far the three-dimensional structures of four PNA complexes have been determined. The PNA-RNA[42] and PNA-DNA[43] duplex structures were determined by NMR methods, while the structures of a PNA_2:DNA triplex[44] and a PNA:PNA duplex[45] were solved by X-ray crystallography. Several general conclusions can be drawn from these structural studies.

In general the PNA is able to adapt to a great extent to its partner. In the PNA-RNA and PNA-DNA duplexes the oligonucleotide adopts close to its natural A conformation and B-conformation receptively in terms of sugar puckering, while the helix parameters have both A- and B-form characteristics. The PNA does, however, prefer a unique, different helix form, the P-form, which is taking over already in the PNA_2DNA triplex and is of course fully developed in the PNA-PNA duplex. This helix is very wide (28 Å diameter) and has a very large pitch (18 base pairs). In terms of base pair conformations it is a very regular helix, and the base pairs are virtually perpendicular to the helix axis.

1.4.4. Solubility of PNA

PNAs are neutral compound with a tendency for self-aggregation and limited water solubility. However the introduction of charged groups at N/C terminus, or introduction of positive charges by modification of PNA backbone greatly improves their properties.[46]

1.4.5. Antigene properties of PNA

PNAs should be capable of arresting transcriptional process by virtue of their ability to form a stable triplex structure or a strand- invaded or strand displacement complex with DNA. Such complexes can create a structural hindrance to block the stable function of RNA polymerase and thus are capable of working as antigene agent[47,48] causing the concomitant complete distortion of the DNA helix and therefore PNA gene targeting at the DNA level should be very efficient. The main obstacle appears to be the access of PNA to the DNA under physiological conditions, which includes the presence of cations that stabilize the DNA double helix and dramatically reduce the rate of helix invasion by the PNA.[49] Several modifications of PNA have shown improvement in terms of binding.[50,51,52] Interestingly, PNA triplex invasion loops are also recognized by RNA polymerases as transcription initiation sites. These findings are highly relevant for the possible future use of PNAs.

1.4.6. Antisense properties of PNA

PNAs have been reported to be less effective as antisense agents than the RNaseH-stimulating phosphorothioates. PNA-RNA hybrids are not substrates for RNaseH,[53,54] the enzyme that is responsible for the antisense effect of phosphodiester and phospsorothioate oligonucleotides. PNAs could exert their antisense effect by the other mechanism that directs steric blocking of ribosomes or essential translation factors. It has been found by *in vitro* translation experiments that mixed purine-pyrimidine sequence PNAs that form PNA-RNA duplexes with the target, did not arrest translation elongation, indicating that the PNA was displaced by the moving ribosome. However, when homopurine targets are present in the translated region of the gene, these can be targeted by homopyrimidine (*bis*)

PNAs, which form extremely stable PNA_2-DNA triplexes that are indeed capable of arresting the ribosome during elongation.

It has been reported that DNA/PNA chimeras are able to stimulate RNA cleavage by RNase H on the formation of a chimeric heteroduplex. RNA cleavage occurs at the ribonucleotides which base pair with the DNA part of the chimera. The cleavage is sequence specific, as random sequence DNA/PNA chimeras do not cleave the RNA under the same condition.[48, 55]

1.5. Biological Applications of PNA.

1.5.1. Inhibition of telomerase activity

Human telomerase, a ribonucleoprotein complex consisting of a protein with DNA polymerase activity and an RNA component, synthesizes $(TTAGGG)_n$ repeats at the 3' end of DNA strands. PNA oligomers that are complementary to the RNA primer-binding site can inhibit the telomerase activity. Studies have shown that the telomerase inhibition activity of PNA is better than that of corresponding activity of phosphorothioate oligonucleotides.[56] This is mainly due to a higher binding affinity of PNA compared to the phosphorothioates. Corey and co-workers have demonstrated an efficient inhibition of telomerase after lipid-mediated delivery of template and non-template-directed PNA into cell.[57]

1.5.2. PNA microarray

The basic requirement for this array system is the ability of all different probes to hybridize to their target sequences with high specificity at single temperature. As PNAs are neutral, they can hybridize with nucleic acids in absence of counterions needed to stabilize pure nucleic acid duplexes. It has been demonstrated that PNA

probes can effectively discriminate between single base mismatches in the target sequence at temperature near the optimum for hybridization.[58] They can also be used to analyze and roughly quantify the amount of target molecules over a considerable concentration range. PNA arrays can be reused much more often than conventional oligonucleotide array, since the PNA molecules are extremely stable under conditions, which natural DNA can not withstand.

1.5.3 Extending Recognition by Peptide Nucleic Acids (PNAs): Binding to Duplex DNA and Inhibition of Transcription by Tail-Clamp PNA-Peptide Conjugates

Peptide nucleic acids (PNAs) are a powerful tool for recognition of double-stranded DNA. Strand invasion is most efficient when pyrimidine PNAs are linked to form a bisPNA in which one strand binds by Watson-Crick base pairing while the other binds by Hoogsteen base pairing to the newly formed PNA-DNA duplex. Within many genes, however, polypyrimidine target sequences may not be located in optimal positions relative to transcription factor binding sites, and this deficiency may complicate attempts to identify potent antigene PNAs. To increase the versatility of strand invasion by PNAs, bisPNAs and bisPNA-peptide conjugates containing a mixed base extension of the Watson-Crick polypyrimidine strand have been synthesized.[59] These tail-clamp PNAs (TC-PNAs) bind duplex DNA and inhibit transcription. DNA recognition occurs with single-stranded or TC-bisPNAs and requires attachment of positively charged amino acids. The ability to bind duplex DNA is not always necessary for inhibition of transcription, possibly because PNAs can bind to accessible DNA within the transcription bubble created by RNA polymerase[60]. These results, expand the range of sequences within duplex DNA that

are accessible to PNAs and suggest that TC-PNA-peptide conjugates are good candidates for further testing as antigene agents.

1.5.4 Antiviral PNAs

One of the key enzymes in the life cycle of retroviruses (such as HIV), reverse transriptase, is very sensitive to PNA "Antisense Inhibition'. Reverse transription of the RNA template is effectively arrested by PNA oligomers bound to the template.[61,62] This finding has raised hope that DNA antiviral drugs could be developed and report has shown that HIV replication in cell culture can be inhibited by PNA targeting gag-pol gene.[63] However, very high PNA concentrations were required emphasizing the need of an efficient cell delivery system for PNA.

1.5.5. Antibacterial PNAs

Inhibiting translation through directly interfering with the ribosome will shut down all protein synthesis, providing a very potent antibiotic strategy. Good and Nielsen demonstrated that PNAs targeted to two regions of ribosomal RNA, the alpha-sarcin loop and the peptidyl transferase center, were capable of causing cell death in bacterial colonies.[64] Homopyrimidine bisPNAs were more effective than the monoPNAs, indicating that triplex formation was important for the observed antibiotic activity. It has been reported that 9-12 mer PNAs attached to the cell wall/membrane –active peptide $(KFF)_3K$, provide improvement in antisense potency in E. Coli amounting to two orders of magnitude while retaining target specificity.[65] Peptide-PNA conjugates targeted to rRNA and to mRNA encoding the fatty acids biosynthesis protein Acp prevented cell growth of Ecoli K12 without any apparent toxicity to human cells, which indicates that peptide, can be used to carry antisense PNA agents into bacteria. Such peptide-PNA conjugates open exciting

possibilities for anti-infective drug development and provide new tools for microbial genetics. These results bear promise that PNA could be developed into 'genetic antibiotics'.

1.6. Cellular uptake of PNA

PNAs do not readily enter cultured cells unless present at high concentrations in the media[66] and unlike DNA/RNA, they cannot be complexed directly with cationic lipids because they are uncharged. However, Corey and co-workers have reported a novel method for *in vitro* cellular delivery of PNAs using cationc lipid.[67] The cationic lipid is capable of associating with the negatively charged phophodiester backbone of DNA and RNA and fusing with the cell membrane to allow the oligonucleotides to enter into the cell. Desired PNA oligomers are hybridized to overlapping oligonucleotides and the complex is mixed with cationic lipid. The cationic lipid-DNA-PNA complex thus formed can be internalized and the partially hybridized PNA is imported into the cell. Cellular uptake of PNAs can also be achieved by the attachment of peptide sequence that promote translocation across cell membranes.[68,69] Peptides such as trojan peptide and penetratin have been used as carriers for cellular delivery of PNA.[70] Another strategy that has been adapted to improvise the delivery of PNA *in vitro* is to incorporate it into delivery vehicles (vesicles), e.g. liposomes. Peptide nucleic acid- nucleic acid chimeras are reported to be taken up even at lower extracellular concentration (1μM), so PNA-DNA chimera may be better antisense agent. At higher concentrations of PNA, cytotoxic effects could also be observed.[71, 72]

1.9. Chemical modification of PNA

The structure of the classical PNA monomer has been subjected to a variety of rational modifications with the aim of understanding the structure-activity relations in this class of DNA mimics as well as obtaining PNA oligomers with specifically improved properties for various applications in medicine, diagnostics and molecular biology. The limitations of PNA for such applications include low aqueous solubility, ambiguity in DNA binding orientation and poor membrane permeability. Structurally, the analogues can be derived from modifications in the ethylenediamine or glycine part of the monomer, linker to the nucleobase, the nucleobase itself or a combination of the above. The strategic rationale behind the modifications are (i) introduction of chirality into the achiral PNA backbone to influence the orientational selectivity in complementary DNA binding, (ii) rigidification of PNA backbone *via* conformational constraint to pre-organize the PNA structure and entropically drive the duplex formation, (iii) introduction of cationic functional groups directly in the PNA backbone, in a side chain substitution or at the N or C terminus of the PNA to improve water solubility (iv) modulate nucleobase pairing either by modification of the linker or the nucleobase itself for effective binding at physiological conditions (v) conjugation with ‘transfer’ molecules for effective penetration into cells. In addition to improving the PNA structure as above for therapeutics, several modifications are directed towards their applications in diagnostics. Some of the modifications are discussed below.

Improvement of aqueous solubility of PNAs has been achieved by the introduction of charges within the molecule or by the introduction of ether linkages in the backbone (Figure 10). Making PNA anionic also aided in increasing the water solubility as in the case of the phosphonate analogs, but was accompanied by a decrease in the binding affinity to complementary nucleic acid sequence[73,74,75,76,77]

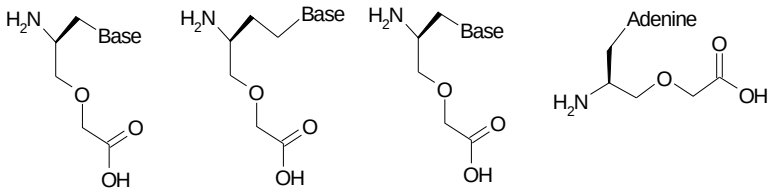


Figure 10 Ether-linked PNA (OPNA)

(Figure 11). The chiral versions of these analogs similar to original PNAs led to excellent aqueous solubility properties. PNAs composed of monomers derived from serine and homoserine coupled by ether linker with glycine or alanine, were able to bind sequence specifically to RNA, though with much weaker affinity.[78, 79]

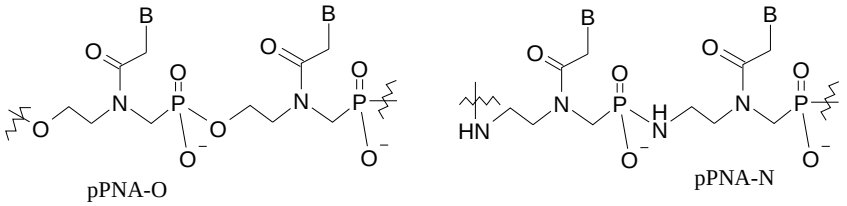


Figure 11 Phosphonate PNA

Novel class of cationic PNA (DNG/PNA) which binds to DNA/RNA targets with high affinity has been also reported[80] (Figure 12a). In another report guanidium functional group was introduced into the PNA backbone, which exhibited remarkable cellular uptake properties while maintaining Watson-Crick recognition with complementray DNA strand[81](Figure12b).

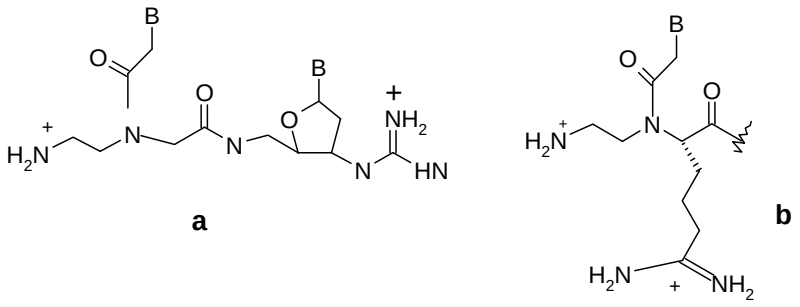


Figure 12 a) PNA-DNG chimera b) GPNA

Another type of modification involved interchange of various CO and NH groups on the peptide linkages leading to retro inverse,[82, 83, 84] peptoid[85] and heterodimeric analogs[86](Figure 13). Except for the heterodimer analogue (Figure 13C), these exhibited a lower potency for duplex formation with complementary

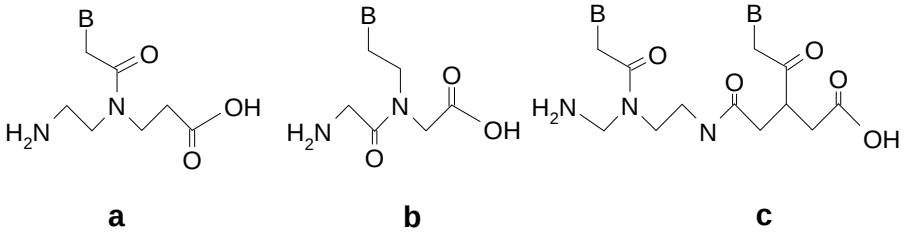


Figure 13 a. Retero-inverso, **b**. Peptoid and **c**. Heterodimeric PNA.

DNA/RNA suggesting that in addition to geometric factors, other subtle requirements such as hydration and dipole-dipole interactions influencing the environment of backbone, may be involved in effecting efficient PNA:DNA hybridization.

In another case, PNA backbone was extended by inserting a methylene group either in aminoethyl part and the linker to the nucleobase (Figure 14). The thermal stability of the hybrids between these PNA oligomers and complementary DNA oligonucleotide was significantly lower than that of the corresponding *aeg*PNA. However, the sequence selectivity was retained. Thymidyl decamers with these

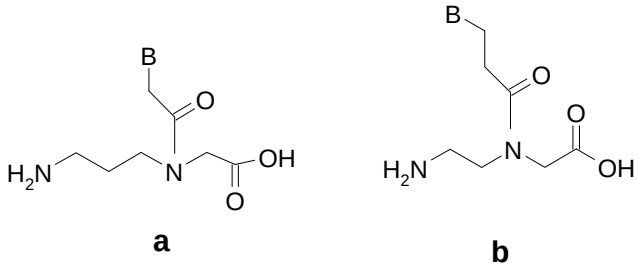


Figure 14 Backbone extended PNA

modified units were unable to hybridize to the complementary $(dA)_{10}$ oligonucleotide, whereas PNA decamer containing only ethylenecarbonyl linkers between the nucleobases showed weak affinity for complementary DNA.[87]

1.9.1. Construction of bridged PNA structures

Any favorable structural reorganization of PNA may trigger the shift in equilibrium towards the desired complex formation because of the reduced entropy loss upon complex formation, provided that the enthalpic contributions suitably compensate. This may be achieved if the conformational freedom in *aeg*PNA is curtailed by bridging the aminoethyl/glycyl acetyl linker arms to give rise to cyclic analogs with preorganized structure. Additionally, the introduction of chemical bridges into *aeg*PNA to provide cyclic structure may help in controlling the rotameric population by fixing the nucleobase orientation. Such modifications also introduce chiral centers into PNA monomeric units with the possibility of further fine-tuning the structural features of PNA to mimic DNA.

PNA with five membered Nitrogen heterocycles

Many researchers have exploited *trans*-4-hydroxy-L-proline for the synthesis of a wide variety chiral, constrained and structurally preorganized PNA.

Aminoprolyl PNA

The introduction of a methylene bridge between β- carbon atom of the aminoethyl segment and the α'carbon atom of the glycine segment of the *aeg*PNA resulted in 4-amino prolyl PNA, with the introduction of two chiral centers[88](Figure

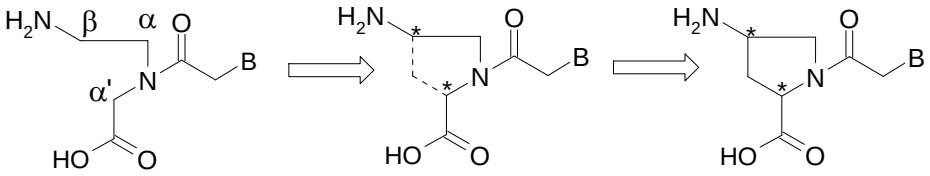


Figure 15 aminoprolyl PNA

15). Upon partial substitution of these monomers in to PNA oligomer, they exhibited tendency to hybridize with nucleic acids similar to that of unmodified PNA. Interestingly inclusion of even a single 4-aminoproline into a PNA sequence, either at the *N*-terminus or in the interior resulted in a very interesting CD profile and led to stabilization of derived PNA-DNA hybrids simultaneously effecting significant discrimination in the orientation of binding. The stability of such complexes decreases with increasing the number of chiral prolyl units and homooligomers derived from each of the diastereomers completely failed to form duplexes. In another report, alternating 4-aminopropyl and glycine units stabilize the complex suggesting that in homo-oligomer, inter-nucleoside distances are too low.

Gly-Pro-Peptide PNA

The methylene bridge was inserted between the α' carbon atom of the glycine unit and the β'- carbon atom of the nucleobase linker of *aeg*PNA[89, 90](Figure 16). Unlike other PNAs this has a tertiary amide group with the amide nitrogen part of a

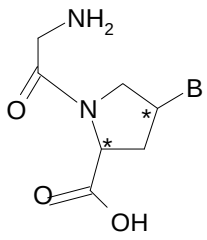


Figure 16 Gly-pro peptide PNA

cyclic ring system on the backbone. This leads to highly rigid structures that are not poised for effective duplex formation.

Aminoethylprolyl PNA

The replacement of the tertiary amide carbonyl on the backbone by a methylene

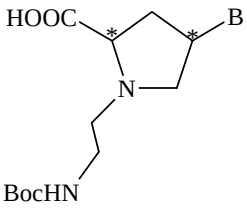


Figure 17 *aep*PNA

group relieves strain to generate *aep*PNA[91](Figure 17). These show remarkable biophysical properties in terms of triplex stabilities.[92] Hitherto unprecedented higher melting of the derived PNA:DNA hybrids reflected very significantly enhanced DNA affinity while retaining the base pair discriminating power. The mixed pyrimidine hairpin sequences with cytosine and N-7 guanine *aep*PNA units exhibited directional discrimination in binding to parallel/antiparallel DNA sequences.[36]

Aepyrrolidinone PNA

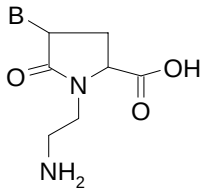


Figure 18 *aepone* PNA

In order to get the best characteristics from both the *aeg*PNA and the *aep*-PNA, the new monomer was synthesized restoring the amide character to the pyrrolidine ring nitrogen *via* selective C5 oxidation of *aep*-proline derivatized intermediate[93](Figure 18). Aepone-PNA oligomers stabilizes the derived triplexes with DNA but destabilizes the complexes formed with poly(rA).

Pyrrolidinone PNA

A methylene bridge was inserted between the α carbon atom of the aminoethyl segment and the β' carbon atom of the acetyl linker to the nucleobase of *aeg*PNA[94] (Figure19). The synthesis of all the four diastereomers of adenin-9-yl-*pyr*PNA was accomplished and the oligomers incorporating the (3*S*,5*R*) isomer were shown to have highest affinity for RNA compared to DNA.

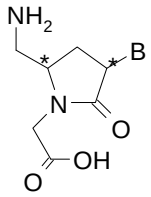


Figure 19 pyrrolidinone PNA

The fully modified decamer bound to rU_{10} with a small decrease in the binding efficiency relative to *aeg*PNA.[95]

Prolyl-(β-amino acid) Peptide PNA

The conformational strain in the alternating proline-glycine backbone was released by replacement of the α amino acid residue by different β amino acid spacers with apropriate rigidity.[96] Novel pyrrolidinyl PNAs comprising alternate

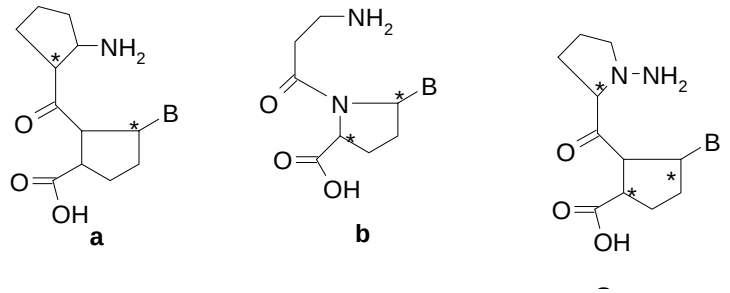


Figure 20 a) prolyl-2-amino cyclopentanecarboxylic acid, b) prolyl-β-alanine, c) prolyl-D/L-aminopyrrolidine carboxylic acid

units of nucleobases modified with D-proline, either D/L aminopyrrolidine 2 carboxylic acid, (1*R*,2*S*) –2-aminocyclopentanecarboxylic acid or β alanine were synthesized[97](Figure 20).

Pyrrolidine PNA and pyrrolidine PNA-DNA chimera

Insertion of a methylene bridge in *aeg*PNA, linking the α-carbon atom of the aminoethyl segment and the β'-carbon atom of the tertiary amide linker, afforded the pyrrolidine PNA.[98] A fully modified (2*R*,4*S*) pyrrolidine PNA decamer formed very stable complexes with both DNA and RNA targets. The incorporation of the (2*S*,4*S*) thymine monomer into oligomers and mixed pyrimidine oligomers resulted in a decreased binding efficiency with the target DNA/RNA sequences. The (2*R*,4*R*) isomer was incorporated into a PNA:DNA dimer amenable to the synthesis of PNA:DNA chimeras. The chimeric PNA:DNA bound to the target DNA with decreased efficiency relative to the native DNA.

A cyclopentane conformational restraint for a Peptide Nucleic Acid:

Based on molecular modelling studies (*S*,*S*) cyclopentadiamine ring was used for

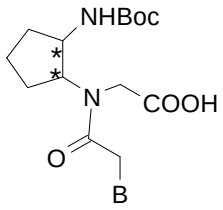


Figure 21 Cyclopentyl PNA

conformational restraint of the C2-C3 dihedral angle of the PNA backbone. The *trans* cyclopentane modification improves the stability of PNA-DNA triplexes and PNA-RNA duplexes for a poly-T PNA.[99] Recently cyclopentyl PNAs[100] having *cis* isomers have been reported (Figure 21). The results suggest that these have a stereochemistry dependent stabilization effect on binding both DNA and RNA. The *cp*PNAs have a better selectivity for mismatch DNA sequence and a higher binding to complementary DNA sequence than the unmodified PNA.

1.9.2. PNA with Six membered ring structures

Six-membered ring structures exhibit unique conformational preferences, and the binding abilities of hexose sugar phosphate containing oligonucleotide have been extensively studied by Eschenmoser etal.[101] The ability of morpholino,[102] hexitol,[103] and cyclohexene oligonucleotides[104] to bind to DNA/RNA are well established and are dictated by the conformational preferences of the six membered ring structures. Conformations in the six membered ring structures are rigid, in contrast to the relatively flexible five membered ring, and hence their influence on the stability of the resulting PNA-DNA/RNA complexes may be expected to make important contributions to the stabilities of the DNA/RNA complexes.

Glucosamine Nucleic Acids , GNAs

The six membered glucosamine ring appeared to fulfill the requirement of

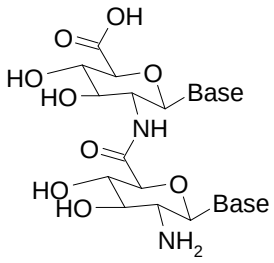


Figure 22 GNA

optically pure and constrained conformational scaffolding for the attachment of nucleobases (Figure 22). The homopyrimidine and mixed base sequences using GNA monomer were constructed. The binding affinities and selectivities of these oligomers to DNA and RNA targets indicated selective recognition of RNA by Watson–Crick hydrogen bonding.[105] The entropy changes were found to be smaller for GNA-DNA/RNA than for DNA-DNA/RNA, consistent with idea that the GNA oligomer was preorganized for binding to the target sequences.

Piperidinone PNA

Introduction of an ethylene bridge between the α carbon atom and β' carbon atom in the ethylene diamine and acetyl linker resulted in a six-membered ring structure – piperidinone PNA[106](Figure 23). (3*R*,6*R*) and (3*S*,6*R*) adenine monomers

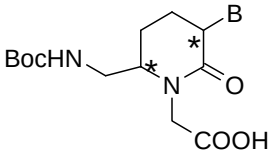


Figure 23 Piperidinone PNA

were synthesized and incorporated into *aeg*PNA which resulted in a large decrease in the duplex stability.

Cyclohexyl PNA

Introduction of conformational constraint in the *aeg*PNA resulted in the chiral cyclohexyl-derived backbone[107](Figure 24). The aminoethyl segment of the *aeg*PNA

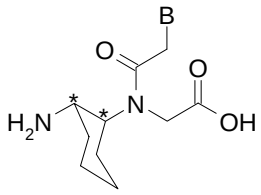


Figure 24 Cyclohexyl PNA

was replaced with a 1,2 diaminocyclohexyl moiety, either in the (*S*,*S*) or (*R*,*R*) configuration. The oligomers with (*S*,*S*)-cyclohexyl residues were able to hybridize with DNA or RNA , with little effect on thermal stability. Molecular modeling studies reveled that (*S*,*S*) isomer can be accommodated more easily in duplex than (*R*,*R*) isomer. In contrast, incorporation of the (*R*,*R*) isomer resulted in a drastic decrease in the stability of PNA-DNA/RNA complexes. The complexes formed by the two isomers were of the opposite handedness, as evident from CD spectroscopy.

Recently, synthesis of ethyl *cis* –(1*S*, 4*R*/1*R*,2*S*)-2-aminocyclohex-1-yl-*N*-(thymin-1-yl-acetyl)glycinate *via* enzymatic resolution of the *trans*-2 azido cyclohexanols has been reported.[108] The crystal structure of intermediate showed equatorial disposition of the tertiary amide group, with the torsion angle β in the range 60°-70°. UV-Tm experiments showed that (1*S*,2*R*) isomer preferred to bind RNA and (1*R*,2*S*) isomer showed higher affinity towards DNA in homothymine sequences leading to stereodiscrimination in recognition of DNA and RNA.[109]

Aminoethyl pipecolyl PNA

α carbon atom of glycyl unit and β carbon atom of linker to a nucleobase are bridged by an ethylene unit to get six membered aminoethylpipecolyl PNA[110](Figure

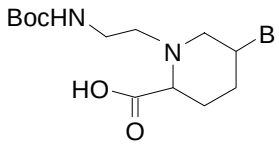


Figure 25 aminoethylpipecolyl PNA

25). UV-Tm studies indicated, (2*S*,5*R*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid, *aepip*PNA, when introduced into PNA oligomers, stabilize the resulting complex with complementary DNA.

[(Aminoethyl)amino]cyclohexanoic acid

Rigidity was introduced into the *aeg*PNA by replacing the glycyl segment in the backbone by α amino cyclohexanoic acid[111](Figure 26). Incorporation of these monomers into oligomers and their DNA/RNA binding properties has not yet been reported.

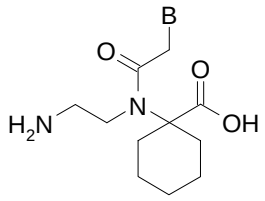


Figure 26 [(Aminoethyl)amino]cyclohexanoic acid

Morpholino PNA

The set of morpholino analogues with phosphonate esters, amide or ester linkages between the morpholino nucleoside residues was synthesized. Preliminary results indicated that amide-linked morpholino PNAs were better accommodated in the complexes than the ester or the phosphonate linked oligomers**.**

1.10. Modified Nucleobases

Non-natural nucleobases could aid in understanding of the recognition process between the natural nucleobase-pairs in terms of factors such as hydrogen bonding and internucleobase stacking. They could also generate new recognition motifs with potential applications in diagnostics. Only a few nucleobase modifications have been reported in the PNA context (Figure 27).

2,6-Diaminopurine offers increased affinity and selectivity for thymine[112]

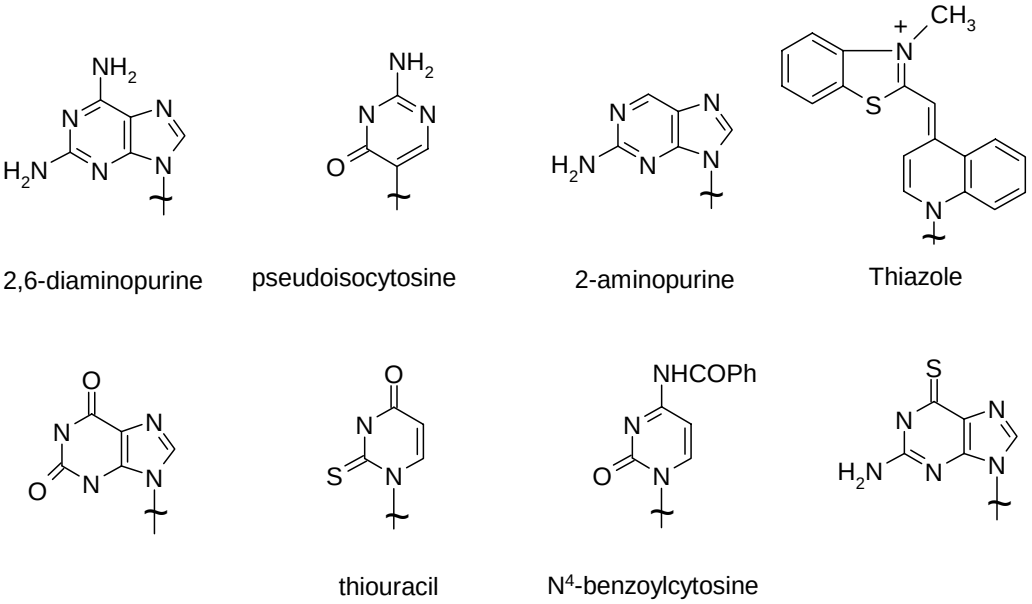


Figure 27 Modified nucleobases

and pseudoisocytosine mimics the C^+ recognition pattern[113] for triplex formation. 2-Aminopurine[114] can hydrogen bond with uracil and thymine in the reverse Watson-Crick mode and being inherently fluorescent, can be used to study the kinetics of the hybridization process with complementary nucleic acids. Replacement of *aeg* PNA with thiazole orange afforded a PNA probe that fluoresced upon hybridization. [115]

The E-base,[116] hypoxanthine,[117] N^4-benzoylcytosine[118] and 6-thioguanine [48]represent some more examples of modified nucleobases. Thiouracil along with 2,6-diaminopurine has been utilized as a non-natural base pair in PNA-DNA recognition and was shown for the first time to lead to a phenomenon termed as 'double duplex invasion'.

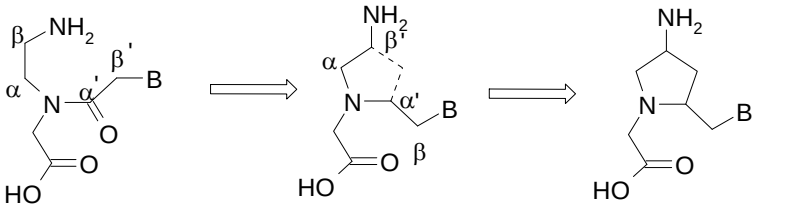
1.11. Present Work

The preceding section gives an overview of the Peptide Nucleic Acids, (PNA) the synthetic imitator of natural nucleic acids. The PNA backbone is radically different, although it is structurally homomorphous to nucleic acid forms. PNA exhibits excellent biochemical stability along with high binding affinity and high recognition specificity. In addition, owing to an electrically neutral pseudopeptide backbone, PNA provides a wider range of hybridization conditions and has an exceptional ability to invade duplex DNA. The major drawbacks like poor water solubility, inefficient cell uptake, self-aggregation and ambiguity in directionality of binding restricts its applications within medicine, diagnostics, molecular biology etc.

In order to overcome these limitations, several modifications of PNA have been reported. The work presented in this thesis involves the design, synthesis and biophysical studies of backbone-modified, chiral charged/ uncharged PNA analogues-Pyrrolidinyl, Pipecolyl and Piperidinyl PNA.

Chapter 2

This chapter describes the synthesis of chiral, positively charged modified pyrrolidine PNA monomer. The modification allows the introduction of rigidity in the PNA backbone while simultaneously allowing a certain degree of flexibility in the linker to the nucleobase. The monomer was derived by bridging the β carbon



*aeg*PNA **I** *pyrrolidine*PNA **II**

Figure 28 Pyrrolidine PNA

atom of the ethylenediamine unit and α' carbon atom of the linker to the nucleobase by a methylene group, with the removal of carbonyl group (Figure 28). The nucleobases are attached to the pyrrolidine ring *via* a flexible methylene group. The synthesis of all four diastereomers with thymine as a nucleobase and (2*R*,4*S*) stereoisomer bearing A, G and cytosine as a nucleobase is described. These monomers have been incorporated into PNA oligomeric sequences by solid phase peptide synthesis. Cleavage and purification of the synthesized oligomers followed by suitable characterization is described. The binding affinities of the PNA oligomers for complementary DNA/RNA sequences have been evaluated by temperature dependent UV-Tm and CD spectroscopic studies. Gel retardation experiments provided additional proof of complexation of the PNA oligomers with complementary DNA. The results of the above studies are discussed.

Chapter 3

This chapter describes the modification of neutral, achiral PNA with an aim to improve its hybridization properties to complementary DNA/RNA sequences. Ethylene bridge was introduced between the β carbon atom of the ethylenediamine unit and α' carbon atom of the glycine unit to get 5-aminopipecolyl PNA monomer,

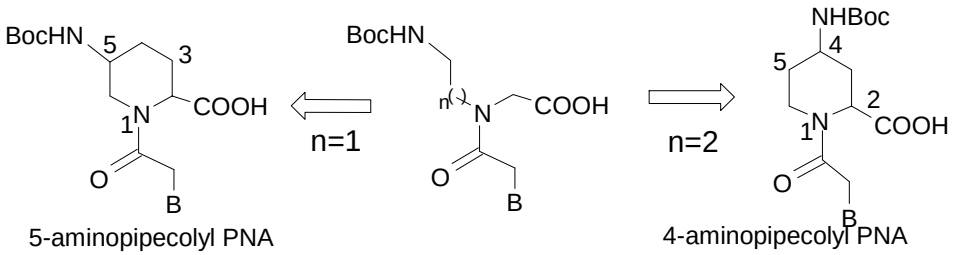


Figure 29 Pipecolyl PNA monomer

while methylene bridge was introduced between γ carbon atom of the aminopropyl segment and α carbon atom of the glycyl segment to get 4-aminopipecolyl PNA (Figure 29). The synthesis of these chiral, neutral monomeric units bearing thymine as a nucleobase is described. Solid phase peptide synthesis of PNA oligomers incorporating these monomeric units at pre-determined positions has been carried out. Oligomers were cleaved from the resin and DNA/RNA hybridization studies were carried out by UV spectroscopy along with gel retardation studies were carried out. These results are discussed.

Chapter 4

The PNA modification described in this chapter is a result of our further efforts to generate positively charged chiral monomers. This monomer was derived from *aeg*PNA by bridging the β carbon atom ethylenediamine and β'carbon atom of linker to the nucleobase (Figure 30). The synthesis of one of the four possible

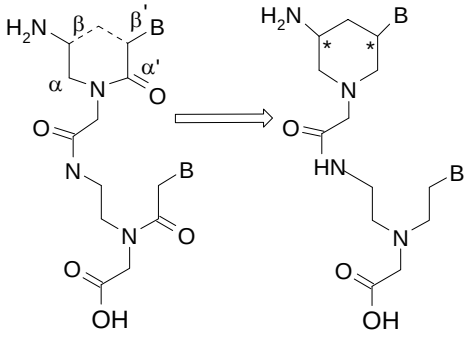


Figure 30 Derivation of the piperidinyl PNA structure from the parent *aeg*PNA structure.

diasteromers with thymine as a nucleobase is described. This monomer was incorporated into PNA oligomeric sequences at predetermined position by SPPS. The oligomers were cleaved from resin and purified by FPLC. The binding affinities of these PNA oligomers for complementary DNA sequence have been carried out. The results of the above studies are discussed.

1.12. References

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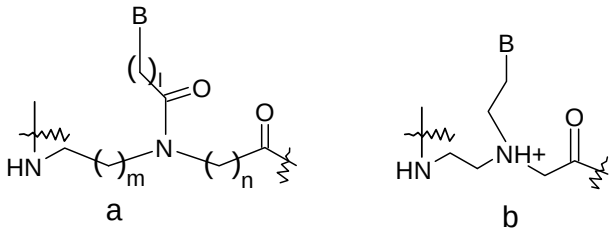
Chapter 2

Chimeric *aeg*-pyrrolidine PNA: Design, synthesis and binding studies with DNA/RNA

.1. INTRODUCTION

Peptide nucleic acids, PNAs,[1] have attracted much attention as nucleic acid mimics due to their exceptionally high affinity and specificity towards complementary RNA and DNA sequences.[2] Since the invention by Nielsen and co-workers in 1991, PNA monomer has been subjected to a variety of rational modifications with the aim to understand the structure activity relations in this class of DNA mimics as well as obtaining PNA oligomers with specifically improved properties such as water solubility, cell penetration and parallel/ anitparallel binding selectivity for various applications in medicine, diagnostics and molecular biology. These modifications include introduction of substituents into *aeg*PNA to create chirality,[3] introduction of positively charged groups onto the PNA backbone[4] and restricting the movement of the backbone or the side chain of PNA.[5,6]

The simplest of the modifications studied involved insertion of a methylene group in each of the structural subunits (aminoethyl,[7] glycine and nucleobase linker segments[8]) of the PNA monomer (Figure 1a). This resulted in significant lowering of thermal



m = n = l = 1 *aeg* PNA
m = 2 n = l = 1*apg* PNA
l= 2 m = n = 1 propionic acid PNA
m = l = 1 n = 2 βalanine backbone

Figure 1 a) *aeg*PNA and its analogs. b) Eth-PNA

stability of the derived PNA:DNA hybrids.

In another attempt, replacement of tertiary amide linker to nucleobase by ethylene linker gave a conformationally flexible backbone as in Eth-PNA (Figure 1b).[4] The resulting tertiary amine in the backbone was protonatable at physiological pH. This change was also not beneficial in terms of thermal stability of complexes of PNA with complementary nucleic acids. The deleterious consequences of such subtle changes to

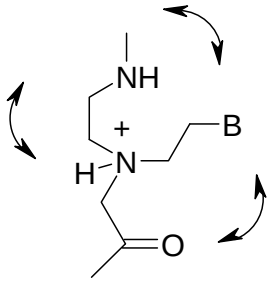


Figure 2 Possible positions for the introduction of alkylene bridges in Eth-PNA

the PNA structure for its binding to the nucleic acids were attributed to the additional flexibility in the *aeg*PNA. In contrast, the efforts were also directed to further freeze the *aeg*PNA conformations by introduction of chemical bridges in *aeg*PNA thus giving preorganized cyclic backbone structures. The structural features of constrained *aeg*PNAs if suitable for complex formation were expected to trigger a shift in equilibrium towards the desired hybrid form because of the reduced entropy loss upon complex formation, provided the enthalpic contributions remain unaffected. Most of the resulting cyclic PNAs with constrained backbone were found to be too rigid and could not improve properties of parent *aeg*PNA.[9,10,11,12, 13,14, 15,16,17] Bridged analogues of PNA arising from Eth-PNA were then studied that led to the design of five and six membered cyclic, chiral PNA analogs with defined configurations and conformational preferences with concomitant installation of a positive charge in the backbone (Figure 2). In this

strategy, the tertiary amide nucleobase linker is being replaced by a ring structure and thus the total elements of unsaturation are conserved in order to strike a balance between rigidity and flexibility of the PNA backbone.[18] The PNA structures thus synthesized are *aep*PNA (Figure 3a),[19]pyrrolidinePNA-DNA chimera (Figure 3b),[20] POM (Figure 3b)[21]and very recently a six membered, aminoethylpipecolyl PNA (Figure 3a).[22] These modifications of *aeg*PNA do bind strongly and specifically to complementary DNA/RNA sequences.

Further, Lowe et al have synthesized conformationally constrained chiral analogues of PNA based on the pyrrolidine core structure derived from alternating 4*R*-thymin-*1*-yl

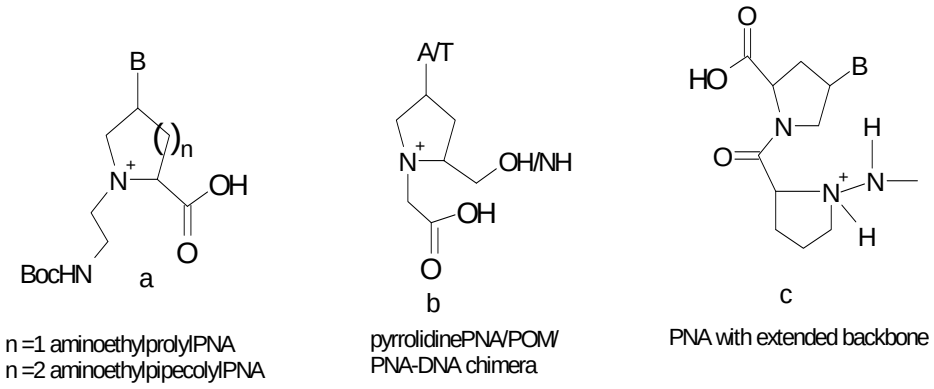


Figure 3 a. aminoethylprolyl/ pipecolyl PNA b. Pyrrolidine/ POMPNA c. PNA with extended backbone

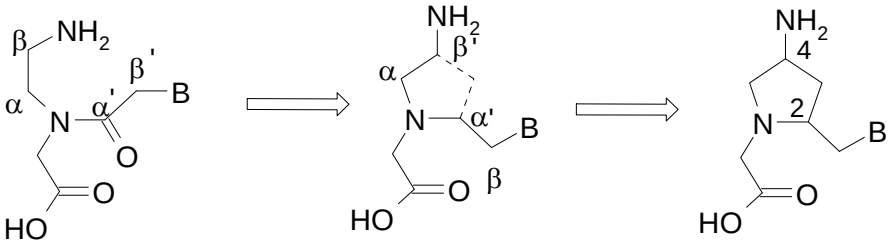
pyrrolidine–2*R*–carboxylic acid and aminopyrrolidine–2*R*-carboxylic acid [23] (Figure 3c). This homopolymeric PNA binds to its complementary oligodeoxynucleotides and displays remarkable preferential affinity for complementary DNA over RNA. In this case, the structural rigidity of the pyrrolidine ring system seems to be compensated by the use of β-amino acid instead of glycyl segment of *aeg*PNA.

The *aep*PNA[19b] where methylene bridge replaces the tertiary amide carbonyl (Figure 3a) shows remarkable biophysical properties with triplex forming

polypyrimidine sequences, enhanced stability of duplexes with DNA having mixed purine-pyrimidine sequences while retaining the base pair discriminating efficiency. The stability of the duplexes of mixed purine-pyrimidine base sequences was found to be dependent on the nucleobase on the pyrrolidine ring.[19b] The reasoning for this could be variable pyrrolidine ring pucker as a result of varying group electronegativities of the nucleobase or the syn/anti conformations of nucleobases with respect to the pyrrolidine ring.

2.1.1. Rationale for the present work

As part of our continuing investigation of conformationally constrained chiral analogues of PNA based on the pyrrolidine core structure,[11,12,19,20,24] there is a recent report from our laboratory describing pyrrolidine PNA **II** (Figure 4) derived by



*aeg*PNA **I** pyrrolidinePNA **II**

Figure 4 Pyrrolidine PNA

introducing a methylene bridge between the α'carbon atom of the nucleobase linker and the β carbon atom of the aminoethyl segment of aminoethylglycyl *aeg*PNA.[25] The design of the PNA **II** is such that the nucleobase attachment to the backbone is through a flexible methylene linker and not directly to the pyrrolidine ring as in *aep*PNA, although the backbone constraint remains in the aminoethyl and nucleobase linkers. Detailed structural studies with this pyrrolidine PNA could be very interesting in light of the recently reported homo-*N*-oligonucleotides (Figure 5) that are the first examples of D-sugar nucleic acids having unrestricted glycosyl conformation.[26]

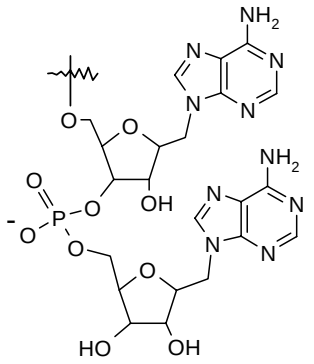


Figure 5 D-sugar nucleic acids

It was found earlier that chimeric *aeg*-pyrrolidine PNA T_8 oligomers derived from either (2*R*,4*S*) and (2*S*,4*S*) pyrrolidine backbone stereochemistry of the ring exhibited stereochemistry-dependent thermal stability of the resulting PNA_2:DNA complexes. The introduction of a single unit of (2*R*,4*S*) pyrrolidine PNA **II** in the T_8 sequence stabilized the PNA_2:DNA complexes (ΔT_m=16^{o}C). The introduction of the (2*S*,4*S*) isomer on the other hand destabilized the complex (ΔT_m= -16^{o}C). The binding efficiency of pyrrolidine PNA oligomers thus seems to be dictated by the stereochemistry of the pyrrolidine backbone that is a part of the acetyl linker connecting the nucleobase in the parent *aeg*PNA **I** (Figure 4).

In view of the above results, it was thought necessary to synthesize all four diastereomeric pyrrolidine PNA monomers of the natural nucleobases. The introduction of nucleobases besides thymine will allow the synthesis of the PNAs with mixed purine-pyrimidine sequences that would elucidate the consequence of the chiral constraint as well as the individual nucleobase on the PNA: DNA/RNA duplex stability.

2.1.2Objectives discussed in this chapter are

1. Synthesis of 4*R*/*S*-(*tert*.butyloxycarbonylamino)-2(*S*/*R*)-(thymin-1-ylmethyl-pyrrolidin-*N*-1-acetic acid diastereomers for PNA synthesis.

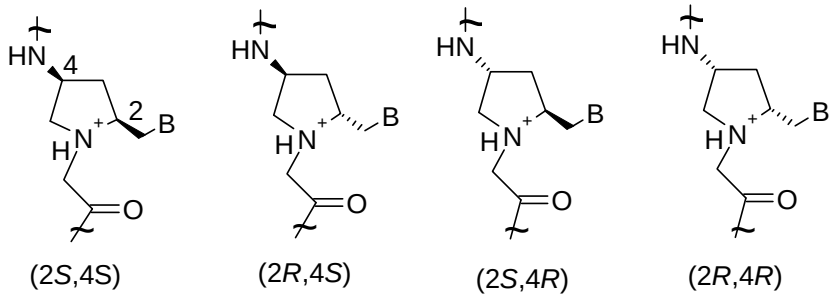


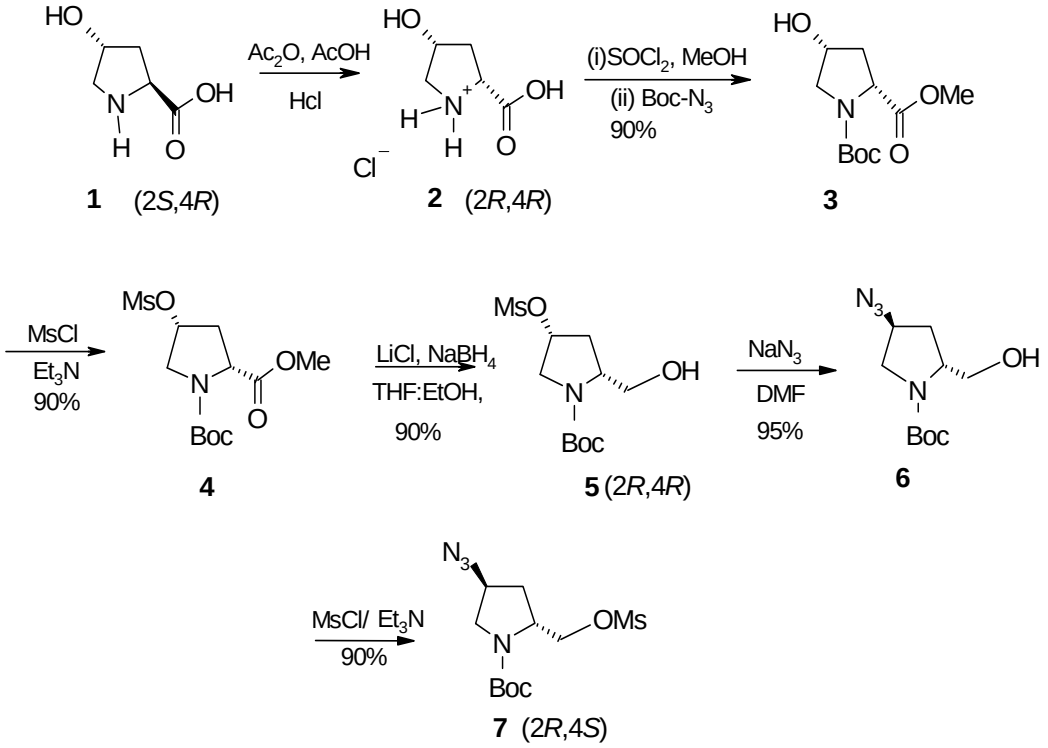
Figure 6 Diastereomers of pyrrolidine PNA

2. Synthesis of (2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino) -2-(N^4-benzyloxycarbonylcytosin/N^6benzoyladenin/guanin/thymin-1-methyl)pyrrolidin-*N*-1-acetic acid monomers for PNA synthesis.
3. Synthesis of *aeg*PNA monomers (A/T/G/C).
4. Solid phase synthesis of chimeric PNA oligomers incorporating pyrrolidine and *aeg*PNA monomers.
5. Cleavage of the oligomers from the solid support and their purification along with their characterization.
6. DNA/RNA hybridization studies using biophysical techniques such as UV-Tm measurements, CD and gel electrophoresis.

2.2. Synthesis of pyrrolidine PNA monomers

*2.2.1 Synthesis of (2R,4S)-4-azido-2-(mesyloxymethyl)-N-(tert.butyloxycarbonyl) pyrrolidine, A common precursor for the synthesis of monomers **7***

The synthesis of (2*R*,4*S*) 4-azido-2-(mesyloxymethyl)-*N*-(*tert*.butyloxycarbonyl) pyrrolidine **7** was achieved starting from naturally occuring 4-hydroxy-L-proline. (Scheme1). The C2-stereocenter in (2*S*,4*R*)-hydroxy-proline **1** was epimerized[27] by treatment with acetic acid and acetic anhydride to yield the *cis* lactone, which was hydrolyzed by refluxing with 2N HCl to get (2*R*,4*R*)-hydroxy-proline hydrochloride **2** in good yield. The purity of **2** was confirmed by ^{13}C NMR and specific rotation.



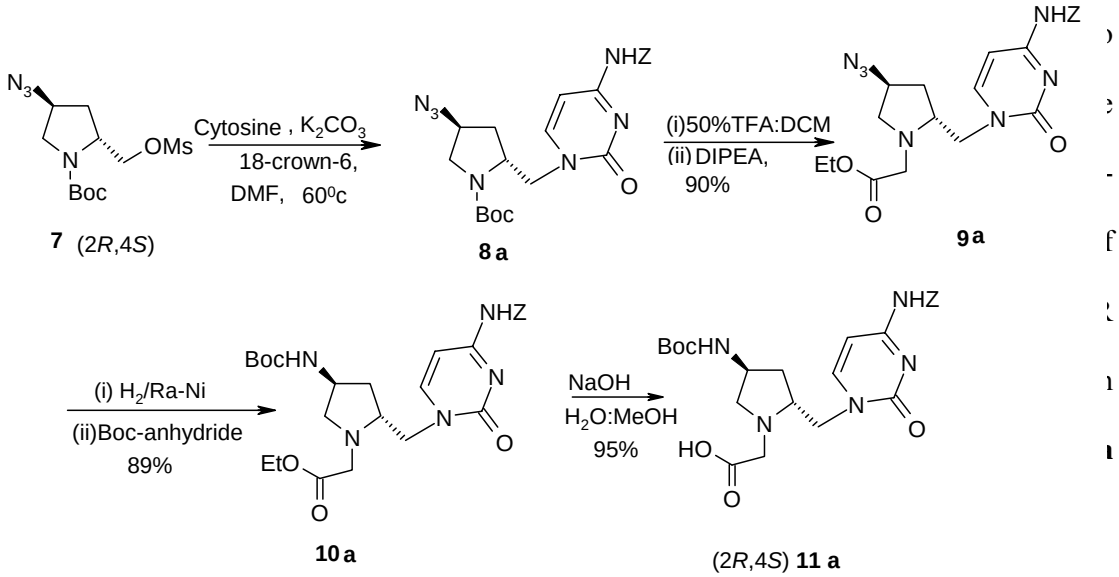
Scheme 1 Synthesis of (2*R*,4*S*) 4-azido-2-(mesyloxymethyl)- *N* -(*tert*.butyloxycarbonyl) pyrrolidine

The *cis* 4-hydroxyproline **2** was then converted to its methyl ester by refluxing in methanol in the presence of thionyl chloride. The resulting (2*R*,4*R*)-4-hydroxyproline-2-methyl ester isolated as a hydrochloride salt, was treated with Boc-azide in dioxane:water in the presence of triethylamine, to yield the carbamate-protected pyrrolidine ring nitrogen (2*R*,4*R*)-*N*-(butyloxycarbonyl)-hydroxy-proline methyl ester **3**. The 4 hydroxyl group in **3** was converted to its mesyl derivative **4** by reaction with mesyl chloride in DCM using triethylamine as a base. The methyl ester was reduced to the corresponding alcohol **5** using a 1:1 mixture of sodium borohydride and lithium chloride in THF:ethanol. The successful reduction was confirmed by the absence of the carbonyl resonance in ^{13}C NMR at 171.0 ppm and the appearance of a broad multiplet corresponding to two additional protons at 3.50-3.90 ppm in the ^{1}H NMR spectrum. SN2 diaplacement of 4*R*-*O*-mesyl group to 4*S* azide was achieved by treating with excess of sodium azide in DMF. The characteristic azide signal at 2100 cm^{-1} in the IR spectrum and an upfield shift of the H4 resonance from 5.18 ppm in the 4(*R*)-*O*-mesyl derivative 5 to 3.70 ppm confirmed the formation of azide **6**. The primary hydroxyl

group at C2 was then converted to its mesyl derivative in presence of mesyl chloride to give derivative **7**. This mesylate derivative was used as a common intermediate for introduction of adenine, cytosine, guanine and thymine nucleobases.

2.2.2. Synthesis of (2R,4S)-4-(tert.butyloxycarbonylamino) -2-(N^4-benzyloxycarbonylcytosin-1-ylmethyl)-pyrrolidin-N-1-acetic acid ***11a***

Compound **7** upon reaction with N^4-benzyloxycarbonylcytosine in the presence of K_2CO_3 and 18-crown-6, gave (2*R*,4*S*)-*N*-(*tert.*butyloxycarbonyl)-azido-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidine **8a**. ^{1}H NMR spectrum of **8a** showed characteristic cytosine peaks at 7.6 ppm for H6 and at 7.1ppm for H5 of cytosine. The pyrrolidine ring nitrogen in **8a** was deprotected with TFA:DCM (1:1) and subsequently alkylated using ethyl bromoacetate to yield (2*S*,4*S*)-4-azido-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidn-*N1*-ethylacetate **9a** in good yield. The signals corresponding to the ethyl ester appeared at 4.14 and1.24 ppm in the ^{1}H

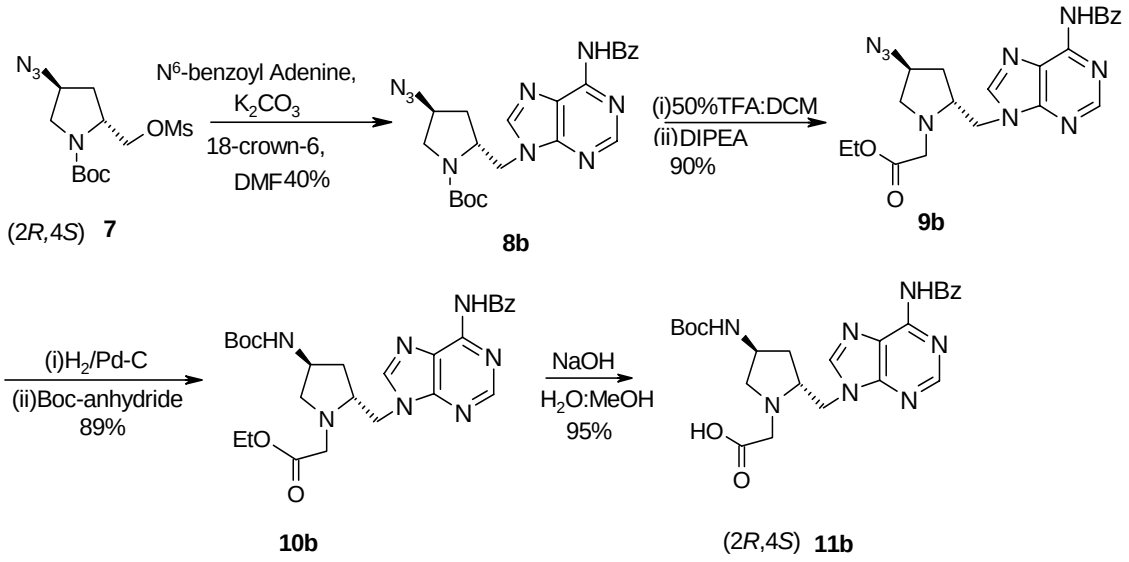


7 (2*R*,4*S*) **8 a** **9 a** **10 a** (2*R*,4*S*) **11 a**

Scheme 2 Synthesis of (2*R*,4*S*)-4-(*tert.*butyloxycarbonylamino) -2- (N^4-benzyloxycarbonylcytosin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid.

2.2.3. Synthesis of (2R,4S)-4-(tert.butyloxycarbonylamino) 2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidin-N-1-acetic acid ***11b***

The mesylate derivative **7** was treated with N^6-benzoyladenine in presence of K_2CO_3 in DMF to obtain (2*R*,4*S*)-4-azido-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidine **8b** (Scheme 3). The product was purified by column chromatography to get pure **8b** in 40 % yield. The product **8b** was confirmed by the characteristic signals at 8.77 and 8.0 ppm for H2 and H8 of adenine. The pyrrolidine ring nitrogen was deprotected with



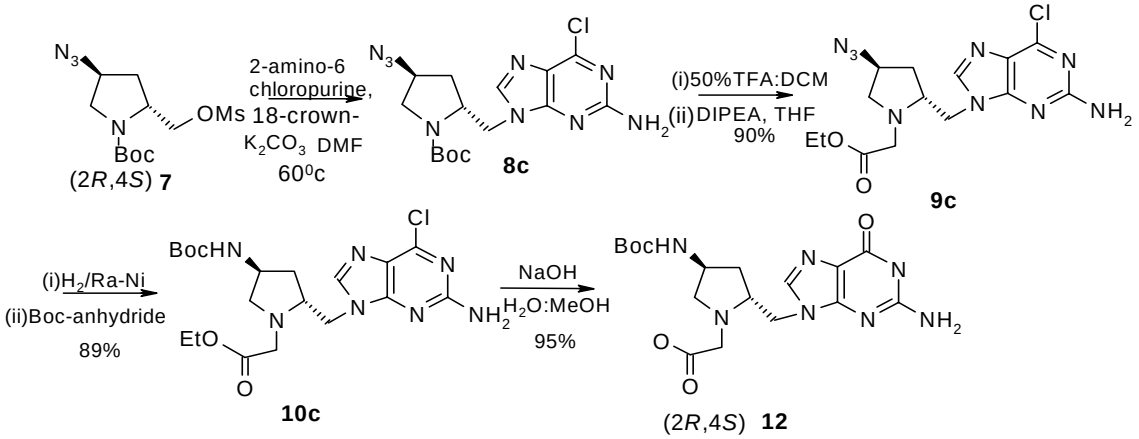
Scheme 3 Synthesis of (2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(N^6-benzoyladenine-9-ylmethyl)-pyrrolidin-*N*-1-acetic acid.

TFA:DCM (1:1) and subsequently alkylated using ethylbromoacetate to yield (2*S*,4*S*)-4-azido-2-(N^6-benzoyladenine-9-ylmethyl)-pyrrolidin-*N1*-ethylacetate **9b** in good yield. The appearance of the signals corresponding to the ethyl ester (4.2 and 1.3 ppm) in the ^{1}H NMR and the carbonyl group 169 ppm in the ^{13}C NMR confirmed the formation of **9b**. The 4(*S*)-azido function was reduced by catalytic hydrogenation using H_2/Pd-C in metahnol, which was converted *in situ* to the corresponding butylcarbamate by Boc-

anhydride in 90% yield. The reduction of amine was confirmed by the disappearance of the azide peak at 2100 cm^{-1} in IR and appearance of a signal at 1.24 ppm in the ^1H NMR spectrum. The ethyl ester function was hydrolyzed using NaOH in aqueous MeOH to yield the protected (2*R*,4*S*) pyrrolidine A^{Bz}- monomer **11b,** suitable for solid phase peptide synthesis. The benzoyl group in **11b** remained unaffected during this hydrolysis.

2.2.4. Synthesis of (2R,4S)-4-(tert.butyloxycarbonylamino) 2-(guanin-9-ylmethyl)-pyrrolidin-N-1-acetic acid ***12***

The (2*R*,4*S*) (*tert*.butyloxycarbonylamino)-2-(guanin-9-ylmethyl)-pyrrolidin-*N*-1-acetic acid was synthesized from mesylate derivative **7** (Scheme 4). The mesylate derivative **7** was treated with 2-amino-6-cholopurine in the presence of K_2CO_3 in DMF to obtain (2*R*,4*S*)-4-azido-2-(2-amino-6-chloropurin-9ylmethyl)-pyrrolidine. The



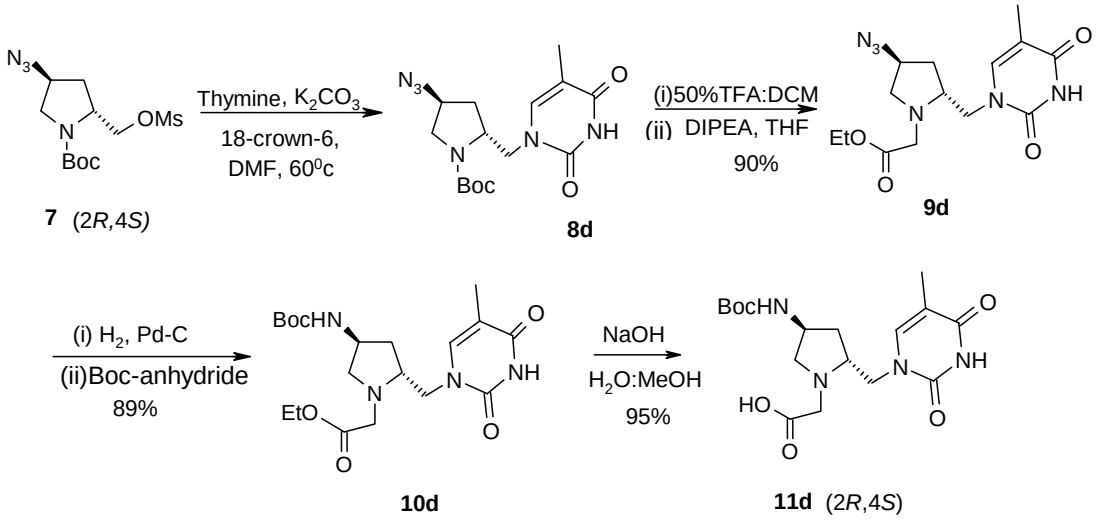
Scheme 4 Synthesis of (2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(guanin-9-ylmethyl)-pyrrolidin-*N*-1-acetic acid

product formation was confirmed by characteristic signal at 8.0 ppm corresponding to H8 proton. The pyrrolidine ring nitrogen was deprotected with TFA:DCM (1:1) and subsequently alkylated using ethylbromoacetate to yield (2*R*,4*S*)-4-azido-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidn-*N-1*-ethylacetate **9c** in good yield. The appearance

of the signals corresponding to the ethyl ester (4.0 and 1.3 ppm) in the ^{1}H NMR and the carbonyl group 170.3 ppm in the ^{13}C NMR confirmed the transformation. The 4(*S*)-azido function was reduced by catalytic hydrogenation using Ra-Ni/H_2 in metahnol, which was converted *in situ* to the corresponding butylcarbamate by Boc anhydride in 90% yield. The reduction of amine was confirmed by the disappearance of the azide peak at 2100 cm^{-1} in IR and appearance of s at 1.37 ppm in the ^{1}H NMR spectrum. The ethyl acetate derivative upon treatment with NaOH in MeOH initially underwent ester hydrolysis within 15 minutes, followed by conversion of the 6-chlorofunction to the 6-oxo function. The transformation of the nucleobase from 2-amino-6-chloropurine to guanine ie 2-amino-6-oxopurine was unambiguously confirmed by NMR as evident from the appearance of a carbonyl resonance 169.0 ppm in ^{13}C NMR spectrum of **12** which was absent in the compound **10c.**

2.2.5. Synthesis of (2R/S,4S)-4-(tert.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-N-1-acetic acid ***11d/13***

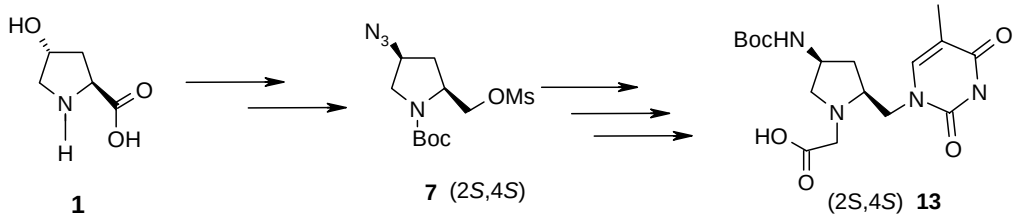
The mesylate derivative **7** was treated with thymine in presence of K_2CO_3 in DMF to obtain (2*R*,4*S*)-4-azido-2-(thymine-1-ylmethyl)-pyrrolidine **8d** (Scheme 5a). The compound was confirmed by the appearance of the characteristic thymine peaks in the ^{1}H NMR spectrum, *viz*. at 7.00 (T-H6) and δ 1.90 (T-CH_3) ppm. The pyrrolidine ring nitrogen was deprotected with TFA:DCM (1:1) and subsequently alkylated using ethylbromoacetate to yield (2*S*,4*S*)-4-azido-2-(thymin-1-ylmethyl)-pyrrolidn-*N1*-ethylacetate **9d** in good yield. The appearance of the signals corresponding to the ethyl ester (4.15 and 1.25 ppm) in the ^{1}H NMR and the ester carbonyl group 169 ppm in the ^{13}C NMR confirmed the formation of **9d**. The 4(*S*)-azido function was reduced by catalytic hydrogenation using H_2/Pd-C in methanol, which was converted *in situ* to the



Scheme 5a Synthesis of (2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-yl-methyl)-pyrrolidin-*N*-1-acetic acid .

corresponding butylcarbamate by Boc-anhydride in 90% yield. The reduction of amine was confirmed by the disappearance of signal at 1.24 in the ^{1}H NMR spectrum.[17] The ethyl ester function was hydrolyzed using NaOH in aqueous MeOH to yield the protected (2*R*,4*S*) pyrrolidine-thymin-monomer **11d** suitable for solid phase peptide synthesis.

Similarly (2*S*,4*S*)–4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid **13** was synthesized from naturally occurring (2*S*,4*R*)-4-hydroxy proline via (2*S*,4*S*)-4-azido-2-(mesyloxymethyl)-*N*-(*tert*.butyloxycarbonyl)

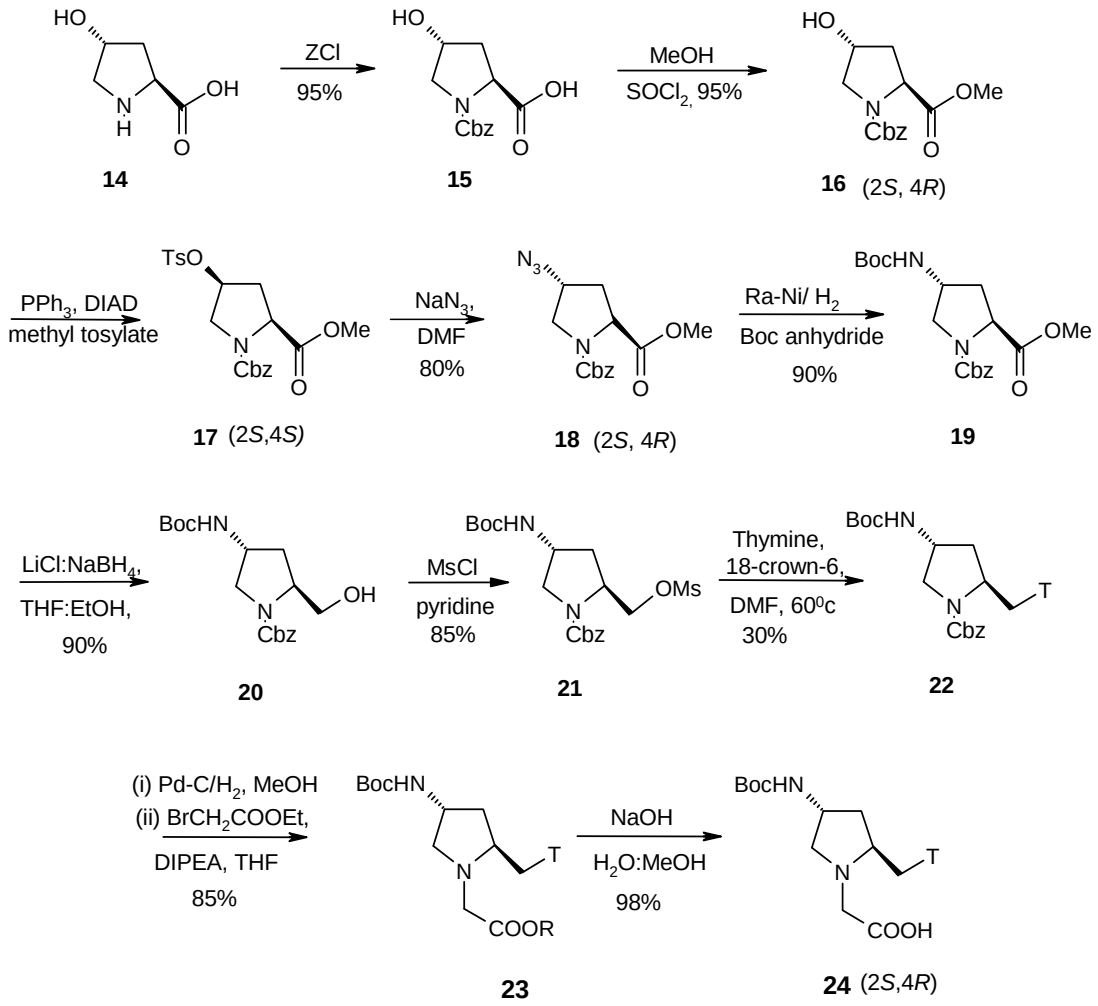


Scheme 5b Synthesis of (2*S*,4*S*) -4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-yl-methyl)-pyrrolidin-*N*-1-acetic acid .

pyrrolidine **7,** following the same synthetic procedure[17](Scheme 5b).

*2.2.6. Synthesis of (2S,4R)-4-(tert.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-N-1-acetic acid **24***

The synthesis of compound **24** was achieved from the naturally occurring (2*S*,4*R*)–4-hydroxy L-proline (Scheme 6). *N*-benzyloxycarbonyl protected proline **15** was obtained in quantitative yield by reacting hydroxy proline with benzyl chloroformate in dioxane water in the presence of $NaHCO_3$ as a base.[28] The N-protected hydroxy proline **15** was then converted to its methyl ester **16** using MeOH/$SOCl_2$ and TEA as a base. Benzyloxycarbonyl group is acid sensitive and TEA was used to neutralize liberated HCl during the reaction. The 4*R*-hydroxy group in **16** was converted to 4*S*-tosylate under Mitsunobu conditions with DIAD/PPh_3 and methyl tosylate effecting inversion of configuration at C-4. The conversion of 4-OH to tosylate was confirmed by appearance of a peak at 2.5 ppm (tosyl-CH_3) and in the aromatic region integrating for nine protons. SN2 displacement of the resulting 4*S*-tosylate by treatment with excess of sodium azide in DMF yielded 4*R* azide **18** again with inversion of configuration. A characteristic peak appearing at 2106 cm^{-1} in the IR spectrum of compound **18** and disappearance of signals corresponding to tosylate group in the ^1H NMR confirmed the formation of azide. Azide group was selectively reduced by catalytic hydrogenation in the presence of Ra-Ni to give free amine, which was then protected as a Boc derivative **19**. The ester function was reduced to alcohol by treatment with LiCl/$NaBH_4$ in THF/EtOH to give **20**. The primary alcohol in **20** was then converted to mesyl derivative **21** with mesyl chloride and triethyl amine. *N*-1 alkylation of thymine was effected by reaction of thymine with **21** in presence of K_2CO_3 and 18-crown-6 to get thymin-1-ylmethyl derivative **22** in 30% yield. The pyrrolidine ring nitrogen in **22** was then deprotected by

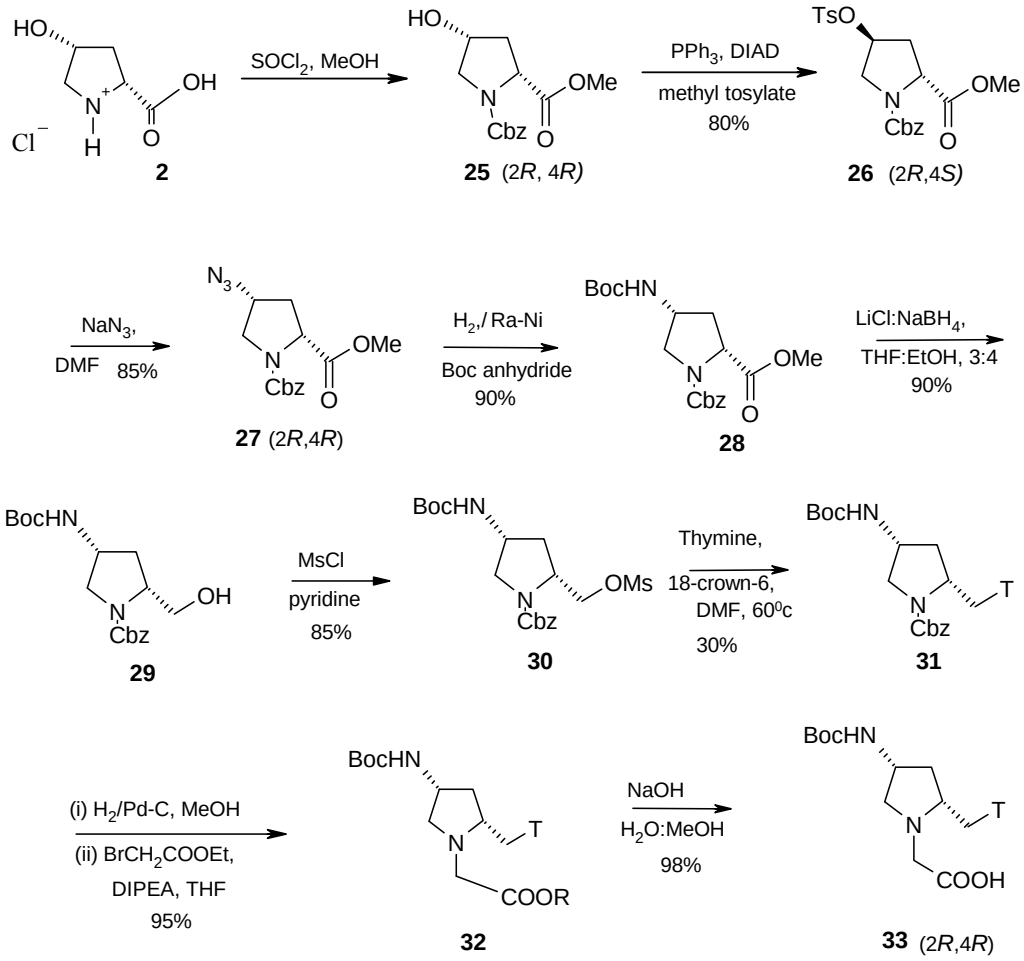


Scheme 6 Synthesis of (2*S*,4*R*)-*(tert*.butyloxycarbonylamino*)*-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid

hydrogenation and alkylated with ethyl bromoacetate in the presence of diisopropyl ethylamine in THF to get **23**. The ethyl ester in **23** was then hydrolyzed using sodium hydroxide in aqueous methanol to give (2*S*,4*R*)-4-(*N-tert*.butyloxycarbonyl-amino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1 acetic acid **24**.

*2.2.7. Synthesis of (2R,4R)-(tert.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-N-1-acetic acid **33***

Synthesis of compound **33** was achieved from (2*R*,4*R*)-hydroxy-L-proline (Scheme 7). Compound **2** was N-protected with benzyloxycarbonyl group prior to esterification to get protected derivative **25**. The 4*R* hydroxyl group in **25** was inverted to 4*S* tosylate under Mitsunobu condition with DIAD/PPh_3 and methyl tosylate. The conversion of OH to tosylate was confirmed by appearance of a peak in ^{1}H NMR spectrum at 2.3 ppm (tosyl-CH_3) and in the aromatic region integrating for 9 protons. SN2 displacement of the resulting 4*S*-tosylate by treatment with excess of sodium azide in DMF yielded 4*R*



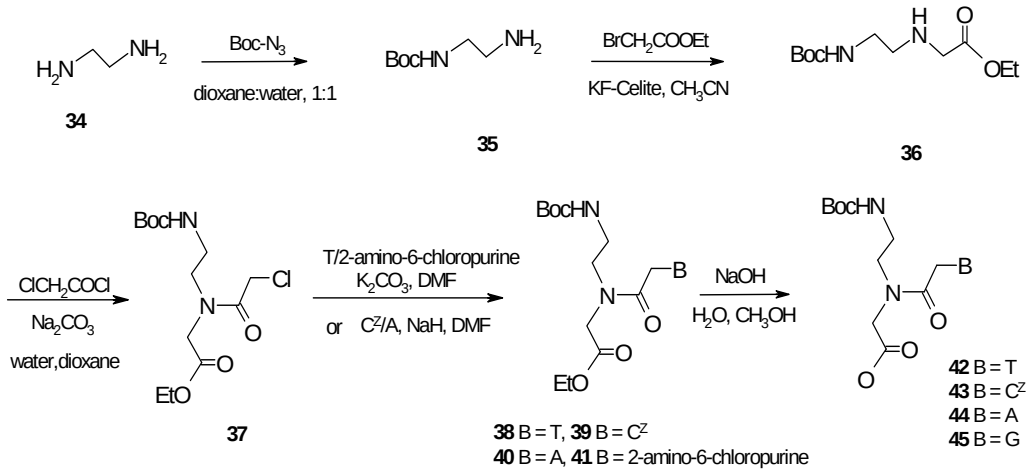
Scheme 7 Synthesis of (2*R*,4*R*)-(*tert*.butyloxycarbonylamino*)*-thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid

azide **27** with inversion of configuration. A characteristic peak appearing at 2100 cm^{-1} in the IR spectrum of compound **27** and disappearance of signals corresponding to tosylate group confirmed the formation of azide. Azide group was selectively reduced by catalytic hydrogenation in the presence of Ra-Ni to give free amine, which was then protected with Boc-anhydride to get **28**. The ester function in **28** was reduced to alcohol **29** by treatment with $LiCl/NaBH_4$ in THF/EtOH. The complete reduction of ester was confirmed by the disappearance of a peak at 3.8-3.4 ppm and appearance of multiplate at 4.27 ppm corresponding to CH_2-OH group in ^1H NMR. The primary alcohol in **29** was then converted to mesyl derivative **30** with mesyl chloride. *N*-1 alkylation of thymine was effected by reaction with mesylate in the presence of K_2CO_3, and 18-crown-6 to give *N*-1 thyminylmethyl derivative **31** in 30% yield. The pyrrolidine ring nitrogen in **31** was then deprotected by hydrogenation and alkylated with ethyl bromoacetate in presence of diisopropyl ethylamine in THF to get **32**. The ethyl ester in **32** was then hydrolyzed using sodium hydroxide in aqueous methanol to give (2*R*,4*R*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid **33**.

2.2.8. Synthesis of aminoethylglycyl (aeg)PNA monomers.

In order to study the effect of the pyrrolidine PNA monomers on hybridization and stability of PNA these monomers were incorporated along with the unmodified *aeg*PNA monomers to get chimeric pyrrolidine-*aeg*-PNA oligomers. Unmodified *aeg*PNA monomers were synthesized from the easily available 1,2-diaminoethane **34** (Scheme 8). On treatment with Boc-azide **34** gave the mono-N-butyloxycarbonate derivative **35**. Optimum yield of **35** was achieved by using a large excess of 1,2-diaminoethane over the Boc-azide under high dilution conditions. The dibutyloxycarbonyl derivative formed in small quantity was removed easily by filtration because of its insolubility in water. *N*-

-*tert*-butyloxycarbonyl-1,2-diamino ethane **35** was *N*-alkylated using ethyl bromoacetate and KF- Celite in dry acetonitrile. The use of KF-Celite[29] was found to be advantageous over K_2CO_3 due to better yield of monoalkylated product and the ease of work-up. After the completion of reaction, KF-Celite was filtered off and acetonitrile was evaporated to get the ethyl-N-(*tert*.butyloxycarbonylaminoethyl)-glycinate **36** in good yield. Compound **36** on treatment with chloroacetyl chloride in presence of Na_2CO_3 in aqueous dioxane gave **37** in good yield. The glycinate **37** thus obtained was



Scheme 8 Synthesis of the *aeg*PNA monomers

purified by column chromatography and used as a common intermediate for the alkylation of nucleobases (Scheme 8).

The alkylation of nucleobases thymine and cytosine using chloroacetyl derivative **37** was found to be regiospecific at N1. Thymine was reacted with **37** using K_2CO_3 as base to obtain ethyl-N-(*tert*.butyloxycarbonylaminoethyl)-(*N*-thymin-1ylacetyl)glycynate **38** in high yield. In the case of cytosine, the exocyclic amino group was protected with benzyloxycarbonyl group and alkylated using **37** and NaH as the base to provide *N*-1-substituted product **39**. Although adenine is known to undergo both *N*-7 and *N*-9 substitution, *N*-7 alkylation product was absent when NaH was used as a base and only

ethyl-*N*-(*tert*.butyloxycarbonylaminoethyl)-(*N*-adenin-9-ylacetyl) glycynate **40** was obtained in moderate yield. Alkylation of 2-amino-6-chloropurine with **37** was facile with K_2CO_3 as base and yielded the corresponding ethyl-*N*-(*tert*.butyloxycarbonylaminoethyl)-(*N*-guanin-9ylacetyl)glycynate **41** in good yield. The exocyclic amino group in adenine, guanine and N^3 of thymine were left unprotected, as these were unreactive under the conditions employed for the subsequent coupling reactions on solid phase. All the compounds exhibited satisfactory ^1H and ^{13}C NMR data consistent with the literature data. All the esters were hydrolyzed with aqueous NaOH to get the corresponding *aeg* monomeric PNA acids **42-45** that were used for the SPPS.

2.2.9. Synthesis of the Protected Nucleobases

Exocyclic amino group in cytosine needs to be protected for its use in peptide synthesis. The benzyloxycarbonyl group can be introduced easily using benzyloxycarbonyl chloride in dry pyridine to get N^4-benzyloxycarbonylcytosine. In case of adenine, when exocyclic amino group was unprotected, yields of adenine alkylation to get monomer **11b** were not satisfactory. Earlier reported Rapaport reagent for Z protection of N^6 of adenine also gave low yield. We then used benzoyl protection. The N^6-benzoyl protected adenine was used for alkylation reaction. The deprotection of N^6-benzoyl group of adenine requires additional step after oligomer cleavage.[19]

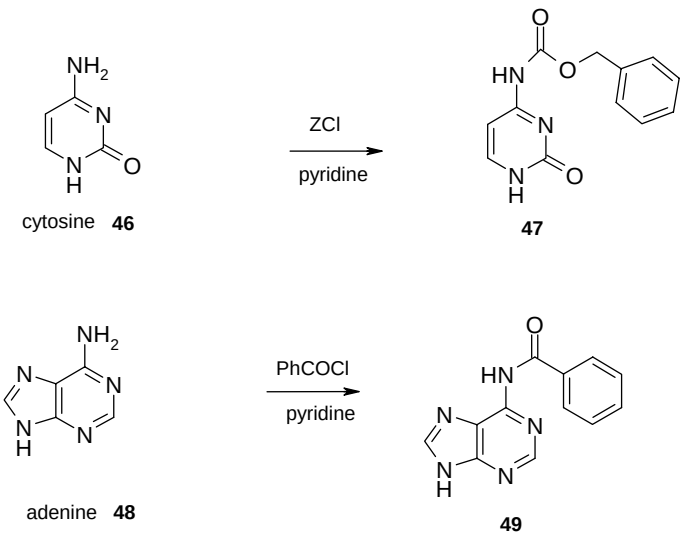
N3 of thymine as well as 2-amino group of guanine did not require protection under peptide coupling conditions and hence are not protected.

N^4-benzyloxycarbonylcytosine 47

Cytosine was treated with benzyloxycarbonyl chloride in dry pyridine to get the desired product, N^4-benzyloxycarbonylcytosine **47** (Scheme 9).

***N*^{6}-benzoyladenine 49**.

Adenine, upon treatment with benzoyl chloride in dry pyridine gave the N^6-benzoyl adenine **49** in good yield, which was obtained as white crystals[30] (Scheme 9).



Scheme 9 Synthesis of protected nucleobases

2.3. Solid Phase PNA Synthesis

2.3.1. General Protocols for PNA Synthesis.

Solid phase peptide synthesis protocols can be easily applied to the synthesis of oligomeric PNAs. The ease of handling and scale-up procedures have made possible the synthesis of PNAs including those incorporating a large number of analogues in an endeavor to improve its favorable binding and biological properties.

As is the case with solid phase peptide synthesis, PNA synthesis is also done conveniently from the 'C' terminus to the 'N' terminus. For this, the monomeric units must have their amino functions suitably protected, and their carboxylic acid functions free. The most commonly used N-protecting groups for solid phase peptide synthesis are the tert.butyloxycarbonyl (Boc) and the 9-flurorenylmethoxycarbonyl (Fmoc)

groups. The Fmoc protection strategy has a drawback in PNA synthesis since a small amount of acyl migration has been observed under basic conditions from the tertiary amide to the free amine formed during piperidine treatmenrt[31,32] in solid phase peptide synthesis. Hence, the Boc-protection strategy was selected for the present work. The amino function of the monomers was protected as the corresponding Boc-derivative and the carboxylic acid function was free to enable coupling with the resin-linked monomer. The HBTU/1-hydroxybenzotriazole (HOBt) activation strategy was employed for the coupling reaction.[33] Merrifield resin (chloromethylstyrene-1%divinylbenzene resin) was selected as the solid polymeric matrix on which the oligomers were built. The first amino acid is linked to this matrix *via* a benzyl ester linkage. This can be cleaved either with a strong acid to yield the C-terminal free carboxylic acid, or with an amine to afford the C-terminal amide.

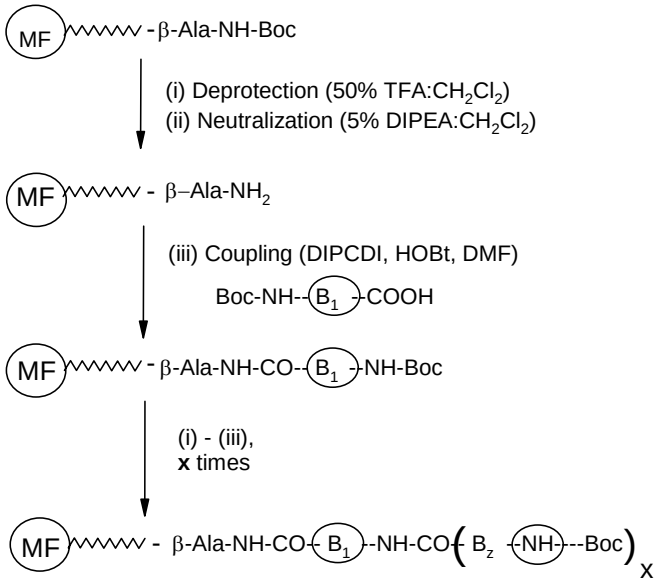
All the oligomers in the present work were synthesized manually on Merrifield resin. β-Alanine was selected as the linker amino acid. Being achiral, it would not interfere with the chirality-induced spectral properties of the pyrrolidine monomers. Its contribution to the hydrophobicity of PNA is also negligible since it has only a short alkyl chain. *N*-Boc-β-alanine was linked to the resin by a benzyl ester linkage *via* the formation of its cesium salt.[34] The loading value of β-alanine on the resin was determined by the picrate assay.[35] The resin loading was suitably lowered by partial capping of the free amino groups obtained after Boc-deprotection as N-acetates. The free uncapped amino groups on the resin were estimated once again by the picrate assay prior to commencing solid phase synthesis.

The PNA oligomers were synthesized using repetitive cycles, each comprising the following steps:

(i) Deprotection of the N-protecting Boc- group using 50% TFA in DCM.

(ii) Neutralization of the TFA salt formed with diisopropylethyl amine (DIPEA) (5% DIPEA in DCM) to liberate the free amine.

(iii) Coupling of the free amine with the free carboxylic acid group of the incoming monomer (3 to 4 equivalents). The coupling reaction was carried out in



Scheme 10 Schematic representation of solid phase peptide synthesis using the Boc-protection strategy

DMF/NMP/DMSO with HBTU as a coupling reagent in the presence of DIPEA and HOBt as catalyst. The deprotection of the *N*-Boc protecting group and the coupling reaction were monitored by Kaiser's test.[36] The Boc-deprotection step gives a positive Kaiser's test, wherein the resin beads as well as the solution are blue in color. On the other hand, upon completion of the coupling reaction, the Kaiser's test is negative, the resin beads remaining colorless.

(iv) Capping of the unreacted amino groups using acetic anhydride in pyridine:DCM

A typical synthesis cycle is depicted in Scheme 10

2.3.2. Synthesis of Chimeric Pyrrolidine-aegPeptide Nucleic Acids

i) Polypyrimidine PNA Sequences

The effect of the pyrrolidine unit on triplex-forming ability was tested by synthesizing poly-pyrimidine poly-thymine octamers. These are known to complex with the complementary DNA A_8 oligomer in a 2:1 PNA:DNA stoichiometry. The control

Table 1 Polypyrimidine and complementary polypurine PNA oligomer sequences

PNA	Sequence Composition	
50	H-T T T T T T T T -β-ala –OH	*aeg*PNA Control
51	H-T T T T T T T **t** $_{(2S,4S)}$ -β-ala–OH	One (2*S*,4*S*) pyrrolidilne unit at C terminus
52	H-T T T T T T T **t** $_{(2R,4R)}$ -β-ala–OH	One (2*R*,4*R*) pyrrolidine unit at C terminus
53	H-T T T T T T T **t** $_{(2S,4R)}$ -β-ala–OH	One (2*S*,4*R*) pyrrolidine unit at C terminus
54	H-T T T T T T T **t** $_{(2R,4S)}$ -β-ala–OH	One (2*R*,4*S*) pyrrolidine unit at C terminus
55	H-T T T **t** $_{(2S,4S)}$ T T T **t** $_{(2S,4S)}$ -β-ala–OH	Two (2*S*,4*S*) pyrrolidine units.
56	H-T T T **t** $_{(2R,4R)}$ T T T **t** $_{(2R,4R)}$ -β-ala–OH	Two (2*R*,4*R*) pyrrolidine units
57	H-T T T **t** $_{(2S,4R)}$ T T T **t** $_{(2S,4R)}$ -β-ala–OH	Two (2*S*,4*R*) pyrrolidine units
58	H -T T T **t** $_{(2R,4S)}$ T T T **t** $_{(2R,4S)}$-β-ala–OH	Two (2*R*,4*S*) pyrrolidine units
59	H-T T T **t** $_{(2S,4S)}$ T T T T -β-ala–OH	One internal (2*S*,4*S*) pyrrolidine unit
60	H-T T T **t** $_{(2R,4R)}$ T T T T -β-ala–OH	One internal (2*R*,4*R*) pyrrolidine unit
61	H-T T T **t** $_{(2S,4R)}$ T T T T -β-ala–OH	One internal (2*S*,4*R*) pyrrolidine unit
62	H-T T T **t** $_{(2R,4S)}$ T T T T -β-ala–OH	One internal (2*R*,4*S*) pyrrolidine unit
63	5'- G C A A A A A A A A –C G-3'	Complementary to PNA –T8 **49-62**
64	5'- G C A A A A **T** A A A –C G-3'	Mismatch DNA for PNAs **49-62**

ii) Mixed purine-pyrimidine sequences

Parallel/antiparallel PNA strands:

Homopyrimidine thymine PNA sequences bind to the complementary homopurine DNA sequence forming PNA_2:DNA triplexes in which it is difficult to distinguish the PNA strand that binds to the central DNA strand by WC hydrogen bonding from that which binds by HG hydrogen bonding. Mixed base sequences form duplexes of antiparallel or parallel orientations that can be selected by proper design of the complementary DNA sequences. By convention, antiparallel PNA:DNA complexes are defined as those in which the 'N' terminal of the PNA faces the 3'-end of the DNA with the 'C' terminal facing the 5'-end and parallel PNA:DNA complexes are those in which the 'C' terminal of PNA faces the 3'-end of DNA with the 'N' terminal towards the 5'-end of the DNA (Figure 7).

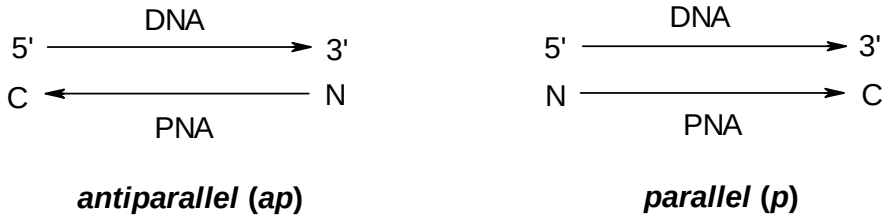


Figure 7 Schematic representation of the antiparallel and parallel modes of complexation of PNA with complementary DNA

In order to study the duplex formation potential of the pyrrolidine PNA backbone, it was imperative to synthesize mixed sequences incorporating both, purines and pyrimidines. The pyrrolidine PNA thymine monomer was therefore incorporated into decamers (Table 2, entry **66- 69**). Pyrrolidine PNA monomers bearing the other natural nucleobases adenine, cytosine and guanine with (2*R*,4*S*) stereochemistry were also efficiently included in PNA oligomer sequences utilizing the same Boc-protection strategy as used for the homothymine sequences (Table 2, Sequences **70-72**). This was

expected to unveil the differences on stability of complexes as a result of pyrrolidine PNA monomers bearing the different nucleobases. Sequence **65** is the unmodified *aeg*PNA and was used as a control.

Table 2 Mixed Base purine-pyrimidine PNA Sequences

PNA	Sequence Composition	
65	H-G T A G A T C A C T -β-ala–OH	*aeg*PNA Control
66	H-G T A G A **t** $_{(2S,4S)}$ C A C T-β-ala–OH	One (2*S*,4*S*) pyrrolidine Unit
67	H-G T A G A **t** $_{(2S,4R)}$ C A C T-β-ala–OH	One (2*S*,4*R*) pyrrolidine Unit
68	H-G T A G A **t** $_{(2R,4R)}$ C A C T -β-ala–OH	One (2*R*,4*R*) pyrrolidine Unit
69	H-G T A G A **t** $_{(2R,4S)}$ C A C T-β-ala–OH	One (2*R*,4*S*) pyrrolidine Unit
70	H-G T A **g** $_{(2R,4S)}$A T C A C T-β-ala–OH	One (2*R*,4*S*) pyrrolidine Unit
71	H-G T **a** $_{(2R,4S)}$ G A T C A C T-β-ala–OH	One (2*R*,4*S*) pyrrolidine Unit
72	H-G T A G A T **c** $_{(2R,4S)}$ A C T-β-ala–OH	One (2*R*,4*S*) pyrrolidine Unit
DNA/RNA		
73	5'- A G T G A T C T A C-3'	*ap* DNA to PNAs **65-72**
74	5'-C A T C T A G T G A-3'	*p*DNA to PNAs **65-72**
75	5'- A G U G A U C U A C-3'	*ap* RNA to PNAs **65-72**
76	5'- A G T G **T** T C T A C-3'	Mismatch in DNA opposite T

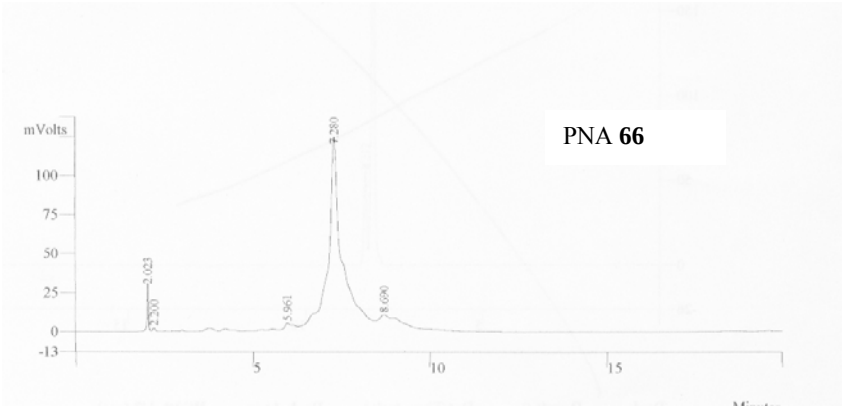
2.4. Cleavage of the PNA oligomers from the solid support

The cleavage of peptides from the Merrifield resin by strong acids like trifluoromethane sulphonic acid (TFMSA)- trifluoroacetic acid (TFA) yields peptides with free carboxylic acids at their 'C' termini. The synthesized PNA oligomers were cleaved from the resin using this procedure to obtain sequences bearing β alanine free carboxylic acids at their 'C' termini (**50-62**, **65-72**). The exocyclic amino groups of

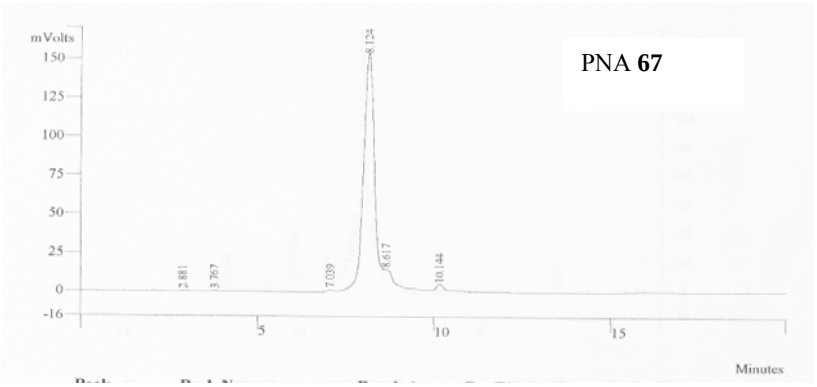
cytosine, adenine or guanine, if protected as benzyloxycarbonyl, are also cleaved during this process. If the N^6-exocyclic amino group of adenine is, however, protected as a benzoyl group, then its deprotection must be carried out under alkaline conditions employing ammonia or ethylenediamine.[15] The ammonia treatment is carried out at 55^{o}C for 16h. These conditions have proved to be harsh on the peptide, as they are conducive to degradation of the peptide by successive 'N'-terminal cleavage. The relatively milder conditions involve treatment with ethylenediamine in ethanol at room temperature overnight. This protocol was followed for oligomers containing N^6-benzoyl adenine pyrrolidine monomeric units, and was carried out prior to their cleavage with TFMSA-TFA.

2.5. Purification of the PNA oligomers

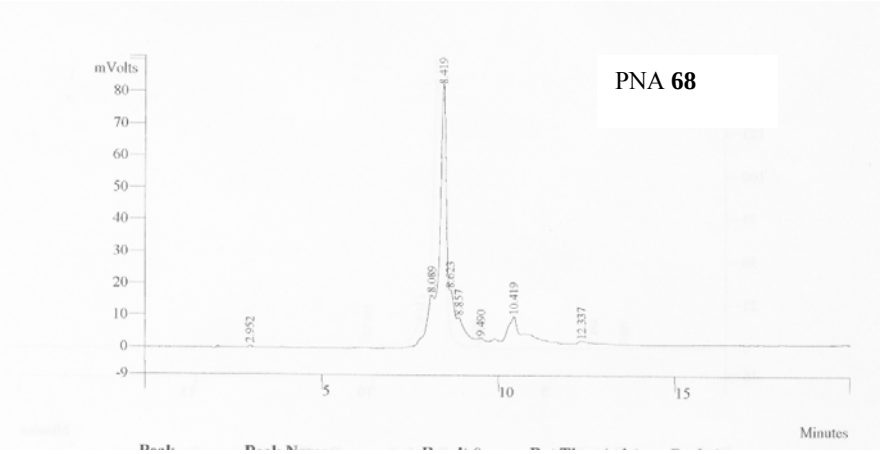
All the cleaved oligomers were subjected to initial gel filtration. These were subsequently purified by reverse phase FPLC on a semi-preparative C8 RP column by gradient elution using an ascending gradient of acetonitrile in water containing 0.1% TFA on a semi- preparative HPLC RP C4 column. The purity of the oligomers was then checked by reverse phase HPLC on a C18 RP column and molecular mass was confirmed by MALDI-TOF mass spectroscopic analysis. Some representative HPLC profiles and mass spectra are shown in Figures 8 and 9.



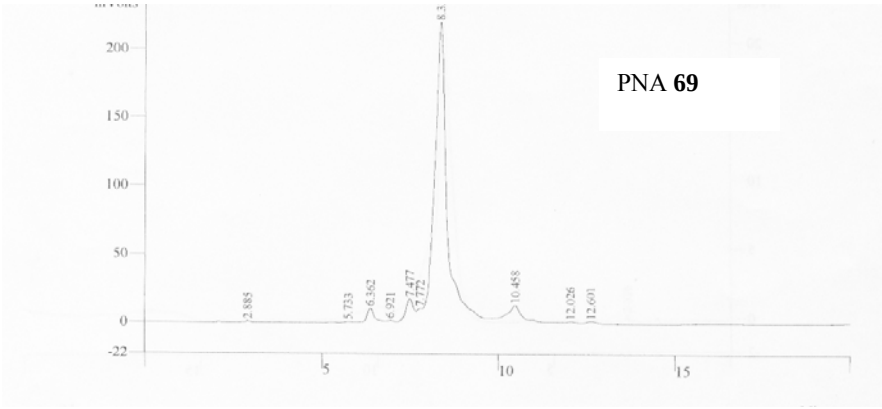
PNA **66**



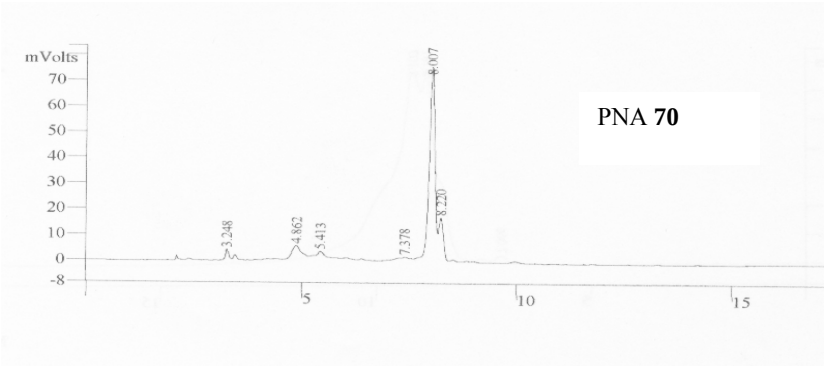
PNA **67**



PNA **68**



PNA **69**



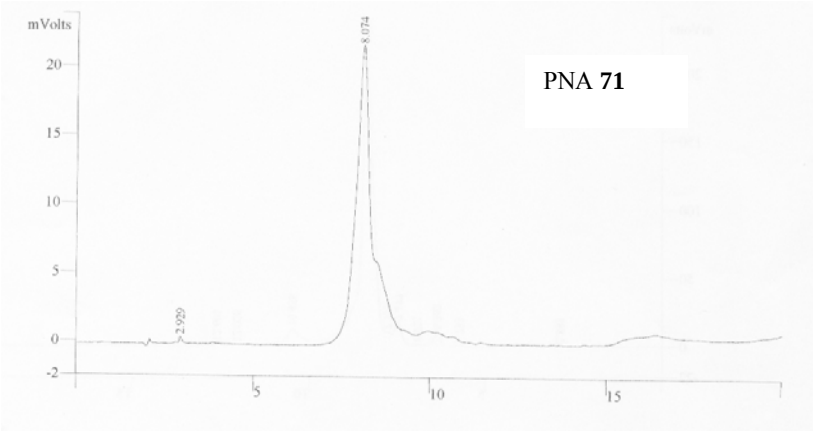
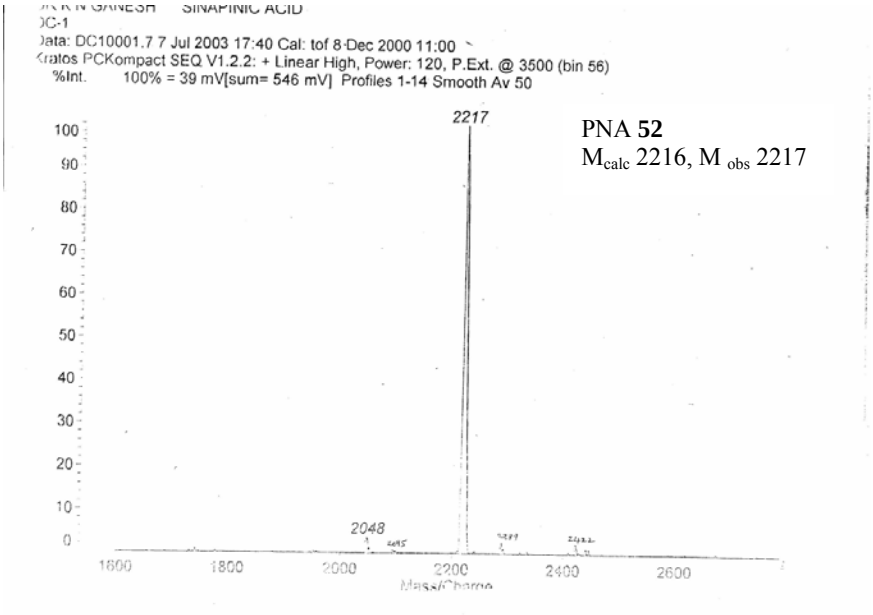


Figure 8 HPLC profiles of representative oligomers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DC-1
Data: DC10001.7 7 Jul 2003 17:40 Cal: tof 8-Dec 2000 11:00
Kratos PCKompact SEQ V1.2.2: + Linear High, Power: 120, P.Ext. @ 3500 (bin 56)
%Int. 100% = 39 mV[sum= 546 mV] Profiles 1-14 Smooth Av 50
2217
2048
PNA 52
M calc 2216, M obs 2217
100
90
80
70
60
50
40
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20
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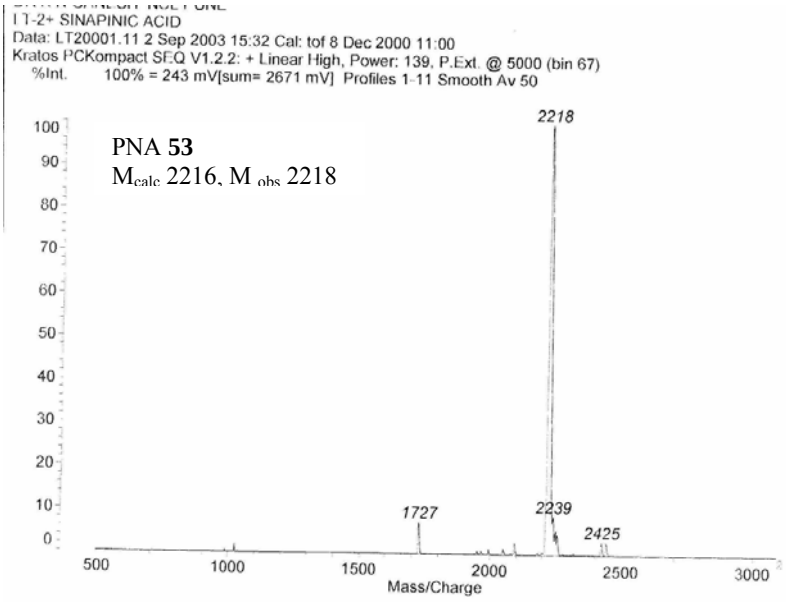
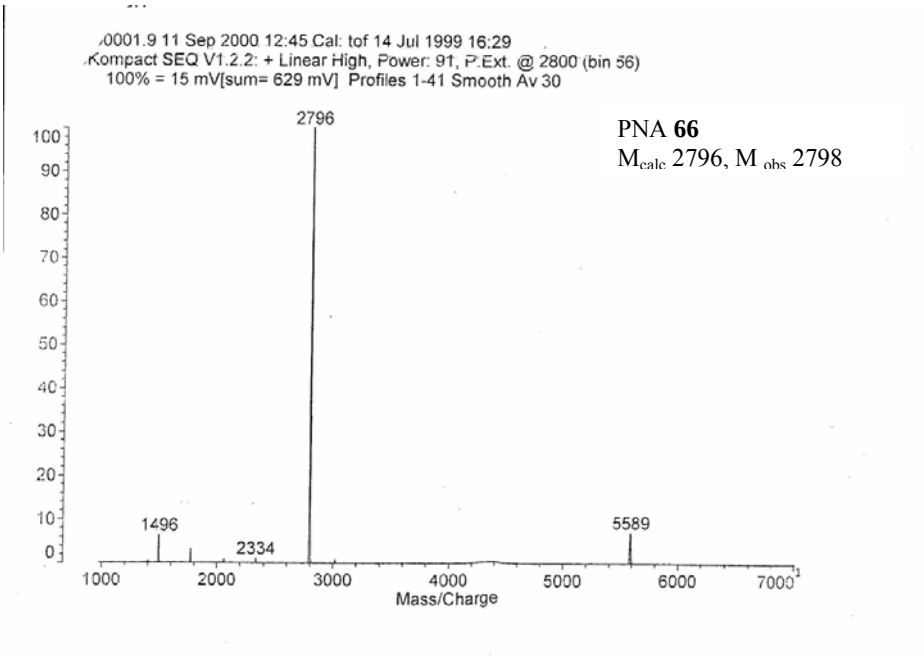
1600
1800
2000
2200
2400
2600


LT-2+ SINAPINIC ACID
Data: LT20001.11 2 Sep 2003 15:32 Cal: tof 8 Dec 2000 11:00
Kratos PCKompact SEQ V1.2.2: + Linear High, Power: 139, P.Ext. @ 5000 (bin 67)
%Int. 100% = 243 mV[sum= 2671 mV] Profiles 1-11 Smooth Av 50
2218
1727
2239
2425
PNA 53
M calc 2216, M obs 2218
100
90
80
70
60
50
40
30
20
10
0
500
1000
1500
2000
2500
3000
Mass/Charge



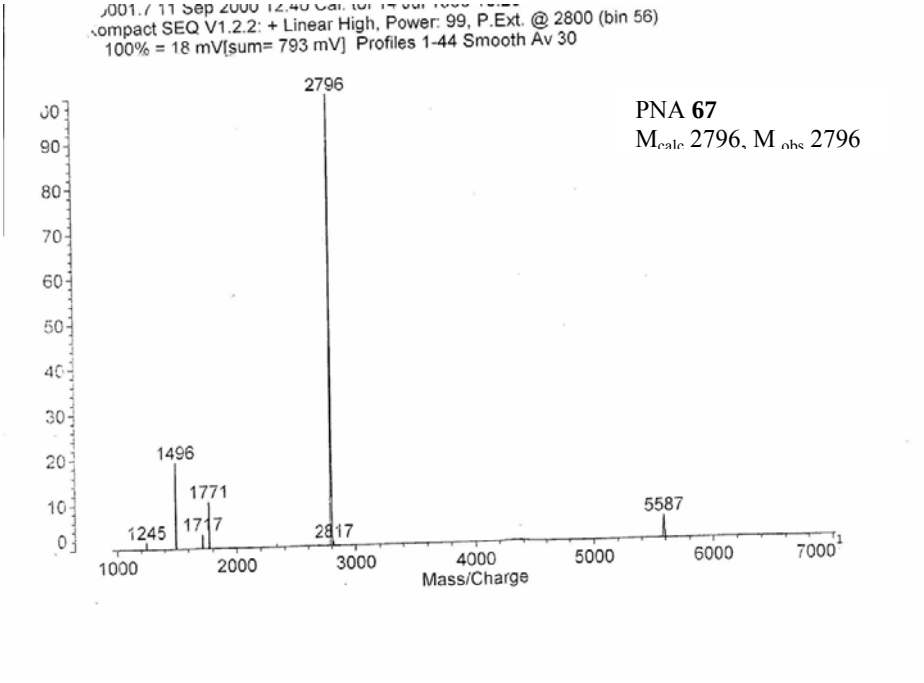


Figure 9 Representative MALDI-TOF spectra of PNA sequences

2.6. Synthesis of complementary oligonucleotides

The oligonucleotides (**63**,**64,73,74** and **76** Table 1 and 2) were synthesized on a ABI DNA synthesizer using the standard β-cyanoethyl phosphoramidite chemistry. The oligomers were synthesized in the 3' → 5' direction on a CPG solid support, followed by ammonia treatment. The oligonucleotides were de-salted by gel filtration, their purity ascertained by RP HPLC on a C18 column. The antiparallel RNA sequence was procured from Genomechanics, U.S.A.

2.7. DNA/RNA complementation studies using biophysical spectroscopic techniques

2.7.1. CD spectroscopy:

CD spectroscopy is a technique available for monitoring structural changes of nucleic acids in solutions or for determining whether a new or unusual structure is formed by a particular polynucelotide sequence. The reliance on CD spectroscopy to study nucleic acid conformations has stemmed from the sensitivity and ease of CD

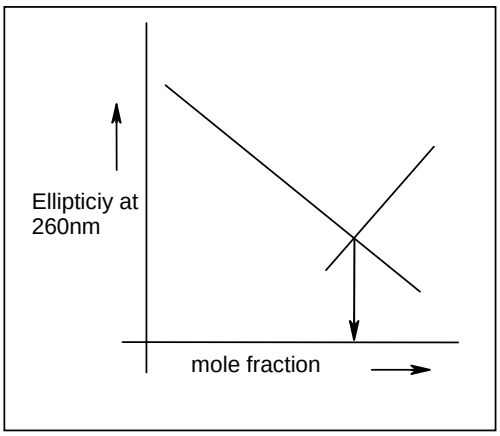


Figure 10 CD mixing showing PNA:DNA binding stoichiometry

measurements, the nondestructive nature of measurements and the fact that conformations can be studied in solution. Although detailed structural information, such as that from X-ray crystallography or NMR spectroscopy is not available from CD spectra, the CD spectrum of nucleic acid in solution can provide a reliable determination of its overall conformational state when compared with the CD spectra of reference sample. In case of nucleic acids, the sugar units of the backbone provide chirality and the bases attached to sugars are chromophore. In the CD spectrum of polynucleotide with stacked bases, the magnitude of CD signals is larger in the 260-280 nm region and significantly higher at 200nm than that of individual bases.

The PNA backbone is inherently achiral and do not exhibit CD signals. The complex formed as a consequence of the binding of achiral PNA and chiral DNA leads to the formation of a chiral complex and thus CD assumes importance in the characterization of such complexes. Job's plot[37] (CD mixing) involving CD spectroscopy, is used to find out binding stoichiometry of PNA:DNA complexes[38] in which one has to follow evolvement of ellipticity at wavelength as a function of mole fraction of added PNA/DNA (Figure 10).

2.7.2. UV-Spectroscopy

Monitoring the UV absorption at 260 nm as a function of temperature has been extensively used to study the thermal stability of nucleic acid system and consequently, PNA:DNA/RNA hybrids as well. Increasing temperature perturbs this system, inducing a structural transition by causing disruption of hydrogen bonds between the base pairs, diminished stacking between adjacent nucleobases and larger torsional motions in the backbone leading to a loss of secondary and tertiary structure. This is evidenced by an increase in the UV absorption at 260 nm, termed as hyperchromocity. The DNA melting

is readily monitored by measuring its absorbance at a wavelength of 260 nm. A plot of absorbance Vs temperature gives a sigmoidal curve in case of duplexes/triplexes and mid point of transition gives the Tm (Figure 11A). In case of triplexes, the first dissociation leads to the duplex (Watson–Crick duplex) and the third strand (Hoogsteen strand) followed by duplex dissociation at higher temperature into two single strands. The DNA triplex melting shows a characteristic double sigmoidal transition with separate melting temperature for each transition.

A non-sigmoidal (e.g., linear) transition with low hyperchromicity is a consequence

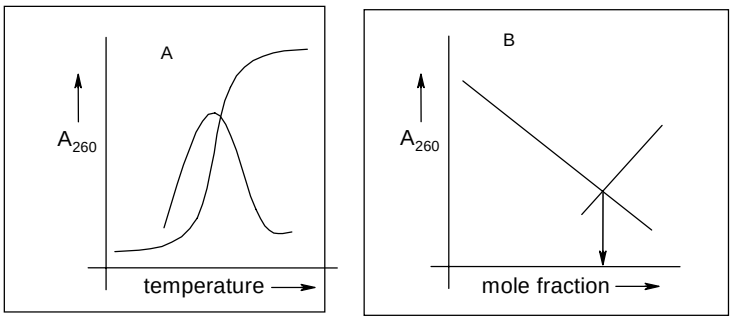


Figure 11 Schematic representation of **A**. UV-melting (thermal stability), **B**. UV-mixing,

of non-duplexation (non-complementation). In many cases, the transitions are broad and the exact Tms are obtained from the peak in the first derivative plots. This technique has provided valuable information regarding complementary interactions in nucleic acid hybrids involving DNA, RNA and PNA.

The UV mixing experiments are carried out by mixing the appropriate oligomers in different mole ratios keeping the total concentration constant. The stoichiometry of paired strands may be obtained from the mixing curves, in which the optical property at given wavelength is plotted against the mole fraction of each strand, known as Job's plot. Two triplex forming oligomers are mixed in various proportions, which can form 1:1, 1:2 or 2:1 complex, depending on the conditions in the medium and binding ability

of the two interacting oligomers. By varying the oligomer proportions, one gets a UV mixing curve provided that the complexes, which are formed, are stable. The hypochromic change in absorbance upon formation of double and triple stranded complexes results in inflection points at either 0.5, 0.33 or 0.67 mole fraction of one strand (Figure 11B). These points correspond to the complete involvement of strands in complexes.

The fidelity of base-pairing in the PNA:DNA complexes can be examined by challenging the PNA oligomer with a DNA strand bearing a mismatch at a desired site, preferably opposite the site of modification. The base mismatch leads to the absence of or incorrect hydrogen bonding between the bases and causes a drop in the measured melting temperature. A modification of the PNA structure is considered good if it gives a much lower Tm with DNA sequences containing mismatches as compared to that with unmodified PNA. It is to be pointed out that in all biophysical experiments described herein, the modified PNAs are always evaluated against the unmodified control PNA.

2.7.3. Gel electrophoresis

Electrophoresis is a technique used to separate, purify and characterize the macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely used techniques in biochemistry and molecular biology. This technique is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is referred as a gel shift or gel retardation assay.

Gel mobility-shift assay is based on the fact that the electrophoretic mobility of a DNA-fragment in a native polyacryalamide gel typically is reduced by the presence of a PNA strand displacement complex. The shift in gel mobility is apparently not due to the increase in mass but rather reflects structural changes.[39] In addition, the gel-shift assay is fast and technically simple and therefore often used to determine the degree of binding. Frequently binding of PNA results in not only one but two or more shifted bands. These distinct bands presumably reflect structural isomers or alternative stoichiometries.

The PNAs modified with one or two units of pyrrolidine PNA and the control PNA were individually treated with oligonucleotide and the complexation were monitored by nondenaturing gel electrophoresis at 10°C. The spots were visualized on a fluorescent TLC background, and the results are shown in figures 17 and 19.

2.8. Results and discussion

2.8.1. Pyrrolidine-PNA monomers and pyrrolidine-PNA oligomers

CD studies: The introduction of chiral amino acids at *C*-terminus of PNA is known to transmit the chirality through the sequence[40] of PNA oligomers and can direct the binding efficacy to the chiral DNA by inducing helical parameters to otherwise achiral PNA backbone.

The transmission of chirality through chiral communication was noted to be through planar chiral amino acids lity in PNA propag to 8-10 base pairs. l at α- carbon

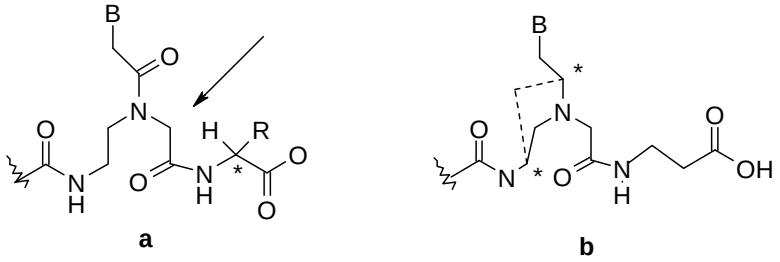


Figure 12 Chiral induction in PNA.

at the sight indicated by arrow in figure 12a, chiral communication is disturbed. As part of the backbone in the chimeric pyrrolidine-*aeg*PNA oligomers, the chirality of pyrrolidene PNA oligomers was envisioned to direct the preferred structure of *ss* PNAs. These monomers were therefore incorporated into triplex forming homopyrimidine PNA T_8 sequences at C- terminus (PNA **51-54**), center of the sequence (**59-62**) and at two positions i.e. center of the sequence and at C terminus (**55-58**) to study the additivity of any structural pre-organization due to preferential base stacking.

In the pyrrolidine monomeric units described here (Figure 12b), the nucleobase orientations are dictated by their intrinsic chirality as evident from CD of the enantiomeric monomer pairs, (2*S*,4*S* and 2*R*,4*R*) and (2*S*,4*R* and 2*R*,4*S*) (Figure 13). The CD profiles of these monomers show mirror image relationship of CD bands at 270 nm, 240 nm and 210 nm and the sign of CD bands is significantly influenced by the

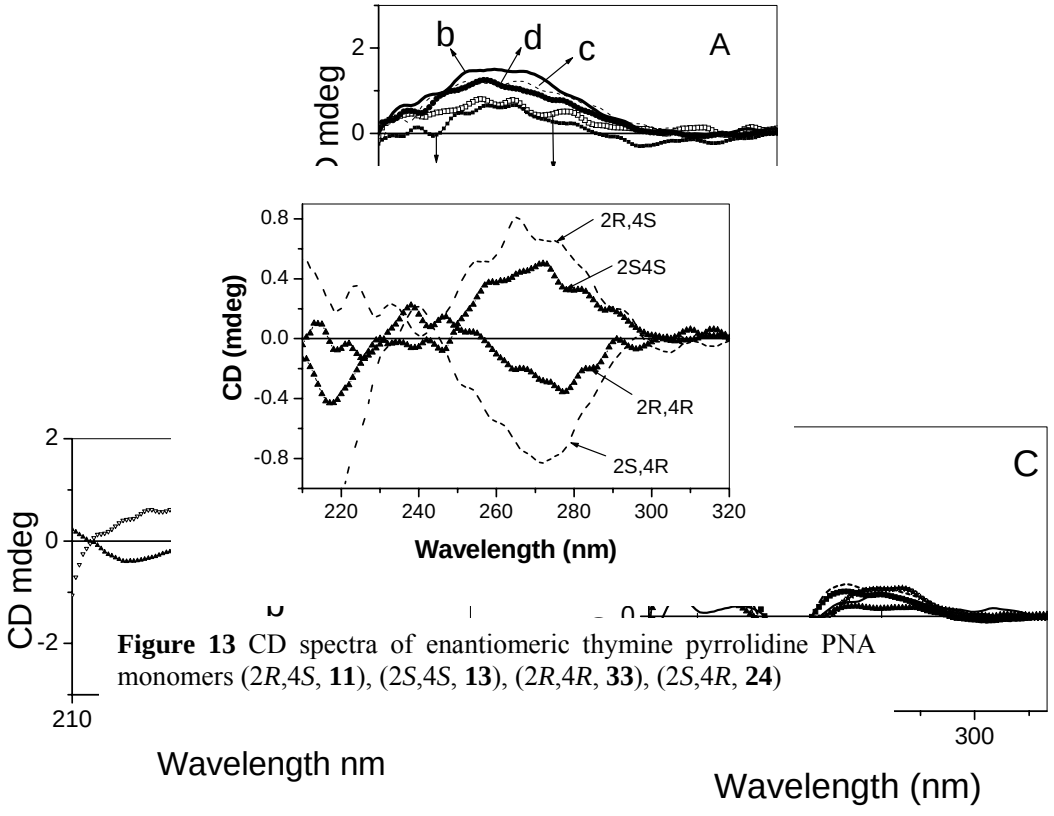


Figure 13 CD spectra of enantiomeric thymine pyrrolidine PNA monomers (2*R*,4*S*, **11**), (2*S*,4*S*, **13**), (2*R*,4*R*, **33**), (2*S*,4*R*, **24**)

Figure 14 A) CD spectra for selected *ss* pyrrolidinePNA a) **52**, b) **53**, c) **59** d) **60**, e) **61** **B),** *ss* pyrrolidinePNA showing mirror image a) **57**, b) **58** **C)** PNA CD profiles of PNA_2:DNA complexes, a) **54:63**, b) **53:63**, c) **52:63**, d) **54:63**, e) **50:63**

stereochemistry of monomes. In single strand pyrrolidine PNA, CD signals arise due to asymmetry induced in base stacking by the chiral pyrrolidine unit. Representative CD spectra of various single stranded pyrrolidine-*aeg*PNA oligomers with a single pyrrolidine at C-/N-termini are shown in Figure 14A. These oligomers exhibited CD signals with maxima at 230-260 nm and minima at 280 nm irrespective of the stereochemistry of the pyrrolidine unit (Figure 14A). Enantiomeric pyrrolidine units (2*S*,4*S* and 2*R*,4*R*) in the central position of the oligomers **59**, **60** did not reflect in CD. When two units of (2*S*,4*R*) and (2*S*,4*R*) pyrrolidine enantiomers were present in the PNA oligomers **57**, and **58**, a mirror image relationship emerged in their CD profiles (Figure 14B). CD spectral data on single strand chiral PNAs thus suggested that propagation of chiral induction could be additive in nature depending upon the stereochemistry and the self-ordering is a function of chirality.

2.8.2. Pyrrolidine-aegPNA$_2$:DNA triplexes

CD studies: Upon complex formation with the complementary DNA, the CD exhibited by the complex was similar to that of the *aeg* control sequence and indicates that DNA CD is the main component (Figure14C). Thus complexes formed by all diastereomers exhibited positive maxima at 260, and 220 nm and negative minima at 280-300 nm, crossover points at ~240 and ~250 nm. The positive band in the region of 250-280 nm seen in the CD spectra of complexes is a characteristic of triplex. The circular dichroism spectroscopy can also be used to study the binding stoichiometry of PNA:DNA complexes and change in elipticity is used to find out the binding stoichiometry of PNA:DNA complexes.[40] The chimeric pyrrolidine-*aeg*PNA single strand sequence **56**, which contains two pyrrolidine units, was used to study the changes in CD signals upon complexation with DNA. Pyrrolidine-*aeg*PNA **56** and DNA **63**

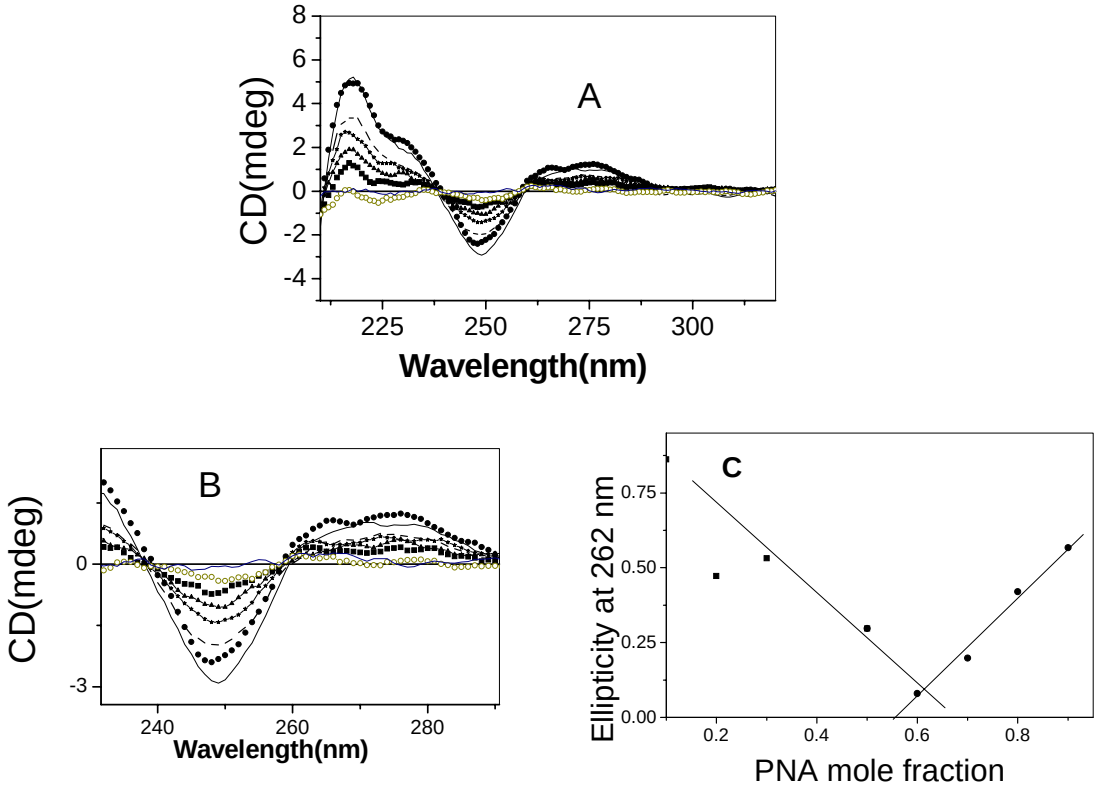


Figure 15 A) CD mixing curve for PNA **56** and DNA **63** mixtures in the various molar proportions. B) Expanded CD spectra of molar mixtures of PNA **56** and DNA **63** C) CD mixing curve for PNA **56** and DNA **63** showing inflection point at 0.61.

were mixed in various proportions and figure 15A shows the CD spectra for these mixtures of pyrrolidine PNA and DNA while Figure 15B shows expanded CD spectra for these mixtures. The ellipticity at 262 nm when plotted against mole fraction of PNA gave an inflection point at 0.61 mol fraction of PNA indicating PNA_2:DNA binding stoichiometry (Figure 15C). Further, the positive maxima at 220, 260 and 280 nm and two isobastic points at 238 nm and 258 nm which are characteristics of PNA_2:DNA triplex confirms the 2:1 binding stoichiometry (Figure 15 A,B).

UV-Tm studies

The investigation of the effect of backbone modifications on the stability of PNA:DNA hybrids were carried out by temperature dependant UV absorbance measurements. The UV-Tm values obtained for PNA: DNA complexes in 1:1 or 2:1 stoichiometry were almost identical and plots of absorbance or percentage hyperchromicity at 260 nm *vs* temperature were sigmoidal, indicative of a two state cooperative transition. The polypyrimidine PNAs prefer to form PNA_2:DNA triplexes

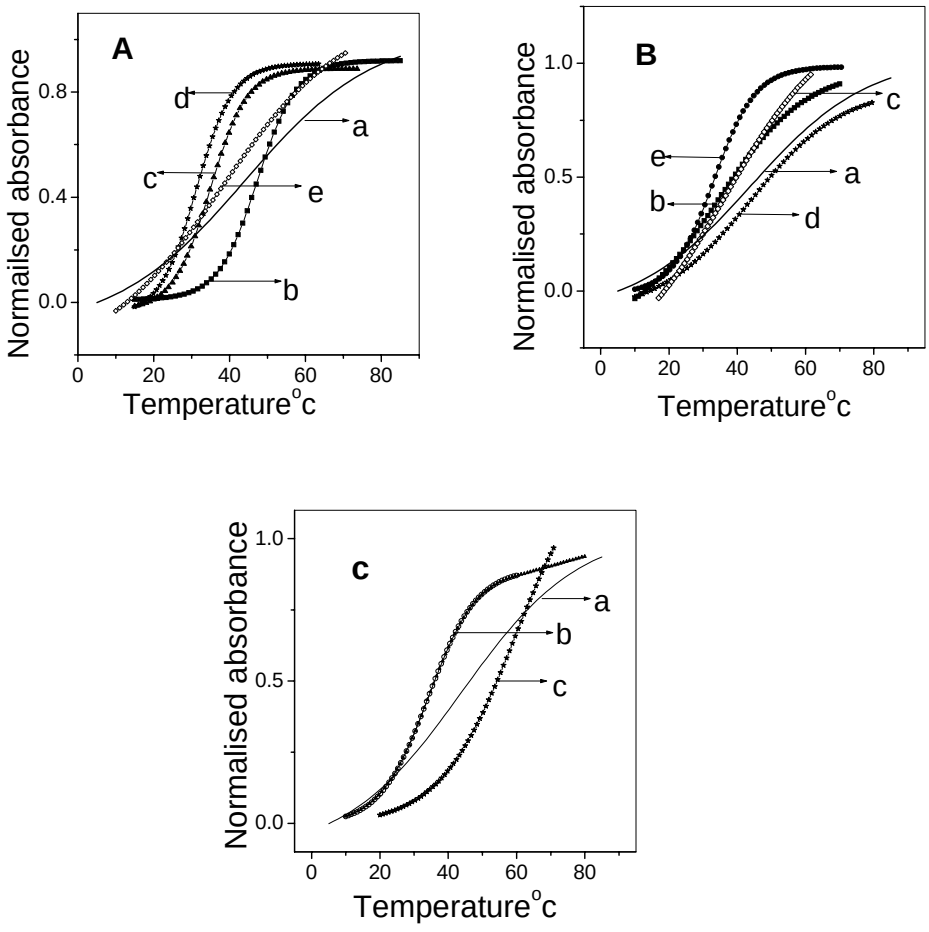


Figure 16 UV-Tm profile of PNA_2:DNA complexes **A**, a) **50:63**, b) **51:63**, c) **52:63**, d) **53:63**, e) **54:63** **B**, a) **50:63**, b) **55:63**, c) **56:63**, d) **57:63**, e) **58:63 C,** a) **50:63**, b) **60:63**, c) **61:63**

even when DNA:PNA stoichiometry is 1:1. The unmodified PNA_2:DNA triplex **50:63** showed a sigmoidal melting curve corresponding to a mid point transition 43°C Tm with ~10% hyperchomicity (Table 3). Thermal stability of modified chimeric pyrrolidine-PNA with DNA was studied employing 2:1 PNA_2:DNA stoichiometry. The results of these studies are summarized in Table 3. The sigmoidal transitions of various PNA_2:DNA triplexes are shown in Figure16.

A single (2*S*,4*S*) pyrrolidine modification at *C*-terminus stabilized the PNA_2:DNA triplex **51:63** compared with the control triplex **50:63** and ΔTm = +4°C with 13% hyperchromicity. The increase in Tm is similar to that observed when a lysine unit is attached at *C*-terminus of the PNA sequence. Additional (2*S*,4*S*) pyrrolidine unit at the center of the sequence did not show additive stability of the complex **55:63**. Other diastreomeric pyrrolidine units at *C*-terminus in **52**, **53**, **54** although showed decrease in Tm, the melting transitions were accompanied by significant hyperchromicity (13-20%) indicating favorable base-stacking interactions in PNA_2:DNA triplexes. A single pyrrolidine modification in the middle of the sequence resulted in the stereochemistry dependant melting temperatures. The triplexes with (2*S*,4*R*) and (2*R*,4*S*) units in **61:63** and **62:63** respectively showed higher Tm values but the transitions were accompanied with low hyperchromicity (4-7%) that signifies weak base-stacking interactions. Interestingly, the (2*S*,4*R*) pyrrolidine unit showed additive stabilizing properties. A single pyrrolidine modification in the center of the sequence stabilized the triplex **61:63** with low % hyperchromicity. When at *C*-terminus, a triplex **53:63** was formed with a lower Tm (ΔTm = - 6°C) that was accompanied with 14 % hyperchromicity. When two units were introduced in the center and at *C*-terminus, a stable triplex **57:53** (ΔTm = +1°C) was formed that is better than the control in both Tm and % hyperchromicity.

Table 3 UV-Tm (°C) of chimeric PNA_2:DNA complexes.

Modified monomer unit **t**	H-TTTTTTT**t**-β-ala-OH **A**		H-TTT**t**TTT**t**-β-ala-OH **B**		H-TTT**t**TTTT-β-ala-OH **C**	
	PNA_2:DNA	Tm°C	PNA_2:DNA	Tm°C	PNA_2:DNA	Tm°C
(2*S*,4*S*)	**51:63**	47 (13)	**55:63**	34 (10)	**59:63**	28 (6) [17]
(2*R*,4*R*)	**52:63**	42 (20)	**56:63**	36 (5)	**60:63**	40 (4)
(2*S*,4*R*)	**53:63**	37 (14)	**57:63**	44 (13)	**61:63**	58 (5)
(2*R*,4*S*)	**54:63**	38 (15)	**58:63**	34 (8)	**62:63**	59 (7) [17]
Control *aeg*PNA_2:DNA triplex **50:63** Tm 43°C (10)						

Values in the parenthesis indicate % hyperchromicity Buffer: 10mM sodium cacodylate, 100mM NaCl, 0.01 mM EDTA. pH 7.3. Melting experiments were performed with a PNA:DNA strand concentration of 1μM each. The concentrations were calculated on the basis of the absorbance at 260nm using the molar extinction coefficients of A,T,G and C for DNA.

Thus a second (2*S*,4*R*) pyrrolidine unit in the PNA sequence effected stabilizing effects that were not observed with other diastereomers.

The results presented here indicate that all the four isomers of the pyrrolidine monomer exert the stabilizing/destabilizing effects on the triplex formation and point out that only the correct stereochemistry of the backbone will eventually be uniformly accepted in the triplex. The stabilization by the (2*S*,4*R*) pyrrolidine stereochemistry reflects both in higher Tm and higher % hyperchromicity that indicate better hydrogen bonding and stacking interactions.

The formation of stable complex **52:63** that showed high % hyperchromicity (20%) was also established by gel shift assay (figure 17, lane 3).

Gel electrophoresis

Under the electrophoretic conditions employed, the single stranded PNAs (PNA52, Lane 2) that carry a net positive charge did not move out of the well. The formation of PNA_2:DNA complexes (Figure 17, Lane 3, **52:63;** Lane 4, **61:63)** was accompanied by

the disappearance of the single strand DNA and appearance of a lower migrating band due to PNA:DNA complexes. The single modified PNA_2:DNA complexes migrated about the same as the unmodified PNA:DNA complex.

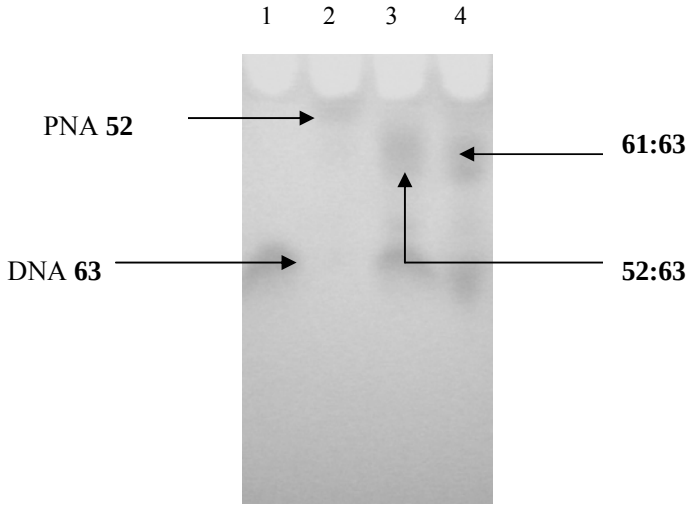


Figure 17 15% Polyacrylamide Gel Electrophoresis of PyrrolidinePNA_2:DNA Lane 1 DNA **63** Lane 2 PNA **52** Lane 3 **52:63.** Lane 4 **61:63**

The sequence specificity of the PNA_2:DNA interactions was studied by introducing mismatched T-T base in DNA oligomer **64** in the center of the sequence and PNAs with modified units. All the oligomers showed decreased stability with DNA 64 with a mismatched base similar to the unmodified *aeg*PNA.

The presence of salt (<100mM NaCl) in the medium is known to destabilize unmodified PNA_2:DNA complexes.[41] The DNA **63** with control PNA **50** exhibited ΔTm- 3°C upon 200mM salt addition, while PNA oligomer with (2*S*,4*S*) pyrrolidine thymine unit **51:63** destabilized the complex to a greater extent (ΔTm -10°C). This result is similar to that seen in other positively charged oligonucleotide analogues.[42] The stability of the hybrids formed between pyrrolidine PNA and DNA could possibly be

aided by the electrostatic attraction between the positively charged protonated pyrrolidine PNA and negatively charged phosphate group of DNA.

2.8.3. Pyrrolidine-*aeg*PNA:DNA duplexes

Uv-Tm studies:

The oligothymine sequences described above were found to form triplexes in which the binding orientation (*p-ap*) of the two PNA strands involved in complex formation remains inconsequential. In order to study orientational preferences of PNA:DNA

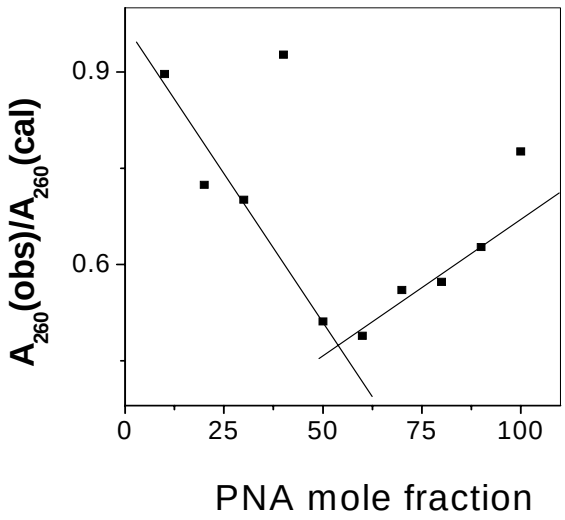


Figure 18 UV-mixing plot for PNA **67**:DNA **75** , showing 1:1 binding stoichiometry

binding mixed purine-pyrimidine sequences were synthesized. The plot of UV absorbance versus molar ratio showed an inflection point at 50, indicating 1:1 binding stoichiometry in mixed sequences (Figure 18). It is also reported in the literature that the mixed base *aeg*PNA sequence containing all the four nuleobases viz, A, T, G and C form 1:1 PNA:DNA duplex.[43] All the pyrrolidine monomers were incorporated into

mix sequence by systematic replacement of central thyminyl-*aeg* unit by thyminyl pyrrolidine diastereomer **11d**, **13**, **24** and **33.**

Also A(**11a**), G(**12**) and C(**11a**) monomers with (2*R*,4*S*) stereochemistry were incorporated in the mixed sequence. The PNA:DNA oligomers utilized in the study of PNA:DNA duplexes are listed in Table 2. The mixed sequence control PNA **65** forms both parallel and antiparallel duplex with DNA and the antiparallel duplex is only slightly stable by 1°C over the parallel duplex (Table 4, Figure 20). All the *aeg*-pyrrolidinePNA oligomers bind preferentially in the *ap* mode (ΔTm (*ap*/*p*)=1-10°C).

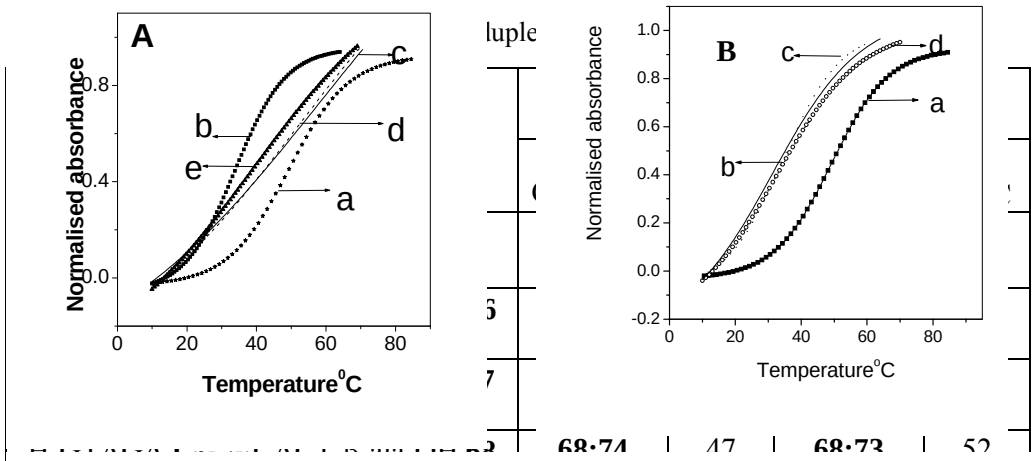


Figure 20 Melting profiles of PNA:DNA complexes **A**: (a) **65:73** (b) **66:73** (c) **67:73** d) **68:73** (e) **69:73** **B**: (a) **65:73** (b) **70:73** (c) **71:74**3(d) **72:73**
Melting experiments were performed with a PNA:DNA strand concentration of 0.5μM each. The concentrations were calculated on the basis of the absorbance at 260nm using the molar extinction coefficients of A,T,G and C for DNA.

Complexes formed by PNA **66** (2*S*,4*S*) and PNA **69** (2*R*,4*S*) with DNA **73** are quite destabilized (Table 4 A, Figure 20, ΔTm = –13° C and -8°C respectively) compared to *aeg*PNA **65**:DNA **73** hybrids. The PNAs **67** (2*S*,4*R*) and **68** (2*R*,4*R*) were found to stabilize the complexes with *ap*DNA **73** (Table 4 A, Figure 20, ΔTm = +11°C and +5° C respectively). The stereochemistry of pyrrolidine ring in (2*S*,4*R*) could thus exercise

preferential structral pre-organisation of *aeg*-pyrrolidine PNA that stabilized PNA:DNA hybrids. The corresponding (2*R*,4*S*) isomer with A, G and C nucleobase, destabilized the *ap* as well as *p* duplexes indicating that this stereochemistry indeed is not suitable for duplex formation independent of the nucleobase (Figure 20 B, Table 5). Thus all isomers were able to differentiate between *ap* and *p* modes of binding. The largest *p*/*ap* differentiation was observed when the (2*S*,4*R*) pyrrolidine-T unit is incorporated in the *aeg*PNA. PNA oligomers **67** and **68** with DNA **76** having a single mismatch at the site complementary to the pyrrolidine unit were destabilized by 14^{o} C and 4^{o} C respectively.

Table 5 UV-Tm data for (2*R*,4*S*) pyrrolidinePNA mixed base PNA:DNA duplexes.

PNA Sequence	DNA (*p*)		DNA (*ap*)	
	PNA:DNA	Tm°C	PNA:DNA	Tm°C
H-GTAGATCACT-β-ala-OH **65**	**65:74**	46	**65:73**	47
H-GTA **g** $_{(2R,4S)}$ ATCACT-β-ala-OH **70**	**70:74**	27	**70:73**	29
H-GT **a** $_{(2R,4S)}$ GATCACT-β-ala-OH **71**	**71:74**	34	**71:73**	36
H-GTAGAT **c**$_{(2R,\ 4S)}$ ACT-β-ala-OH **72**	**72:74**	31	**72:73**	34

This indicates that the recognition process could be primarily through WC base pairing aided by steric fit and not overruled by steric fitting or electrostatic interactions.

The overall data indicates the stereochemistry dependent stability effect, in turn modulating by orientational and stereochemical factors. The most interesting results were obtained with the (2*S*,4*R*) and (2*R*,4*R*) pyrrolidine thymine units. The UV melting profiles of the pyrrolidine PNA:DNA duplexes are shown in figure 20.

Gel electrophoresis

The formation of PNA:*ap*DNA duplexes was also confirmed by the non-denaturing

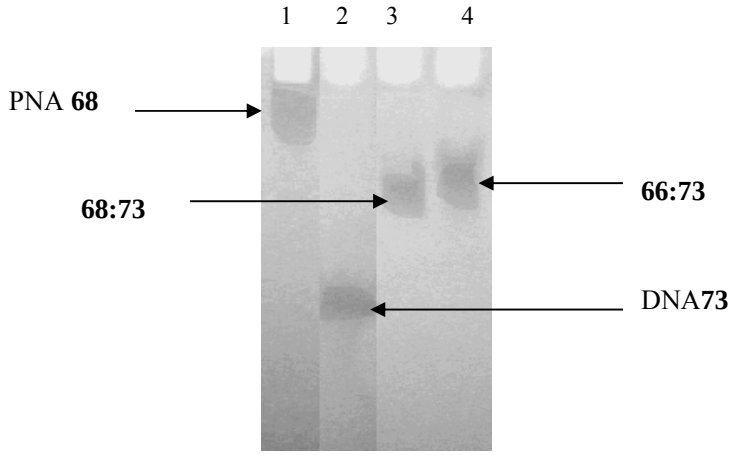


Figure 22 A Duplex PNA:DNA 15% Polyacrylamide Gel Electrophoresis of Pyrrolidine PNA:DNA complexes. Lane 1 PNA **68**; Lane2, DNA **73;** Lane3 **68:73;** Lane 4, **66:73.**

gel-shift assay (Figure 22). The PNA:DNA complexes derived from pyrrolidinePNA with single modified unit were significantly retarded in the gel (Lane 3 and 4 PNA:DNA ,**68:73**, **66:73**). The PNA_2:DNA triplexes showed more retardation as compared to PNA:DNA duplexes.

2.8.4. Pyrrolidine-aegPNA:RNA duplexes

UV-Tm studies: We also studied the binding properties of the modified PNAs with *ap* RNA considering the differential structural requirements for PNA:RNA as compared to PNA:DNA.[44] Interestingly with RNA, PNAs comprising all the four thymine diastereomers form stable complexes than *aeg*PNA (Table 6, Figure 21).

Very significantly, PNA oligomers with *cis* diasteromers as in (2*S*,4*S*) **66** and

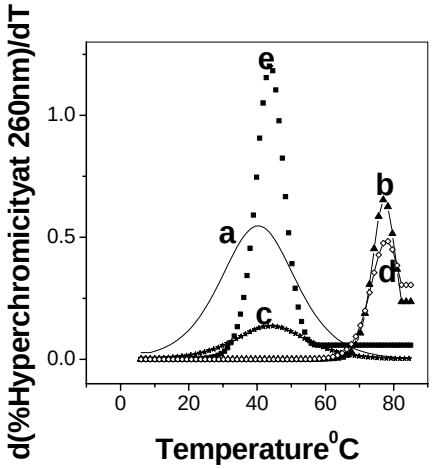


Figure 21 Melting profile and first derivative plots of PNA:RNA complexes. (a) **65:75** (b) **66:75** (c) **67:75** (d) **68:75** (e) **69:75**

(2*R*,4*R*) **68** which destabilized the corresponding DNA duplexes, conferred very large stabilization (Figure 21, Table 6,ΔTm = +35°C and +37°C) of the resulting RNA duplexes. This is an interesting observation and the reason could be either effective better steric fitting of PNA stereochemistry with RNA or other complex intrinsic structural differences in DNA and RNA.

Table 6 UV-Tm (°C) of PNA:RNA complexes containing the nucleobase Thymine.

PNA	PNA:RNA.	Tm°C
H-GTAGATCACT-β-ala-OH	**65:75**	41
H-GTAGA **t** $_{(2S,4S)}$ CACT-β-ala-OH	**66:75**	76
H-GTAGA **t** $_{(2S,4R)}$ CACT-β-ala-OH	**67:75**	44
H-GTAGA **t** $_{(2R,4R)}$ CACT-β-ala-OH	**68:75**	78
H-GTAGA **t** $_{(2R,4S)}$ CACT-β-ala-OH	**69:75**	43

The (2*R*,4*S*) pyrrolidne PNA oligomers (**70-72**) incorporating other nucleobases G, A and C hybridized with RNA **75** but with decrease in Tm compared to control *aeg*PNA **65** (Table 7, ΔTm= -8°C, -3°C and -5°C).

Table 7 UV-Tm data for the (2*S*,4*R*) pyrrolidine PNA mixed base PNA:RNA complexes

PNA	PNA:RNA	Tm°C
H-GTAGATCACT-β-ala-OH	**65:75**	41
H-GTA **g** $_{(2R,4S)}$ ATCACT-β-ala-OH	**70:75**	33
H-G T ***a*** $_{(2R,4S)}$ GATCACT-β-ala-OH	**71:75**	38
H-GTAGAT **c** $_{(2R,\ 4S)}$ ACT-β-ala-OH	**72:75**	36

Apart from the stereochemical requirements, the reason for the destabilization could also be the position of the modification in the sequence as observed in case of fluorinated olefinic PNAs.[45] The (2*R*,4*S*) stereochemistry that stabilized the PNA_2:DNA triplex structures was thus found to be unsuitable to form duplexes with RNA in *ap* orientation. The PNAs with L-lysine unit at *C*-terminus is known to bind better to RNA over DNA.[46] In our studies with the unmodified *aeg*PNA **16**, presence of β-alanine at *C*-terminus did not significantly differentiate binding between DNA and RNA.

2.8.5. Parallel/antiparallel (p/ap) orientation selectivity:

The largest *ap/p* differentiation (ΔTm = +12°C) was observed for PNA **67** having (2*S*,4*R*) pyrrolidine-thymine unit. This stereochemistry was thus found to be better suited for *ap* binding than binding in *p* orientation. The diastereomer in **68** (**t** 2*R*,4*R*) also stabilized *ap* duplex with DNA **73** with comparatively less discrimination of *ap/p* orientation selectivity. The results reported here show the differential *p/ap* DNA and RNA binding properties conferred by a single pyrrolidine unit in an *aeg*PNA sequence as a consequence of the stereochemistry of the pyrrolidine ring. Such effect was earlier observed when a chiral cyclic monomer was present in the middle of the sequence along with L-lysine unit at the *C*-terminus or *N*-terminus of the sequence.[47] We have used β-alanine at *C*-terminus so that the *C*-terminus remains achiral and the results obtained are solely because of the presence of the chiral pyrrolidine unit in the middle of the PNA sequence and not due to the positive charge or chirality of the terminal lysine unit.

2.9. Summary

*aeg*PNA homopyrimidine sequences are known to form triplexes. CD-mixing experiments indicated a 2:1 binding stoichiometry (PNA_2:DNA) for homopyrimidine pyrrolidine PNA:DNA complex while 1:1 stoichiometry was observed for purine-pyrimidine sequences. The % hyperchromocity *vs* temperature plots derived from the UV-melting data indicated a single transition characteristic of PNA_2:DNA melting, wherein both PNA strands dissociate from the DNA strand simultaneously, in a single step.

For triplexes, (2*S*,4*R*) stereochemistry with thymine as a nucleobase stabilizes the triplexes while (2*R*,4*S*) highly destabilizes the triplexes. The complex formation was stringent as evident from the melting studies with mismatch DNA sequence introducing a T-T mismatch at a centrally located thymine unit in the PNA. The complexes so

formed were unstable leading to the conclusion that although the positive charge on the pyrrolidine ring nitrogen does play a role in enhancing the binding to target DNA/RNA sequence, the main cause of the stabilization appears to be specific hydrogen bonding between the complementary nucleobases in PNA and DNA.

The binding of these modified oligomers with DNA was also seen in diagnostic gel mobility shift experiments, where a single modification effected significant retardation.

The presence of higher salt concentration in the medium caused a greater destabilization of the complex compared to the control *aeg*PNA:DNA complex. This perhaps must be due to protonated proline ring nitrogen in pyrrolidine unit, leading to electrostatic destabilization under increasing ionic strength.

In case of duplexes mix sequence with (2*R*,4*S*) A,G, C and T destabilizes the complex with complementary DNA while with RNA it shows marginal effect. The trend observed for (2*S*,4*S*) and (2*R*,4*S*) in the Tm of the PNA-DNA duplexes was reversed to that observed with PNA-RNA complexes. It is found that 1) *Trans* (2*S*,4*R*) pyrrolidine thymine monomer in PNA oligomers stabilized PNA_2:DNA triplexes and *ap* PNA:DNA duplex. 2) c*is* (2*S*,4*S*) and (2*R*,4*R*) remarkably enhance PNA:RNA duplex stability 3) All pyrrolidine modifications show preference for antiparallel DNA/RNA binding and 4) RNA hybrids are more stable than DNA hybrids. Thus, the different structural features of the PNA_2:DNA, PNA:DNA versus PNA:RNA complexes seem to have impact on the selection of the stereochemistry of the pyrrolidine unit while binding to either RNA or DNA complementary sequence, in duplex binding mode. It is interesting to note that the pyrrolidine backbone with (2*R*,4*S*) stereochemistry in homopyrimidine sequence stabilized the triplex with complementary DNA, but was found to destabilize the derived DNA/RNA duplexes.

An added and important asset of this positively charged chiral backbone is its enhanced solubility in aqueous media. All the diastereomers studied in this case, have the different special disposition of the attachment of the nucleobase to the proline ring at C-2 and C-4 position. Changing the stereochemistry does play crucial role in affecting the stability of the pyrrolidine PNA:DNA complexes.

The interesting stability differences seen in the complexes of different pyrrolidine PNAs with DNA/RNA and stereochemical preferences need to be further explored for different sequences to study the sequence dependence.

2.10. EXPERIMENTAL

The chemicals used were of laboratory or analytical grade. All the solvents used were purified according to the literature procedures.[51] All the reactions were monitored for completion by TLC. Usual work-up implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulphate and evaporation under vacuum.

Column chromatography was performed for purification of compounds on LOBA chemie silica gel (100- 200 mesh). TLCs were carried out on pre-coated silica gel GF_{254} aluminium sheets (Merck 5554). TLCs were run in either dichloromethane with an appropriate quantity of methanol or in petroleum ether with an appropriate quantity of added ethyl acetate for most compounds. Free acids were chromatographed on TLC using a solvent system of *iso*-propanol: acetic acid: water in the proportion 9: 1: 1. The compounds were visualized with UV light and/ or by spraying with ninhydrin reagent subsequent to Boc-deprotection (exposing to HCl vapors) and heating.

^{1}H (200 MHz/300MHz) and ^{13}C (50MHz) NMR spectra were recorded on a Bruker ACF 200 spectrometer fitted with an Aspect 3000 computer and all the chemical shifts are referred to internal TMS for ^{1}H and chloroform-d for ^{13}C. The chemical shifts are quoted in δ (ppm) scale. Optical rotations were measured on a JASCO DIP-181 polarimeter and CD spectra were recorded on a JASCO J715 spectropolarimeter.

Mass spectra were recorded on a Finnigan-Matt mass spectrometer, while MALDI-TOF spectra were obtained from a KRATOS PCKompact instrument.

(2*S*,4*R*)-(*N*-butyloxycarbonyl)-4-*O*-mesyl-proline methyl ester 4

To an ice-cooled solution of (2*R*,4*R*)-(*N*-butyloxycarbonyl)-4-*O*-mesyl-proline methyl ester **3** (2.0g, 8.2mmol) in dry pyridine (15ml) was added dropwise, methane sulfonyl chloride (1.3ml, 16.3mmol). Stirring was continued at room temperature. The reaction was complete in 4h. The solvent was completely removed *in vacuo* and the residue, taken up in water. The product was extracted in chloroform. The chloroform extracts were dried over sodium sulfate and evaporated to get the product **14**, which was obtained in the pure form after silica gel column chromatography (2.5g, 95%).

^{1}H NMR ($CDCl_3$) δ: 5.32 - 5.22 (m, 1H, H4), 4.52 - 4.35 (m, 1H H2), 3.85 - 3.73 (m, 2H,H5, H5'), 3.75 (s, 3H, $COOC\underline{H}_3$), 3.05 (s, 3H, $SO_2C\underline{H}_3$), 2.72 - 2.52 (m, 1H, H3), 2.35 - 2.17 (m, 1H, H3'), 1.48 & 1.43 (s, 9H, $(CH_3)_3$).

(2*R*,4*R*)-*N*-(butyloxycarbonyl)-4-*O*-mesyl-pyrrolidine-2-methyl alcohol 5

To a stirred mixture of dry ethanol (8ml) and dry THF (6ml), was added lithium chloride (0.05g, 11.6mmol) and sodium borohydride (0.43g, 11.6mmol) and stirring continued for ~ 10 min under Argon atmosphere. A solution of (2*R*,4*R*)-*N*-(butyloxycarbonyl)-4-*O*-mesyl-2-proline methyl ester **4** (1.50g, 4.6mmol) in THF (1ml)

was added dropwise and stirring was continued at room temperature under argon atmosphere for 6h, when TLC indicated the disappearance of the starting material. The reaction was then cooled on ice and acidified till pH 4.0 with a 10% aqueous solution of citric acid. The reaction mixture was stirred in an ice-bath for ~10min, after which, the solvents were removed under vacuum. The residue was taken up in water and the product extracted in ethyl acetate. The organic layer was then dried over sodium sulphate and evaporated to get the desired product **5** as a white solid in quantitative yield (1.34g, 98%).

^{1}H NMR ($CDCl_3$) δ: 5.18 (m, 1H, H4), 4.10 (m, 1H, H2), 3.90 - 3.55 (m, 4H, H5, H5', CH_2OH), 3.25 (br s, CH_2OH), 3.05 (s, 3H, SO_2CH_3), 2.40 (m, 1H, H3), 2.07 (m, 1H, H3'), 1.45 (s, 9H, $(CH_3)_3$).

^{13}C NMR ($CDCl_3$) δ: 157.0, 80.7(*C*4), 68.93, 58(*C*2), 51(*C*5), 33.8(*C*3), 28.4((*C*H_3)$_3$), 21.76

HRMS calcd for $C_{11}H_{21}NO_6S$ (M+) 295, Obs. 296

(2*R,4S)*-*N*-(butyloxycarbonyl)-4-azido-pyrrolidine-2-methyl alcohol 6

A mixture of (2*R*,4*R*)-*N*-(butyloxycarbonyl)-4-*O*-mesyl-pyrrolidine-2-methyl alcohol **5** (1.40g, 4.7mmol) and sodium azide (1.1g, 16.6mmol) in dry DMF (15ml) was stirred at 60^{o}C overnight, when the reaction was found to be complete by TLC inspection. The solvent was completely removed *in vacuo* and the residue, taken up in water. The product **6** was extracted in ethyl acetate and was obtained after the organic layer was dried over sodium sulfate and evaporated to dryness under vacuum (1.12g, 97.5%).

^{1}H NMR ($CDCl_3$) δ: 4.60 (br s, 1H, OH), 4.05 (m, 2H, CH_2OH), 3.70 (m, 1H, H4), 3.50 (m, 3H, H5, H5', H2), 2.10 (m, 1H, H3), 1.85 (m, 1H, H3'), 1.45 (s, 9H, $(CH_3)_3$).

^{13}C NMR ($CDCl_3$) δ: 154.8 ($COOC(CH_3)_3$), 79.5 ($C(CH_3)_3$), 63.8 (CH_2OH), 58.4 (C4), 57.4 (C2), 51.3 (C5), 33.4 (C3), 27.6 (($CH_3)_3$).

(2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(*O*-mesyl methyl)-pyrrolidine 7

To a stirred, cooled mixture of (2*S*,4*S*)-*N*-(butyloxycarbonyl)-4-azido-pyrrolidin -2-methanol (1.0g, 4.1 mmol) and triethylamine (1.44ml, 10.3mmol) in dry dichloromethane (10ml), methane sulfonyl chloride (0.8ml, 10.3mmol) was added drop wise. Stirring was continued at room temperature overnight. The solvent was completely removed under vacuum. The residue was taken up in water and the crude product obtained by extraction in dichloromethane, followed by drying of the organic layer sodium sulfate and evaporation under vacuum. Silica gel column chromatography yielded the pure product in the form of gum (0.6g, 90% yield).

^{1}H NMR $CHCl_3$-d δ: 5.0 (m, 1H, H4), 4.30-4 (m, 3H, H2, $CH_2OSO_2CH_3$), 3.8-3.5 (m, 2H, H5, H5'), 3.45-3.3 (m, 1H), 3.00 (s, 3H, SO_2CH_3), 2.40-2.20 (m, 2H, H3, H3'), 1.45 (s, 9H, $(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 152.29 ($COO(CH_3)_3$), 80.5 ($C(CH_3)_3$, 68.9 ($CH_2OSO_2CH_3$), 58.3(C2), 55.8 (C4), 51.4 (C5), 44.5(SO_2CH_3), 34.4 (C3), 27.9($C(CH_3)_3$.

ESIMS calcd for $C_{22}H_{25}N_9O_3$ (M+) 463.6, found 464 (M+1)

(2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidine 8a

A mixture of (2*R*,4*S*)-*N*-(butyloxycarbonyl)-4-azido-2-(*O*-mesyl methyl)-pyrrolidine **7** (0.80g, 2.50mmol), anhydrous potassium carbonate (0.86g, 6.25mmol), N^4-benzyloxycarbonyl-cytosine (0.60g, 3.00mmol) and 18-crown-6 (0.13g, 0.75mmol) in

dry DMF was stirred at 60°C under argon atmosphere overnight. The solvent was completely removed under vacuum and the crude residue was purified by column chromatography subsequent to filtering and evaporation of the filtrate to obtain the pure product in the form of liquid gum **8a** (0.27g, 30% yield)

$[\alpha]^{24}{}_{D}$ –25.0 (c 0.04, MeOH)

^{1}H NMR $CHCl_3$-*d* δ: 7.65 (m, 1H, H6-Cyt), 7.30 (s, 5H, CH_2-Ar), 7.15 (m, 1H,Cyt-H5), 5.20 (s, 2H, CH_2-Ar), 4.4-4.20 (m, 3H,CH_2-Cyt), 3.8-3.3(m, 2H, H4), 3.52 (m, 1H, H5), 3.35 (m, 1H, H2), 2.15 (m, 2H, H3), 1.45 (s, 9H,$(CH_3)_3$).

^{13}C NMR $CHCl_3$-*d* δ: 162.0 (CO-Ar), 154.3 (C-Ar), 152.1 (Boc-CO), 152.6 (C-C4), 148.9(C-C6), 134.8, 128.3, 128.0,(CH_2Ar), 94.3 (C5), 80.6 ($CCH_3)_3$, 67.5(CH_2-Ar), 59.6(C4), 52(CH_2-Cyt), 51.3 (C5), 36.0 (C2), 34.3 (C3), 27.8$(CH_3)_3$.

ESIMS calcd for $C_{22}H_{28}N_7O_5$ (M+) 470.5, found 470.0

(2*R*,4*S*) 4-azido-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid ethyl ester 9a

To a solution of (2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidine (0.27g, 0.57mmol) in dry CH_2Cl_2 (1ml) was added TFA (1ml) and the reaction was stirred at room temperature for 10min, when TLC indicated a complete absence of starting material. The resulting free amine was immediately subjected to alkylation using ethylbromoacetate (0.07ml, 0.63mmol) in dry THF (2ml) in the presence of DIPEA (0.060ml, 0.35mmol). The amine was completely consumed within 3h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to get the pure product **3a** in the form of gum. (0.24g, 93%).

^{1}H NMR $CHCl_3$-d δ: 7.92 (d, 1H, J = 7.32Hz), 7.35 (s, 5H), 7.16 (d, 1H, J = 7.33Hz), 5.19 (s, 2H), 4.14 (m, 4H), 3.89 (m, 1H), 3.70 (m, 1H), 3.40 (m, 2H), 3.29 (m, 1H), 2.58 (m, 1H), 2.04 (m, 1H), 1.85 (m, 1H), 1.24 (t, 3H, J = 7.32Hz).

^{13}C NMR $CHCl_3$-d δ: 170.4, 162.4, 155.5, 152.7, 149.8, 135.0, 128.2, 128.0, 127.7, 94.4, 67.2, 60.5, 59.2, 58.3, 55.4, 51.6, 40.3, 34.3, 13.7.

ESIMS calcd for $C_{21}H_{25}N_7O_4$ (M+) 439.4, Found 440 (M+1).

(2*R*,4*S*) 4-(*tert.*butyloxyoxycarbonylamino)-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid ethyl ester 10a

(2*R*,4*S*)-4-azido-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidin-*N*1-ethylacetate (0.24g, 0.55mmol) was reduced to the (4*S*)-amine by hydrogenation using Raney-Ni as a catalyst (0.16g) in ethyl acetate at room temperature for 2h, which was converted in situ to the corresponding tert-butyl carbamate by Boc-anhydride (0.14g, 0.66mmol). The product was purified by silica gel column chromatography to get pure **4a** in the form of gum (0.25g, 90% yield).

$[\alpha]^{24}{}_{D}$ –33.1(c 0.024, MeOH)

^{1}H NMR $CHCl_3$-d δ: 7.72 (br s, 1H), 7.4-7.2 (m, 4H, Ar), 5.20 (m, 2H,C$\underline{H}_2$Ar), 4.4-4.12 (m, 2H,C$\underline{H}_2$-C),4.36 (m, 1H, H4), 3.6-3.2 (m, 2H, H5), 3.42 (m, 2H, C$\underline{H}_2$COOEt), 3.31 (m, 1H), 2.2-1.7 (m, 2H, H3'), 1.41 (s, 9H, $(CH_3)_3$), 1.4 (br m, 3H, $CH_2C\underline{H}_3$).

^{13}C NMR ($CDCl_3$) δ: 171.1(C$\underline{O}$OEt), 162.8(Ar-$\underline{C}$O), 156.0(Cyt-C4), 155.6(Cyt-C2), 152.9(Cyt-C6), 150.0, 135.3, 128.7, 128.5, 128.3(Ar), 94.8(C-C5), 79.6($\underline{C}(CH_3)_3$), 67.8 ($\underline{C}H_2$-Ar), 60.8($\underline{C}H_2$-CH_3), 59.1(C4), 58.7($\underline{C}H_2$-N), 54.1($\underline{C}H_2$-C), 53.5(C5), 49.8(C2), 36.1(C3), 28.4,($\underline{C}H_3)_3$). 14.2 ($CH_2C\underline{H}_3$).

ESIMS calcd for $C_{26}H_{36}N_5O_7$ 530.6, found 530.0

(2*R*,4*S*)-4-(*tert.b*utyloxyoxycarbonyl -amino)-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid 11a

To a solution of (2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(N^4-benzyloxycarbonyl-cytosin-1-ylmethyl)-pyrrolidin-*N*-1-ethylacetate 4a (0.1 g, 0.44mmol) in methanol:water, 1:1 (3ml) was added 1M NaOH (1ml) and stirred for 20min at room temperature. The excess alkali was neutralized using Dowex H^+ resin, which was then filtered off to get the product as white foam. (90%yield)

$[\alpha]^{24}_D$ -5.0 (c 0.002 MeOH)

^{1}H NMR D_2O δ: 7.41-7.31(m , 2H) 6.99-6.91 (m, 1H) ,5.18(m, 1H), 2.55 (s 1H), 2.31-1.68 (m,2H)1.46 (s, 2H), 1.45-1.32 ,2.3 (s, 2H).

^{13}C NMR D_2O δ: 172.13(COOH), 170.97(NCO), 158.79, 155.63, 155.28, 153.95, 150.84, 142.66, 138.64, 137.54, 128.63, 128.48, 128.29, 128.17, 128.06, 127.97, 127.85, 127.80, 126.77, 126.58, 85.06, 63,07, 62.97, 59.61, 58.59, 57.72, 53.85, 52.56, 51.66, 40.38, 34.33, 28.37, 27.97($C(CH_3)_3$).

ESIMS calcd for $C_{25}H_{35}N_5O_6$ 501.5, found 502 (m+)

λ_{max} = 270nm, ε =7.8cm^2/μmol

(2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidine 8b

A mixture of N^6-benzoyladenine (0.80g, 3.15mmol) and K_2CO_3 (0.86g, 6.25mmol) in dry DMF was stirred at 60°C for 1h. A solution of (2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(O-mesyl methyl)-pyrrolidine (0.84g, 2.63mmol) in DMF was added drop wise to this mixture and stirring was continued at 60°C for 5h, when TLC inspection

indicated absence of the starting material. The solvent was completely removed under vacuum and the crude residue, purified by column chromatography to obtain the pure product **8**b in the form of gum. (0.26g, 40% yield)

$[\alpha]^{24}{}_{D}$ +35.7(c 0.014, MeOH)

^{1}H NMR $CHCl_3$-d δ: 8.77 (s, 1H, H8-Adenine), 8.02 (m, 2H, H2-Ade), 7.54 (m, 3H, Ar), 4.60 (m, 2H,C$\underline{H}_2$-Ade), 4.31 (m, 1H, H4), 3.79 (m, 1H, H5'), 3.50 (m, 1H, H5), 3.2 (m, 1H, H2)2.06 (m, 2H, H3), 1.49 (s, 9H, $(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 164.9(COAr), 154.2(Boc-CO), 152.2(A-C2,), 152.1(A-C6), 149.4 (A-C4), 143.2 (A-C8), 133.3, 132.2, 128.2, 127.7 (Ar), 122.4 (A-C5), 80.5 ($C(CH_3)_3$), 58.3, 55.8 (C5), 51.4 (C2), 36.03, 34.4 (C3), 30.09, 27.9 $(CH_3)_3$).

ESIMS calcd for $C_{22}H_{25}N_9O_3$ (M+) 463.5, found 464.0 (M+1)

(2*R*,4*S*)-4-azido-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidin-*N*-1-ethylacetate 9b

To a solution of (2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(*O*-mesyl methyl)-pyrrolidine **7** (0.39g, 0.84mmol) in dry CH_2Cl_2 (1ml) was added TFA (1ml) and the reaction was stirred at room temperature for 10min, when TLC indicated a complete absence of starting material. The resulting free amine was immediately subjected to alkylation using ethylbromoacetate (0.1ml, 0.93mmol) in dry THF (4ml) in the presence of DIPEA (0.22ml, 1.26mmol). The amine was completely consumed within 2.5h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to get the pure product **9b** in the form of gum. (95%yield).

$[\alpha]^{24}{}_{D}$ +35.7(c 0.004, MeOH)

^{1}H NMR $CHCl_3$-d δ: 8.70, (s, 2H,), 8 (m, 3H), 7.6 (m, 4H), 4.2, (m, 2H), 3.5 (m, 2H), 3.4 (m, 2H), 2.7, (m, 1H), 2, (m, 1H), 1.3 (t, 3H).

^{13}C NMR $CHCl_3$-d δ: 176.5, 170.2, 146.7, 146.3, 145.5, 140.9, 135.2, 131.6, 129.4, 127.7, 120.7, 60.8, 60.5, 58.6, 58.1, 54.1, 45.4, 34.3, 13.8.

ESIMS calcd for $C_{22}H_{25}N_9O_3$ 463.6, found 464.0 (M+)

(2*R*,4*S*)-4-(*tert.*butyloxyoxycarbonylamino)-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidin-*N*-1-ethylacetate 10b

(2*R*,4*S*)-4-azido-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidin-*N*-1-ethylacetate **9b** (0.34g, 0.76mmol) was reduced to the (4*S*)-amine by hydrogenation using 10% Pd-C as a catalyst at room temperature for 2h. The resulting amine (0.30g, 0.71mmol) was converted *insitu* to the corresponding tert.Butyl carbamate by Boc-anhydride (0.l9g, 0.85mmol). The crude product was purified by silica gel column chromatography to get pure product **10b** in the form of gum. (0.37g, 95%).

^{1}H NMR $CHCl_3$-d δ: 8.10 (m, 2H, Ade-H8), 7.90 (s, 1H,Ph), 7.45 (m, 3H, Ar), 7.25 (s, 1H, Ar), 5.10 (m, 1H), 4.20 (m, 4H,N-C$\underline{H}_2$, C$\underline{H}_2$-A), 4.05 (m, 1H,H4), 3.75 (m, 1H,H2), 3.40 (m, 4H,H5), 2.60 (m, 2H,H3), 1.45 (s, 9H,$(CH_3)_3$), 1.28 (t, 3H,$CH_2C\underline{H}_3$).

^{13}C NMR $CHCl_3$-d δ: 171.2 ($\underline{C}$OOEt), 162.7 (Ar), 155.5(Boc-$\underline{C}$O), 152.4(A-C2), 152.0(A-C6), 149.7(A-C4), 144.6(A-C8) 133.7, 132.4, 128.4, 128.2, 12.8.0, 122.5(Ar), 78.9($\underline{C}(CH_3)_3$, 60.7, 60.1($\underline{C}H_2$-A), 58.8 (N-CH_2), 53.1(C2), 49.0 (C5), 45.9 ($\underline{C}H_2CH_3$), 35.7 (C3), 28.2 (C($\underline{C}H_3)_3$), 14.1 ($CH_2\underline{C}H_3$). ESIMS calcd for $C_{26}H_{25}N_7O_5$ 515.5, Found 515.0

(2*R*,4*S*)-4-(*tert.*butyloxyoxycarbonylamino)-2-(N^6-benzoyl-adenin-9-ylmethyl)-pyrrolidin-*N*-1-acetic acid 11b

To a solution of (2*R*,4*S*)-4-(*tert.*butyloxyoxycarbonylamino)-2-(N^6-benzoyladenin-9-ylmethyl)-pyrrolidin-*N*-1-ethylacetate **10b** (0.34g, 0.76mmol) in methanol:water, 1:1 (3ml) was added 1N NaOH (1ml) and reaction mixture was stirred for 20min at room temperature when the TLC indicated complete absence of starting material. The excess alkali was neutralized using Dowex H^+ resin which was then filtered off. The filtrate was then evaporated to get the product as a white foam. (90%yield)

$[\alpha]^{24}_D$ +20 (c 0.002, MeOH)

ESIMS calcd for $C_{24}H_{29}N_7O_5$ 495.5, Found 497 (m+1)

λ_{max} = 270nm, ε = 16.0$cm^2/\mu mol$

(2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidine 8c

A mixture of (2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(*O*-mesyl methyl)-pyrrolidine **77** (0.10g, 0.31mmol), anhydrous potassium carbonate (0.22g, 1.56mmol), 2-amino-6-chloropurine (0.13g, 0.78mmol) and 18-crown-6 (0.03g, 0.09mmol) in dry DMF was stirred at 65°C under argon atmosphere overnight. The solvent was completely removed under vacuum and the crude residue, purified by column chromatography subsequent to filtering and evaporation of the filtrate to obtain the pure product 2c in the form of gum. (0.06g, 45% yield)

$[\alpha]^{24}_D$ +5.83 (c 0.012, MeOH)

^{1}H NMR $CHCl_3$-d δ: 8.0 (s, 1H, 2-amino-6chloropurine-H8), 7.60 (s, 1H), 5.15 (br, 2H, C<u>H</u>$_2$-N), 4.6-4.4 (m, 1H, H4), 4.35-4.1 (m, 3H, C<u>H</u>$_2$-G), 3.9-3.6 (m, 2H,H5), 3.5-3.3 (m, 1H, H5'), 3.2-3 (m, 1H, H2), 2.1-1.8 (m, 2H, H3), 1.40 (s, 9H,$(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 159.2(2-amino-6chloropurine-C2), 153.9(2-amino-6chloropurine-C4), 152 (BocCO), 150.6(2-amino-6chloropurine-C6), 142.4(2-amino-6chloropurine-H8), 123.9(2-amino-6chloropurine-H5), 80.3($C(CH_3)_3$), 58.2(C2), 55.5(C4), 51.4(CH_2-G), 44.1(C5), 34.1(C3), 27.6($C(CH_3)_3$).

ESIMS calculated for $C_{15}H_{20}N_9O_2Cl$ 393.3 Found 394(M+1)

(2*R*,4*S*)-4-azido-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidin-*N*-1-ethylacetate 9c

To a solution of (2*R*,4*S*)-*N*-(butyloxyoxycarbonyl)-4-azido-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidine **8c** (0.08g, 0.20mmol) in dry CH_2Cl_2 (1ml) was added TFA (1ml) and the reaction was stirred at room temperature for 10min, when TLC indicated a complete absence of starting material. The resulting free amine was immediately subjected to alkylation using ethyl bromoacetate (0.025ml, 0.20mmol) in dry THF (1ml) in the presence of DIPEA (0.053ml, 0.31mmol). The amine was completely consumed within 2h, upon which, the solvents were removed under vacuum and the crude product purified by silica gel column chromatography to get the pure product 3c in the form of gum. (89% yield)

$[\alpha]^{24}_{D}$ +36.66 (c 0.012 , MeOH),

^{1}H NMR $CHCl_3$-d δ: 8.05 (s, 1H), 5.25 (s, 2H), 4.20 (m, 2H), 4.05 (m, 2H), 3.70 (m, 1H), 3.40 (m, 2H), 2.65 (m, 1H), 2.00 (m, 1H), 1.75 (m, 1H), 1.30 (t, 3H).

^{13}C NMR $CHCl_3$-d δ: 170.3, 159.4, 150.7, 143.4, 124.2, 60.9, 60.5, 58.8, 58.3, 54.7, 45.4, 34.4, 31.1, 13.8.

ESIMS calculated for $C_{15}H_{20}O_2N_9Cl$ 393.8 Found 394(M+)

(2*R*,4*S*)-4-(*tert.*butyloxyoxycarbonylamino)-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidine-*N*-1-ethylacetate 10c

(2*R*,4*S*)-4-azido-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidine-*N*-1-ethylacetate **9c** (0.07g, 0.18mmol) was reduced to the (4*S*)-amine by hydrogenation using 10% pd-C as a catalyst (0.05g) in methanol, which was converted in situ to the corresponding tert-butyl carbamate by Boc-anhydride (0.08g, 0.46mmol) to get the crude product that was purified by silica gel column chromatography to get pure 4c in the form of gum.(85% yield).

^{1}H NMR $CHCl_3$-d δ: 7.95 (s, 1H), 5.28 (s, 2H), 5.13 (m, 1H, G-NH2), 4.14 (m, 5H,N-CH2, C$\underline{H}_2$CH$_3$), 3.48 (m, 1H, H4), 3.28 (m, 2H, CH_2-G), 2.93 (m, 2H, H5), 2.51 (m, 1H, H2), 2.28 (m, 2H, H3), 1.37 (s, 9H), 1.26 (t, 3H, $CH_2C\underline{H}_3$).

^{13}C NMR ($CDCl_3$) δ: 171.2 (C$\underline{O}$OEt), 159.3 (2-amino-6chloropurine, C2), 155.4 (2-amino-6chloropurine-C4), 154.3 (BocCO), 151.2 (2-amino-6chloropurine-C6), 143.5 (2-amino-6chloropurine-C8), 124.7 (2-amino-6chloropurine-C5), 77.9 (C(CH_3)$_3$), 61.0, 59.8, 59.2, 58.8 ($\underline{C}H_2CH_3$), 55.1(N-$\underline{C}H_2$), 46.0 (C5), 36.5 (C3), 28.4 (C($\underline{C}H_3$)$_3$), 14.2 ($CH_2\underline{C}H_3$).

ESIMS calculated for $C_{19}H_{27}O_4N_2Cl$ 439.8 Found 456(M+17, M+NH_3)

(2*R*,4*S*)-4-(*tert.*butyloxyoxycarbonylamino)-2-(guanin-9-ylmethyl)-pyrrolidin-*N*-1-acetic acid 12

To a solution of (2*R*,4*S*)-4-(*tert.*butyloxycarbonl-amino)-2-(2-amino-6-chloropurin-9-ylmethyl)-pyrrolidine-*N*-1-ethylacetate (0.02g, 0.04 mmol) in methanol: water, 1:1 (5 ml) was added 1M NaOH (2 ml) and stirred overnight at room temperature. The excess alkali was neutralized using Dowex H^+ resin, which was then filtered off. The filtrate was then concentrated to get the pure product as white foam. (88% yield)

$[\alpha]^{24}_D$ –5.0 (c 0.002, MeOH) ,

^{1}H NMR D_2O δ: 8.37-8.16 (m, 1H), 4.47-4.19 (m, 1H, H4), 4.26-3.96 (m, 5H, H5, N-CH2, CH2-G), 3.71-3.66 (m, 1H, H5), 3.38-3.21 (m, 1H, H2), 2.36-2.21 (m, 1H, H3), 2.15-1.89 (m, 1H, H3), 1.57-1.30 (m, 9H, $(CH_3)_3$).

^{13}C NMR D_2O δ: 170.6 (-COOH), 169.9 (CO-G), 159.5 (GC2), 156.9 (G-C4), 154.2 (BocCO), 140.5 (G-C8), 139.7 (G-C5), 81.06 ($C(CH_3)_3$), 69.5, 65.4, 65.0, 63.0, 62.4(N-CH_2), 59.6 (C4), 59.2, 58.6, 57.3 (CH_2-G), 54.8, 47.1 (C5), 42.9 (C3), 30.3 (C2), 29.5, 27.3 ($C(CH_3)_3$

ESIMS calculated for $C_{16}H_{24}O_5N_7$ 406.2 Found 408 (m+1)

λ_{max} = 270nm, ε = 11.9cm^2/μmol

(2*S*/*R*,4*S*) *N*-1-(benzyloxycarbonyl)-4-(*p*-toluenesulphonyloxy) proline methyl ester (17,26)

(2*R*/*S*,4*R*) *N*-1(benzyloxycarbonyl)-4-(*p*-toluenesulphonyloxy) proline methyl ester (5gm,17.85 mmol), PPh_3(3.97 gm,19.65 mmol)and methyl-p-toluene sulphonate(3.65 gm,19.40 mmol) were taken in dry THF(100ml) and the mixture was cooled to –10ºC in an ice salt bath. To this, a solution of diisopropylazodicarboxylate (5.14ml, 25 mmol) in dry THF (10ml) was added using syringe over a period of 1 h. The reaction mixture was further stirred for 1 hr. at –10ºC followed by 12 h at room temperature, when the

TLC indicated the complete conversion of starting material. The reaction mixture was concentrated and was subjected to silica gel column chromatography. The separation proved to be difficult as the diisopropylhydrazodicarboxylate moves very closely with the product.

17 $[\alpha]^{24}_{D}$ -1.1 (c 0.012 , MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.9- 7.10 (m 2H), 7.4-7.1 (m 2H), 5.4-4.8 (m, 2H), 4.6-3.9 (m , 2H), 3.8-3.49 (m, 4H), 2.5 (s, 3H), 2.2-1.9 (m, 2H).

^{13}C NMR $CHCl_3$-d δ: 173.5, 156.7, 145.0, 136.2, 133.6, 132.1, 131.5, 129.8, 127.3, 127.6, 67.36, 62.0, 52.2, 21.5, 20.6, 14.3.

ESIMS calculated for $C_{20}H2_3O_8NS$ 433.4 Found 434.0 (M+1)

26$[\alpha]^{24}_{D}$ +15.31 (c 0.322 , MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.8- 7.6 (m 2H, Ar) 7.3-7.1 (m 2H, Ar) 5.1-4.8 (m, 2H, NHBOC, H4) 4.4-4.2 (m,1H, H2),4.2-3.9 (m, 2H, H5, 3.7 (s, 3H, COOCH3), 3.6-3.5 (d, 2H, CH3 1), 3.1 (s, 1H, H1), 2.49-2.3 (s, 3H, OTS), 1.4 (s, 9H, BOC)

^{13}C NMR $CHCl_3$-d δ 172.5, 156.0, 143.0, 136.2, 132.6, 132.1, 130.4, 129.9, 126.3, 125.6, 65.36, 62.0, 51.2, 22.5, 20.1, 13.4.

ESIMS calculated for $C_{20}H2_3O_8NS$ 433.4 Found 434.0 (M+1)

(2*S*/*R*,4*R*) *N*-1 (benzyloxycarbonyl)-4-azido-proline methyl ester (18, 27)

The mixture from the above step was taken in dry DMF (50 ml) and to this was added NaN_3 (7.64 gm, 117 mmol). The mixture was stirred at 60^{o}C for 12 h. when TLC indicated complete conversion of starting material, DMF was removed under vacuum on a rotary evaporator, 100 ml of water was added to the mixture and extracted with

ethyl acetate. (30ml x 3). Organic layers were pooled, washed with water (20 ml) and concentrated to give the crude product that was further purified by column chromatography. (85% yield)

18 $[\alpha]^{24}_{D}$ –50.9 (c 0.022 , MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.4 (s, 5H, Ar), 5.35-5 (m, 2H, Ar-CH2), 4.5-4.32 (m, 1H), 4.25-4.15 (m, 1H, H4), 3.75 (s, 3H, $COOCH_3$), 3.6-3.4 (m, 2H, H5), 2.4-2.3 (m, 2H, H3), 1.42 (s, 9H, $(C(CH_3)_3$

^{13}C NMR $CHCl_3$-d δ: 171.5 (CO-Ar), 171.2 ($COOCH_3$), 154.2, 153.8, 136.0, 128.1, 127.6 (Ar), 80 ($C(CH_3)_3$), 67.1, 67.0, 58.9, 57.5, 57.2, 52.2, 51.1 ($COO\underline{C}H_3$), 50.8 (C5), 35.8 (C3), 34.8, 28.6 ($C(CH_3)_3$

ESIMS calculated for $C_{14}H_{16}O_4N_4$ 304.2, Found 321 (M+17,M+NH_3)

27 $[\alpha]^{24}_{D}$ +20.45 (c 0.022,MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.8-7.4 (m, 2H), 7.3-7.2 (m, 2H), 5.2-4.7 (m, 3H), 4.45 (m, 2H), 3.66 (s, 3H), 3.42 (m, 2H), 2.13-1.48 (m, 2H).

^{13}C NMR $CHCl_3$-d δ: 172.5, 154.2, 153.8, 128.3, 128.2, 127.8, 127.8, 127.6, 69.5, 58.9, 52.2, 51.1, 50.8, 35.8, 21.7.

ESIMS calculated for $C_{14}H_{16}O_4N_4$ 304.3, Found 305.0 (M+1)

(2*S*/*R*,4*R*) *N*-1-(benzyloxycarbonyl)-4-(*tert*.butyloxycarbonylamino) proline methyl ester (**19,28**)

Compound **15** (4.74 gm, 15 mmol) was dissolved in a dry ethyl acetate and to this was added Ra/ Ni (4ml) and Boc-anhydride (4 ml, 18 mmol). The mixture was subjected to

hydrogenation for 2 hrs. at a pressure of 40 psi in a parr- hydrogenation apparatus when TLC indicated complete conversion of starting material. The crude product was purified by column chromatography to get **16** in the form of gum. (95 %yield)

19 $[\alpha]^{24}_{D}$ –17.20 (c 0.022 MeOH)

^{1}H NMR $CHCl_3$-d δ: 7.4-7.29 (m, 5H), 5.22-5.11 (m, 2H), 5.08-5.04 (m, 1H), 4.66 (bs, 1H), 4.88-4.40 (m, 1H), 4.37-4.26 (m, 1H), 3.89-3.84 (m, 1H), 3.76 (s, 2H), 3.58 (s, 1H), 3.45-3.34 (m, 1H), 2.28-2.15 (m, 2H), 1.45 (s, 9H).

^{13}C NMR $CHCl_3$-d δ: 172.2, 172.0, 154.8, 154.5, 153.8, 135.9, 128.1, 127.7, 127.6, 77.0, 76.7, 67.0, 57.5, 52.1, 51.9, 51.5, 36.6, 35.5, 28.0.

ESIMS calculated for $C_{19}H_{26}N_2O_6$ 378.2 Found 379.0(m+1)

28 $[\alpha]^{24}_{D}$ +9.0 (c 0.02 MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.5 (s, 5H), 5.20-5.1 (m, 2H), 4.27 (m, 1H), 3.87 (m, 1H), 3.6-3.4 (m, 3H), 3.39-3.32 (m, 1H), 2.46-2 (m, 2H), 1.44 (s, 9H).[1]

^{3}C NMR $CDCl_3$ δ: 173.5, 154.8, 154.4, 153.7, 136.0, 128.1, 127.7, 127.5, 79.36, 67.0, 57.7, 57.3, 53.0, 52.8, 51.9, 49.7, 48.7, 36.6, 35.5, 28.0.

ESIMS calculated for $C_{19}H_{26}N_2O_6$ 378.2 Found 378.0

(2*S*/*R*,4*R*)- *N*[1](benzyloxycarbonyl)-4-(*tert*.butyloxycarbonylamino) pyrrolidine-2-methanol (**20,29**)

To a stirred mixture of dry ethanol (20ml) and dry THF (30ml), was added lithium chloride (2.16g, 50mmol) and sodium borohydride (1.88g, 50mmol) and stirring continued for ~ 10 min under argon atmosphere. A solution of (2*R*,4*R*) *N*-1-(benzyloxycarbonyl)-4-(tert-butoxycarbonylamino) -proline methyl ester (5.49

gm,14mmol) in THF (10ml) was added drop wise and stirring was continued at room temperature under argon atmosphere for 6h. When TLC indicated complete disappearance of starting material, pH of the reaction mixture was adjusted to 4 with aqueous ammonium chloride. The reaction mixture was stirred for 10 minutes and the solvents were removed on rotary evaporator. The residue was taken up in water and extracted with ethyl acetate. (4x 20 ml) The organic layer was then dried over sodium sulphate and evaporated to get crude product. Silica gel column chromatography yielded the pure product in the form of gum. (80% yield)

20$[\alpha]^{24}_{D}$ –27.5 (c 0.004 , MeOH),

^{1}H NMR $CHCl_3$-d δ 7.32 (s, 5H, Ar), 5.16 (s, 2H, Ar-CH_2), 4.72-4.64 (m, 1H, H4), 3.8-3.0 (m, 3H,CH_2-OH), 3.79-3.6 (m, 3H, H5), 3.5-3.4 (m, 2H, H2, OH), 2.01-1.75 (m 2H, H3), 1.44 (s, 9H,($C(CH_3)_3$)

^{13}C NMR $CHCl_3$-d δ: 156.5, 154.9 (N-CO), 136.0, 128.2, 127.9, 127.6(Z), 79.5 ($C(CH_3)_3$), 66.0 (CH_2-OH), 58.8 (C4), 52.8 (C5), 48.9 34.4 (C3), 28.0 ($C(CH_3)_3$).

ESIMS calculated for $C_{18}H_{26}O_5N_2$ 350.4 Found 350.0

29 $[\alpha]^{24}_{D}$ +5.0 (c 0.024 MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.42-7.30 (s, 5H,Ar), 5.18-5.12 (m, 2H,CH_2-Ar), 4.8 (bs, 1H, H4), 4.27-3.96 (m, 4H, CH_2-OH, H5), 3.87-3,79 (m, 1H), 3.76-3.52 (m, 3H,), 3.49-3.39 (m 1H, H2), 2.46-2.0 (m 2H, H3), 1.44 (s, 9H, ($C(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 156, 135.0 128.2, 127.8, 127.5(Ar), 79.5($C(CH_3)_3$, 67.0 (CH_2-Ar), 65.4 (CH_2-OH) 58.7 (C4), 52.7(C5), 51.9, 48.8 (C2), 34.2, 33.6 (C3), 28.0 ($C(CH_3)_3$.

ESIMS calculated for $C_{18}H_{26}O_5N_2$ 350.4 Found 350.0

(*2S/R,4R*)-*N*--(benzyloxycarbonyl)-4-(*tert*.butyloxycarbonylamino)-2-(-O-mesylmethyl)-pyrrolidine (**21,30**)

To a stirred, cooled mixture of alcohol **20** (1.64 gm,4.6 mmol) and triethylamine(1.95 ml,19.30mmol) in dry dichloromethane (10 ml) was added drop wise methane sulphonyl chloride (0.64 ml, 5.5 mmol). Stirring was continued for further 30 min. when TLC indicated absence of starting material. The solvent was removed under vacuum, residue was taken up in water and extracted with ethyl acetate (2x10ml). The organic layer was dried over sodium sulphate, and then evaporated to dryness. Silica gel column chromatography yielded the pure product in the form of gum. (95% yield)

21 ^{1}H NMR $CHCl_3$-d δ: 7.4 (s, 5H, Ar), 5.0 (s, 2H,CH_2-Ar), 4.9-4.8 (m, 1H, H4), 4.35-3.7 (m, 4H,H5,CH_2-OMs), 2.8 (s, 3H, O-Mesylate), 2.49-2.3 (m, 1H, H3), 1.8-1.7 (m,1H, H3), 1.4 (s, 9H,($C(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 154.8, 155.6 ($NCOOCH_2Ar$), 154.1, 136.0, 128.6, 127.88, 127.7(Ar), 79.7 ($C(CH_3)_3$),68.9 (CH_2-OMS), 66.8 (CH_2-Ar), 60.1, 54.8, 54.3 , 52.2 (C4) , 36.9 (C2), 36.7 (C3), 33.7 (SO_2CH_3) , 28.0 ($C(CH_3)_3$

ESIMS calculated for $C_{19}H_{28}O_8N_2S$ 446.3 Found 448.0 (M+1)

30 $[\alpha]^{24}_D$ +25.5 (c 0.02,MeOH),

H^1 NMR $CHCl_3$-d δ: 7.4 (s, 5H, Ar), 5.0 (s, 2H), 4.9-4.8 (m, 1H), 4.35-3.7 (m, 4H), 2.8 (s, 3H), 2.49-2.3 (m, 1H), 1.8-1.7 (m,1H), 1.4 (s, 9H).

^{13}C NMR $CHCl_3$-d δ: 154.0, 155.0 (N-COO), 136.0, 128.2, 127.7 (Ar), 80.3 ($C(CH_3)_3$), 68.9 (CH2-Ar), 68.8 (CH_2-Oms), 60.1, 55.1(C4), 52.2 (C5), 48.5, 36.7 (C2), 33.2, 28.0($C(CH_3)_3$).

ESIMS calculated for $C_{19}H_{28}O_8N_2S$ 446.3 Found 448.0 (M+1)

(2*S/R*,4*R*)-*N*-(benzyloxycarbonyl)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidine (**22,31**)

To the compound **18** (1gm, 2.7 moml) in dry DMF was added anhydrous potassium carbonate (0.6gm, 5mmol), thymine (0.51gm, 4mmol) and 18-crown–6 (0.26gm, 0.98 mmol). The reaction mixture was kept for stirring at 60 ^oC for 4 h. When TLC indicated complete conversion of starting material, solvent was removed under vacuum. Silica gel column chromatography yielded the pure product in the form of gum. (0.4 gm, 35%).

22 $[\alpha]^{24}_D$ –10.0 (c 0.01,MeOH),

^1H NMR $CHCl_3$-d δ 7.49 (s,5H, Ar), 5.2 (s,2H,CH_2-Ar), 4.57 (m, 1H, H4), 4.3 –3.6 (m, 4H, H5, CH_2-T,), 3.7(m,1H, H5), 3.49(m, 1H, H5) 2.3-2.1 (m,1H, H3), 2.1 (s, 3H, Thy-CH_3), 1.8(s, 2H, H3)1.48 (s, 9H, ($C(CH_3)_3$).

^{13}C NMR $CHCl_3$-d δ: 170.5 (CO-NH), 162.5, 154.4 (Thy-C4), 153.7 (Thy-C2), 150 (Z-CO), 136.6(Thy-C6), 134(Thy-C5), 128.4, 127.9, 127.7 (Ar), 79.4 ($C(CH_3)_3$, 66.9, 64.5(CH_2-Ar), 55.0 (C4), 52.4 (CH_2-Thy), 52.2, 49.1 (C5), 36.9, 36.3, 34.2 (C2), 31.3 (C3), 29 ($C(CH_3)_3$, 12 (Thy-CH_3).

ESIMS calculated for $C_{23}H_{30}O_6N_4$ 458.7 Found 458.0

31 $[\alpha]^{24}_D$ +41.25 (c 0.008,MeOH),

^1H NMR $CHCl_3$-d δ: 7.3 (s, 5H, Ar), 4.9 (m, 2H,CH_2-Ar), 4 –3.6 (m, 5H,CH_2-T, H5), 3.49-3.1 (m, 1H, H5), 2.4-2.1 (m, 1H, H2), 1.9-1.5 (m, 5H,T-CH3, H3), 1.45 (s, 9H ($C(CH3)_3$).

^{13}C NMR $CHCl_3$-d δ: 163.8 (CO-NH), 155.4 (Thy-C4), 154.9 (ThyC2), 151.7(Z-CO), 140.1(Thy-C6), 135.9 (Thy-C5), 128.2, 127.9, 127.6, 127.5(Ar), 80 ($C(CH3)_3$, 66.9 (CH_2-Z), 56.6 (C4), 52.7 (CH_2-Thy), 49.5 (C5), 48.5, 36.1(C2), 32.8 (C3), 28.0 ($C(CH_3)_3$, 11.8 (Thy-CH_3).

ESIMS calculated for $C_{23}H_{30}O_6N_4$ 458.7 Found 458.0

(2*R/S*,4*R*)–4-(*tert*.butyloxycarbonylamino)-2-(thymin-1ylmethyl)-pyrrolidin-*N*-1-ethyl acetate (**23,32**)

Compound **22** (0.35g, 0.8mmol) was taken in methanol and to this 10% pd-C (0.3g) was added. The mixture was subjected to hydrogenation at a pressure of 50 psi for 6 h. when TLC indicated complete absence of starting material. This free amine was immediately used for alkylation using ethyl bromoacetate (0.2g, 1.6mmol) in the presence DIPEA (0.08ml, 0.35mmol). The reaction was complete after 2 h. as evident from TLC. The solvent was completely removed under vacuum and crude product was purified by silica gel column chromatography to get pure product in the form of gum. (90 % yield)

23 $[\alpha]^{24}_D$ –12.01(c 0.004 MeOH),

^{1}H NMR $CHCl_3$-d δ: 7.49-7.1 (s, 2H,Thy-$\underline{H}_6$), 5.2-5 (m, 1H), 4.8-4.6 (q, 2H, $OC\underline{H}_2CH_3$), 4.33-4.0 (m, 5H, H4, N-CH2, H5,), 2 (s, 2H, Thy-CH3), 1.8-1.6 (m, 2H, H3), 1.49 (s, 9H,($C(CH_3)_3$) 1.2 (t, 3H,$CH_2C\underline{H}_3$)

^{13}C NMR $CHCl_3$-d δ: 170,167.5 (COOEt), 162.8 (Thy-C2), 154.9 ($COO(CH_3)_3$, 151.1 (Thy-C4), 139.7 (Thy-C6), 63.4, 61.2, 60.9 (N-CH_2), 59.4, 58.6 (C5), 52.8, 52.1(CH2-Thy), 49.1 (C2), 41.8, 36.4 (C3), 28.1 ($C(CH_3)_3$, 13.8 (OCH_2CH_3), 12.6 (Thy-CH_3).

ESIMS calculated for $C_{19}H_{30}N_4O_6$ 410.4, Found 410.0

32 $[\alpha]^{24}_{D}$ +5.0 (c 0.002 MeOH)

^{1}H NMR $CHCl_3$-d δ: 9.39 (s, 1H), 7.29(s, 1H), 4.96 (s, 1H), 4.18 (q, 2H), 4.02-3.94 (m, 1H), 3.6-3.37 (m, 2H), 3.26-3.23 (s, 1H), 3.14-3.04 (m, 1H), 3.00-2.91 (m, 1H), 2.80-2.69 (m, 1H), 2.34-2.20 (m, 1H), 1.89 (s, 3H), 1.63-1.52 (m, 1H), 1.39 (s, 9H), 1.28 (t, 3H).

^{13}C NMR $CHCl_3$-d δ: 154.9, 128.3, 127.9, 127.6, 66.9, 55.8, 51.9, 48.9, 34.4, 29.4, 28.0, 13.8,11.8.

ESIMS calculated for $C_{19}H_{30}N_4O_6$ 410.4 Found 411

(2*R*/*S*,4*R*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid (24,33)

To a solution of (0.11 gm, 0.27 mmol) in methanol (1 ml) was added 2M aqu. NaOH (1 ml). The reaction was stirred at room temperature for 10 min, when TLC indicated complete conversion of stating material. The excess of alkali was neutralized by Dowex H^+ resin, which was then filtered off. The filtrate was then evaporated to get the product as a white foam. (0.10 gm, 90%)

(2*S*,4*R*)-4-(*tert*.butoxycarbonylamine)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid 24

^{1}H NMR D_2O δ: 7.31 (s, 1H), 4.45 (s, 1H), 4.12-3.9 (m, 5H), 3.04 (m, 1H), 2.78 (s, 1H), 2.06 (m, 2H), 1.70 (s, 3H), 1.20 (s, 9H).

^{13}C NMR D_2O δ: 172.18, 169.52, 164.79, 156.97, 152.76,140.83, 110.88, 81.88, 65.20, 62.98, 60.10, 57.47, 57.18, 55.20, 48.53, 43.06, 36.72, 32.87, 29.50, 27.41, 11.75.

ESIMS calculated for $C_{17}H_{26}N_4O_6$ 382.4, Found 383.0 (M+1)

λ_{max} 270 nm, $\varepsilon = 9.0$

(2*R*,4*R*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid 33

^{1}H NMR D_2O δ: 7.28 (s, 1H, T-H6), 4.47-4.39 (m, 1H, N-CH_2COOH), 4.12-4.09 (m, 1H, N-CH_2COOH), 4-3-4.91 (m, 2H, N-CH2), 3.77-3.64 (m, 3H, CH2-Thy, H5), 3.49-3.39 (m, 1H, H4), 3.22 (bs, 1H, H2), 2.54-2.48 (m, 1H, H3), 1.64 (s, 4H, Thy-CH3, H3), 1.14 (s, 9H, $(CH_3)_3$).

^{13}C NMR D_2O δ: 170.04 (COOH), 166.51(Thy-C2), 157.43 $(COOCH_3)_3$, 153.63 (Thy-C4), 143.07 (Thy-C6), 111.26 (Thy-C5), 81.65 $(C(CH_3)_3$, 65.70 (C4), 60.37 (N-CH2COOH), 55.92 (C5), 47.75 (C2), 47.28 (CH2-Thy), 46.55, 34.44, 32.72, 31.83 (C3), 29.53, 27.34 $(C(CH_3)_3$, 11.10 (Thy-CH_3).

ESIMS calculated for $C_{17}H_{26}N_4O_6$ 382.4, Found 383.0(M+1)

λ_{max} = 270nm, $\varepsilon = 8.6 cm^2/\mu mol$

(2*R*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1-acetic acid 11d

^{1}H NMR δ: 7.50 (s, 1H, T-H6), 4.30 (m, 2H, N-CH_2COOH), 3.95 (m, 4H, CH_2-T, H5, H5'), 3.65 (m, 1H, H4), 3.40 (m, 1H, H2), 2.75 (m, 1H, H3), 2.00 (m, 1H, H3'), 1.85 (s, 3H, T-CH_3), 1.35 (s, 9H, $(CH_3)_3$).

^{13}C NMR D_2O δ: 171.1 (COOH), 167.3 (T-C2), 158.3 $(COO(CH_3)_3)$, 154.5 (T-C4), 144.0 (T-C6), 112.0 (T-C5), 82.4 $(C(CH_3)_3)$, 66.5 (C4), 61.3 (N-CH_2COOH), 57.0 (C5), 48.1 (C2), 47.4 (CH_2-T), 32.7 (C3), 28.3 $(C(CH_3)_3)$, 12.1 (T-CH_3).

(2*S*,4*S*)-4-(*tert*.butyloxycarbonylamino)-2-(thymin-1-ylmethyl)-pyrrolidin-*N*-1ethyl acetate 13

^{1}H NMR ($CDCl_3$) δ: 9.30 (s, 1H, T-N3H), 7.32 (s, 1H, T-H6), 5.00 (d, 1H, Boc-NH), 4.20 (q, 2H, OCH_2CH_3), 4.10 (m, overlapping signal, 1H, H4 & 1H, N-CH_2), 3.55- 2.85 (m, 4H, CH_2-T, H5, H5'), 2.75 (m, 1H, H2), 2.30 (m, 1H, H3), 1.95 (s, 3H, T-CH_3), 1.60 (m, 1H, H3'), 1.45 (s, 9H, $(CH_3)_3$), 1.30 (t, 3H, OCH_2CH_3).

^{13}C NMR ($CDCl_3$) δ: 170.8 (COOEt), 164.7 (T-C2), 155.5 ($COOC(CH_3)_3$), 151.8 (T-C4), 141.9 (T-C6), 110.2 (T-C5), 79.5 ($C(CH_3)_3$), 61.2 (C4), 60.9 (OCH_2CH_3), 60.0 (N-CH_2), 54.8 (C5), 49.9 (CH_2-T), 49.6 (C2), 35.9 (C3), 28.4 ($C(CH_3)_3$), 14.3 (OCH_2CH_3), 12.2 (T-CH_3).

Synthesis of PNA monomers

***N*-1-(butyloxycarbonyl)-1,2-diaminoethane 35**

1,2-diaminoethane **34** (20g, 0.33mol) was taken in dioxane: water (1:1, 500ml) and cooled in an ice-bath. Boc-azide (5g, 35mmol) in dioxane (50ml) was slowly added with stirring and the pH was maintained at 10.0 by continuous addition of 4N NaOH. The mixture was stirred for 8h and the resulting solution was concentrated to 100ml. The N1, N2-di-Boc derivative not being soluble in water, precipitated, and it was removed by filtration. The corresponding N1-mono-Boc derivative was obtained by repeated extraction from the filtrate in ethyl acetate. Removal of solvents yielded the mono-Boc-diaminoethane **35** (3.45g, 63%).

^{1}H NMR $CHCl_3$-d δ: 5.21 (br s, 1H, NH), 3.32 (t, 2H, J=8 Hz), 2.54 (t, 2H, J=8 Hz), 1.42 (s, 9H).

Ethyl *N*-(2-butyloxycarbonylamino)ethyl-glycinate 36

The *N1*-(Boc)-1,2-diaminoethane **35** (3.2g, 20mmol) was treated with ethylbromoacetate (2.25ml, 20mmol) in acetonitrile (100ml) in the presence of K_2CO_3 (2.4g, 20mmol) and the mixture was stirred at ambient temperature for 5h. The solid that separated was removed by filtration and the filtrate was evaporated to obtain the ethyl *N*-(2-Boc-aminoethyl)-glycinate **37** (4.3g, 83%) as a colourless oil.

^{1}H NMR $CHCl_3$-d δ: 5.02 (br s, 1H, NH), 4.22 (q, 2H, J=8Hz), 3.35 (s, 2H), 3.20 (t, 2H, J=6Hz), 2.76 (t, 2H, J=6Hz), 1.46 (s, 9H), 1.28 (t, 3H, J=8Hz).

Ethyl *N*-(butyloxycarbonyl-aminoethyl)-*N*-(chloroacetyl)-glycinate 37

The ethyl *N*-(2-butyloxycarbonylaminoethyl)-glycinate **36** (4.0g, 14mmol) was taken in 10% aqueous Na_2CO_3 (75ml) and dioxane (60ml). Chloroacetyl chloride (6.5ml, 0.75mmol) was added in two portions with vigorous stirring. The reaction was complete within 5 min. The reaction mixture was brought to pH 8.0 by addition of 10% aqueous Na_2CO_3 and concentrated to remove the dioxane. The product was extracted from the aqueous layer with dichloromethane and was purified by column chromatography to obtain the ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)-glycinate **37** as a colourless oil in good yield (4.2g, 80%).

^{1}H NMR $CHCl_3$-d δ: 5.45 (br s, 1H), 4.14 (s, 2H), 4.00 (s, 2H), 3.53 (t, 2H), 3.28 (q, 2H), 1.46 (s, 9H), 1.23 (t, 3H, J=8Hz).

Ethyl-*N*-(*tert.*butyloxycarbonylaminoethyl)-(*N*-thymin-1-ylacetyl)glycinate 38

Ethyl *N*-(butyloxycarbonylaminoethyl)-*N*-(chloroacetyl)-glycinate **35** (4.0g, 11.6mmol) was stirred with anhydrous K_2CO_3 (1.56g, 11.8mmol) in DMF with thymine (1.4g,

11.2mmol) to obtain the desired compound **38** in good yield. DMF was removed under reduced pressure and the oil obtained was purified by column chromatography.

^{1}H NMR $CHCl_3$-d δ: 9.00 (br s, 1H, T-NH), 7.05 (min) & 6.98 (maj) (s, 1H, T-H6), 5.65 (maj) & 5.05 (min) (br s, 1H, NH), 4.58 (maj) & 4.44 (min) (s, 1H, T-CH_2), 4.25 (m, 2H, OCH_2), 3.55 (m, 2H), 3.36 (m, 2H), 1.95 (s, 3H, T-CH_3), 1.48 (s, 9H), 1.28 (m, 3H).

^{13}C NMR ($CDCl_3$) δ: 170.8, 169.3, 167.4, 164.3, 156.2, 151.2, 141.1, 110.2, 79.3, 61.8, 61.2, 48.5, 48.1, 47.7, 38.4, 28.1, 13.8, 12.2.

Ethyl-*N*-*(tert*.butyloxycarbonylaminoethyl)-(*N*^{4}-benzyloxycarbonyl cytosine--1ylacetyl)glycinate 39

A mixture of NaH (0.25g, 6.2mmol) and N^4-benzyloxycarbonyl cytosine **46**(1.24g, 6.2mmol) was taken in DMF and stirred at 75^{o}C till the effervescence ceased. The mixture was cooled and ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)-glycinate **37** (2.0g, 6.2mmol) was added. Stirring was then continued at 75^{o}C to obtain the cytosine monomer, *N*-(Boc-aminoethylglycyl)-(*N*^{4}-benzyloxycarbonyl cytosine)ethyl ester **39**, in moderate yield (1.62g, 50%).

^{1}H NMR $CHCl_3$-d δ: 7.65 (d, 1H, J=8Hz), 7.35 (s, 5H), 7.25 (d, 1H, J=8Hz), 5.70 (br s, 1H), 5.20 (s, 2H), 4.71 (maj) & 4.22 (min) (br s, 2H), 4.15 (q, 2H), 4.05 (s, 2H), 3.56 (m, 2H), 3.32 (m, 2H), 1.48 (s, 9H), 1.25 (t, 3H).

Ethyl-*N*-*(tert*.butyloxycarbonylaminoethyl)-(adenine-1ylacetyl)glycinate 40

NaH (0.25g, 6.1mmol) was taken in DMF (15ml) and adenine (0.8g, 6.1mmol) was added. The mixture was stirred at 75^{o}C till the effervescence ceased and the mixture

was cooled before adding ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)-glycinate **37**(2.0g, 6.1mmol). The reaction mixture was heated once again to 75^{o}C for 1h, when TLC analysis indicated the disappearance of the starting ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)-glycinate. The DMF was removed under vacuum and the resulting thick oil was taken in water and the product, extracted in ethyl acetate. The organic layer was then concentrated to obtain the crude product, which was purified by column chromatography to obtain the pure **40.**

^{1}H NMR $CHCl_3$-d δ: 8.32 (s, 1H), 7.95 (min) & 7.90 (maj) (s, 1H), 5.93 (maj) & 5.80 (min) (br, 2H), 5.13 (maj) & 4.95 (min), 4.22 (min) & 4.05 (maj) (s, 2H), 4.20 (m, 2H), 3.65 (maj) & 3.55 (min) (m, 2H), 3.40 (maj) & 3.50 (min) (m, 2H), 1.42 (s, 9H), 1.25 (m, 3H).

Ethyl-*N*-*(tert.*butyloxycarbonylaminoethyl-1ylacetyl)glycinate 2-amino-6-chloropurine 41

A mixture of 2-amino-6-chloropurine (1.14g, 6.8mmol), K_2CO_3 (0.93g, 7.0mmol) and ethyl *N*-(Boc-aminoethyl)-*N*-(chloroacetyl)-glycinate **37** (2.4g, 7.0mmol) were taken in dry DMF (20ml) and stirred at room temperature for 4h. K_2CO_3 was removed by filtration, and the DMF, by evaporation under reduced pressure. The resulting residue was purified by column chromatography to obtain the **41** in excellent yield (2.65g, 98%).

^{1}H NMR $CHCl_3$-d δ: 12.25 (s, 1H, iBu-N*H*), 10.13 (s, 1H, G-N^{1}-*H*), 7.90 (s, 1H, G-*H*8), 5.85 (m, 1H, Boc-N*H*), 5.33 (maj) & 5.13 (min) (s, 2H, CH_2-G), 4.40 - 4.05 (m, 4H, N-CH_2-COOC$H_2$$CH_3$), 3.60 (m, 2H, Boc-NH-C$H_2$), 3.40 (maj) & 3.30 (min) (m, 2H, Boc-

NH-CH_2-CH_2), 2.55 (m, 1H, CH(CH_3)$_2$), 1.45 (min) & 1.40 (maj) (s, 9H, (CH_3)$_3$), 1.25 (m, 9H, CH_2CH_3, CH(CH_3)$_2$).

^{13}C NMR $CHCl_3$-d δ: 180.2 (*C*OOCH(CH_3)$_2$), 169.4 (*C*OOEt), 167.2 (G-CH_2-*C*O), 162.9 (G-*C*2), 156.4 (G-*C*4), 153.3 (*C*OOC(CH_3)$_3$), 148.0 (G-*C*6), 145.2 (G-*C*8), 112.2 (G-*C*5), 79.6 (*C*(CH_3)$_3$), 61.5 (*C*H(CH_3)$_2$), 48.7 (N-*C*H_2-COOEt), 47.1 (O*C*H_2CH_3), 38.7 (Boc-NH-*C*H_2), 35.9 (Boc-NH-CH_2-*C*H_2), 28.4 ((*C*H_3)$_3$), 18.9 ((*C*H_3)$_2$), 14.1 (CH_2*C*H_3).

Hydrolysis of the ethyl ester functions of PNA monomers

General method

The ethyl esters were hydrolyzed using 2N aqueous NaOH (5ml) in methanol (5ml) and the resulting acid was neutralized with activated Dowex-H^+ till the pH of the solution was 7.0. The resin was removed by filtration and the filtrate was concentrated to obtain the resulting Boc-protected acid (**42 - 45**) in excellent yield (>85%).

N^4-benzyloxycarbonylcytosine 47

Cytosine (1.0g, 9.0mmol) was suspended in dry pyridine (100ml) at 0 ^oC. CBz-Cl (3.2ml, 22.5mmol) was added and the reaction was stirred under nitrogen overnight. The pyridine suspension was evaporated to dryness. Water (10ml) and dilute HCl was added to bring the pH to 4.0. The resulting white precipitate was filtered off, washed with water and partially dried under vacuum. The wet precipitate was boiled in absolute ethanol (10ml), cooled to 0 ^oC, filtered, washed thoroughly with ether and dried under vacuum (Yield = 1.1g, 50%).

N^6-Benzoyl Adenine 49

Benzoyl chloride (12.7ml, 110mmol) was added to a suspension of adenine (5.0g, 37mmol) in dry pyridine (50ml). The reaction mixture was refluxed for 2h. The solvent was removed under vacuum. The crude product was triturated with warm $NaHCO_3$ solution, which caused the separation of an oily phase. When the aqueous suspension was shaken with $CHCl_3$, the amide precipitated. The crystals were filtered off and washed with water (Yield = 7.5g, 85%)

Picric Acid Estimation of Resin Functionalization

The typical procedure for estimation of the loading value of the resin was carried out with 5mg of the resin and comprised the following steps:

The resin was swollen in dry CH_2Cl_2 for at least 30min. The CH_2Cl_2 was drained off and a 50% solution of TFA in CH_2Cl_2 was added (1ml x 2), 15 min each. After washing thoroughly with CH_2Cl_2, The TFA salt was neutralized with a 5% solution of DIPEA in CH_2Cl_2 (1ml x 3, 2 min each). The free amine was treated with a 0.1M picric acid solution in CH_2Cl_2 (2ml x 2, 3min each). The excess picric acid was eliminated by extensively washing the resin with CH_2Cl_2. The adsorbed picric acid was displaced form the resin by adding a solution of 5% DIPEA in CH_2Cl_2. The eluant was collected and the volume was made up to 10 ml with CH_2Cl_2 in a volumetric flask. The absorbance was recorded at 358nm in ethanol and the concentration of the amine groups on the resin was calculated using the molar extinction coefficient of picric acid as 14,500 $cm^{-1}M^{-1}$ at 358 nm.

Kaiser's Test

Kaiser's test was used to monitor the Boc-deprotection and amide coupling steps in the solid phase peptide synthesis. Three solutions were used, viz. (1) Ninhydrin (5.0 g) dissolved in ethanol (100 ml), (2) Phenol (80 g dissolved in ethanol (20 ml) and (3) KCN: 2 ml of a 0.001M aqueous solution of KCN in 98 ml pyridine).

To a few beads of the resin to be tested taken in a test tube, were added 3-4 drops of each of the three solutions desribed above. The tube was heated at 100 ^{o}C for ~5 min, and the colour of the beads was noted. A blue colour on the beads and in the solution indicated successful deprotection, while colourless beads were observed upon completion of the amide coupling reaction. The blank solution should remain yellow.

Cleavage of the PNA oligomers from the solid support

A typical cleavage reaction was carried out with 5 or 10mg of resin-bound PNA oligomer. The resin-bound PNA oligomer (10mg) was stirred in an ice-bath with thioanisole (20µl) and 1,2-ethanedithiol (8µl) for 10min, TFA (120µl) was added and stirring was continued for another 10min. TFMSA (16µl) was added and stirring continued for 2h. The reaction mixture was filtered through a sintered funnel. The residue was washed with TFA (3 x 2ml) and the combined filtrate and washings were evaporated under vacuum and co-evaporated with ether, avoiding heating during this process. The residue was precipitated using dry ether and centrifuged to obtain a white pellet. The pellet was re-dissolved in methanol (~0.1ml) and re-precipitated by adding ether. The pellet collected after centrifugation was subjected to this re-precipitation process at least thrice, when a white precipitate was obtained of the crude PNA oligomer.

Gel Filtration

The crude PNA oligomer obtained after ether precipitation was dissolved in water (~0.5ml) and loaded on a gel filtration column. This column consisted of G25 Sephadex and had a void volume of 1ml. The oligomer was eluted with water and ten fractions of 1ml volume each were collected. The presence of the PNA oligomer was detected by measuring the absorbance at 260nm. The fractions containing the oligomer were freeze-dried. The purity of the cleaved crude PNA oligomer was determined by RP HPLC on a C18 column. If found to be above 90%, the oligomers were sued as such for experiments without further purification. If the purity was not satisfactory, the oligomers were purified by HPLC/FPLC.

FPLC

The crude PNA oligomers were dissolved in water containing 0.1% TFA, the starting buffer for injection. The polypyrimidine T8 sequences were purified using a gradient of 0 to 50% buffer B in 30 min at a flow rate of 1.0 ml/min, where buffer A = water with 0.1% TFA and buffer B = 60% CH3CN in water containing 0.1% TFA. The mixed sequence PNAs eluted earlier and hence had to be purified using a gradient of 0 to 30% B in 30 min at a flow rate of 1.0 ml/min, when good resolution of the peaks was obtained. The purity of the oligomer after FPLC was ascertained by HPLC on a C18 RP column.

HPLC

The purity of the PNA oligomers was ascertained on an analytical RP C18 column using a gradient of 5 to 80% CH_3CN in water containing 0.1% TFA at a flow rate of 1.5 ml/min.

HPLC purification of the mixed sequence decamers was carried out on a semi-preparative RP C4 column using isocratic elution at a flow rate of 6.0 ml/min. The eluent was varied between 8 to 12% CH3CN in water containing 0.1% TFA to obtain optimum separation of the constituent peaks. The oligomers so collected were re-checked for purity by analytical HPLC as described above.

MALDI-TOF Mass Spectrometry

Literature reports the analysis of PNA purity by MALDI-TOF mass spectrometry[52] in which several matrices have been explored, viz. sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid). Of these, sinapinic acid was found to give the best signal to noise ratio with all the other matrices typically producing higher molecular ion signals.

UV-Melting

The concentration of the PNA oligomers was calculated on the basis of the absorption at 260nm, assuming the molar extinction coefficients of the nucleobases to be as in DNA, i. e., T, 8.8 $cm^2/\mu mol$; C, 7.3 $cm^2/\mu mol$; G, 11.7 $cm^2/\mu mol$ and A, 15.4 $cm^2/\mu mol$. The hairpin PNA oligomer (**6**-**9**) and the relevant complementary DNA oligonucleotide (**10**/**11**) were mixed together in a 1:1 molar ratio in 0.01M sodium

phosphate buffer, pH 7.4 to get a final strand concentration of 1μM. The samples were annealed by heating at 85 ^{o}C for 1-2 min, followed by slow cooling to room temperature, kept at room temperature for ~30 min and then, refrigerated overnight. The samples were heated at a rate of 0.5 ^{o}C rise per minute and the absorbance at 260nm was recorded at every minute. The percent hyperchromicity at 260nm was plotted as a function of temperature and the melting temperature was deduced from the peak in the first derivative plots.

Gel shift experiments

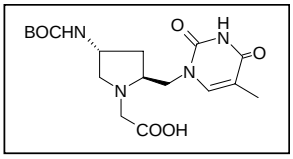
The PNA and DNA oligomers utilized in the gel mobility shift assays were mixed together in the desired ratios in water. The samples were lyophilized to dryness and re-suspended in 10μl tris-EDTA buffer, pH 7.0. The samples were annealed as described earlier by heating at 85^{o}C for 5min. followed by slow cooling to room temperature and refrigeration overnight. Prior to loading, 10μl glycerol in TBE buffer, pH 7.0, the gel-running buffer, was added and the sample, loaded on the gel. Bromophenol blue was used as the tracer dye, but was loaded in an adjacent well and not mixed with the sample.

Gel electrophoresis was performed on a 15% non-denaturing polyacrylamide gel (acrylamide:*bis*-acrylamide, 29:1) until the BPB migrated to three-fourths of the gel length. During electrophoresis, the temperature was maintained ~10^{o}C.

Bands of the single stranded PNA/DNA and the PNA:DNA complexes were visualized as dark bands by UV-shadowing, i.e., by illuminating the gel placed on a fluorescent thin-layer silica gel chromatographic plate, F_{254}, 20cm x 20cm using UV light.

2. 11. Appendix

♦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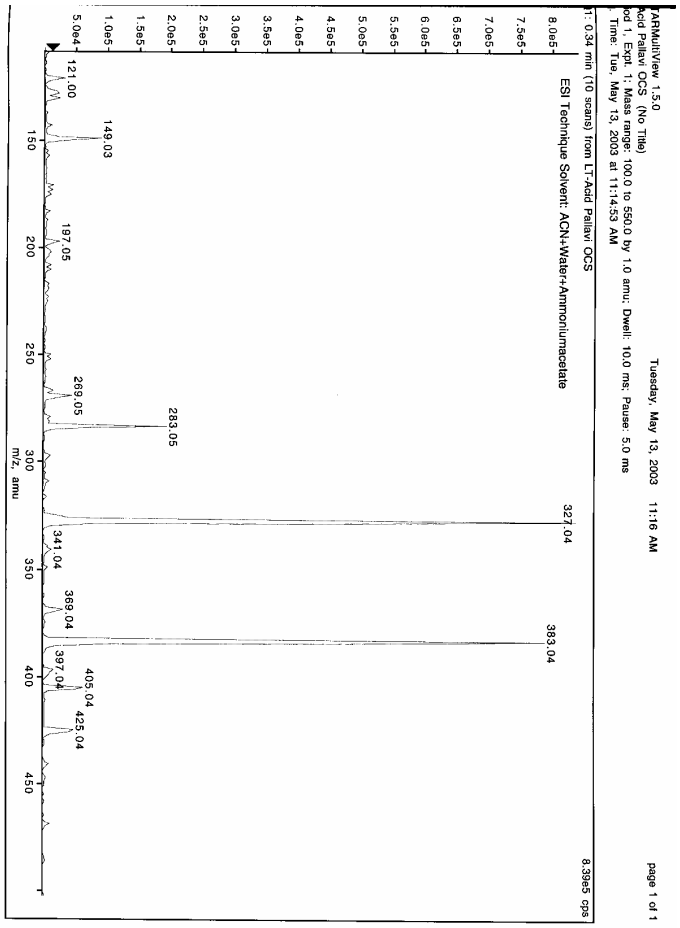
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page 1 of 1
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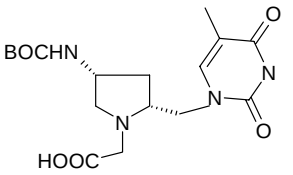
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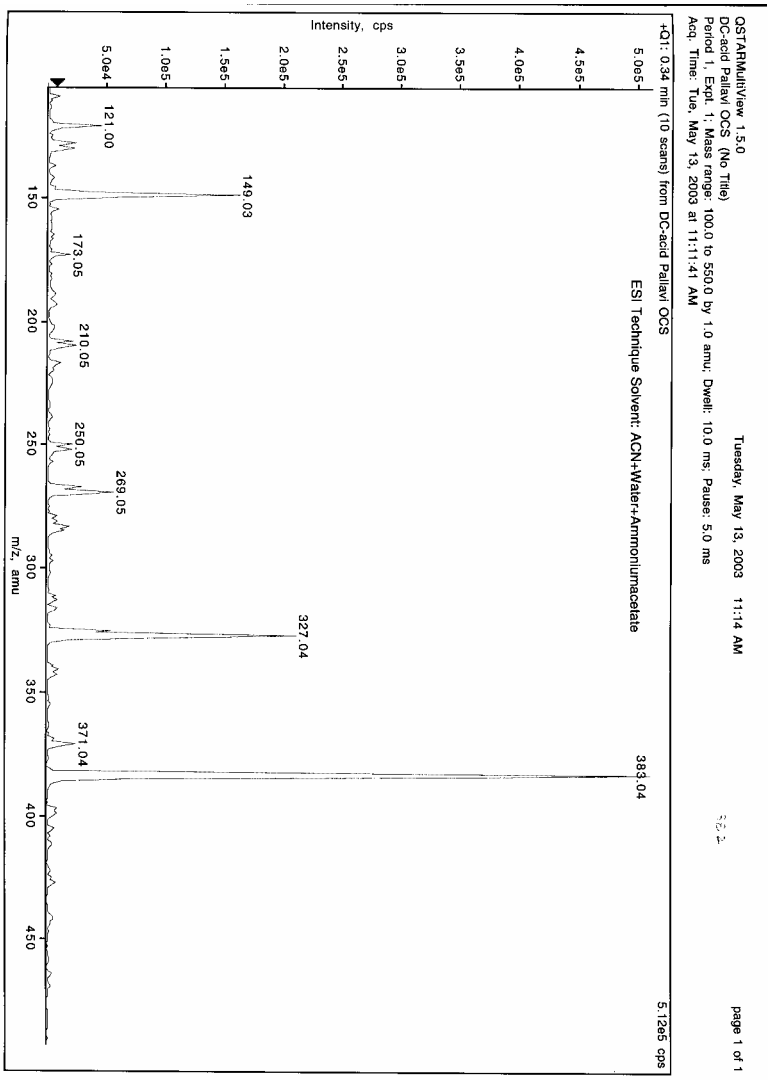
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page 1 of 1
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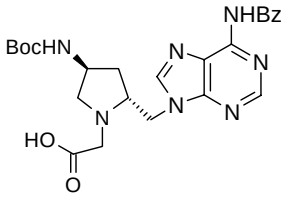
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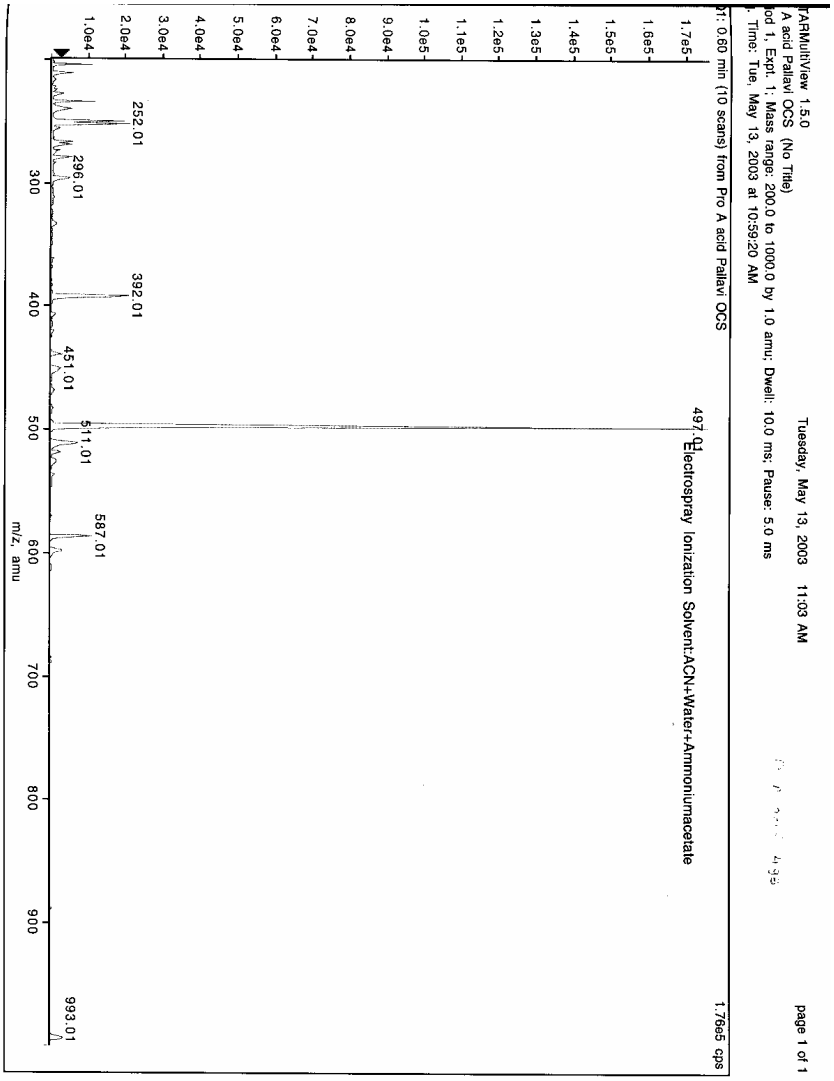
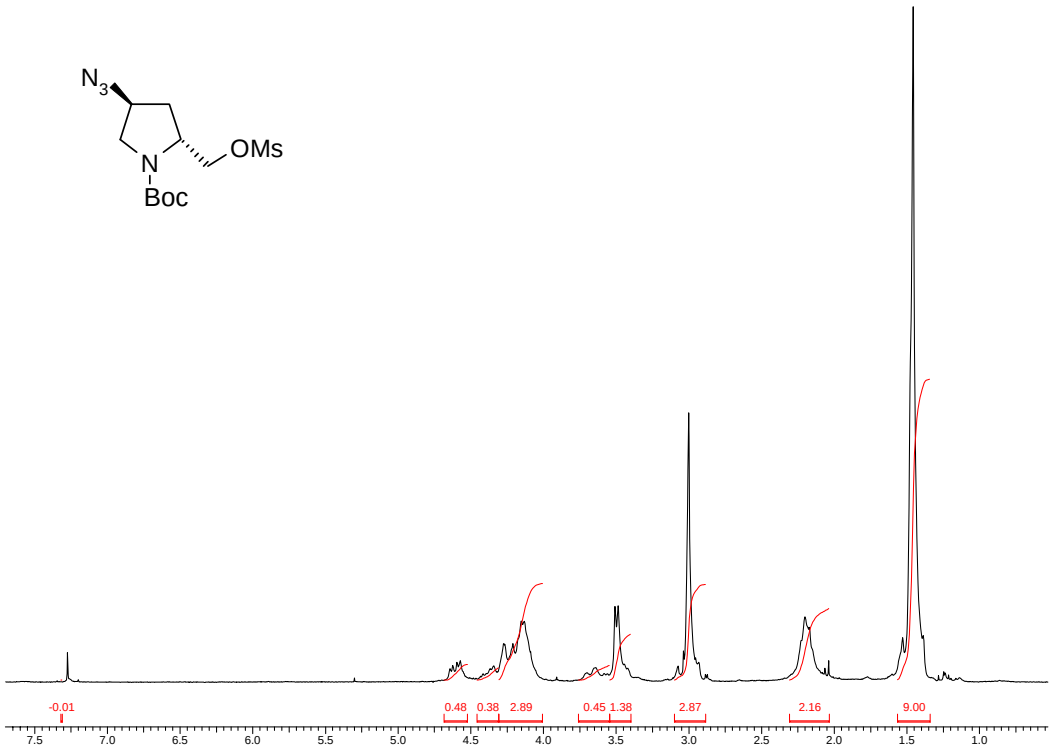
HO
O


TARMultiView 1.5.0
A acid Pallavi OCS (No Title)
od 1, Expt. 1: Mass range: 200.0 to 1000.0 by 1.0 amu; Dwell: 10.0 ms; Pause: 5.0 ms
Time: Tue, May 13, 2003 at 10:59:20 AM
Tuesday, May 13, 2003 11:03 AM
page 1 of 1
1: 0.60 min (10 scans) from Pro A acid Pallavi OCS
Electrospray Ionization Solvent:ACN+Water+Ammoniumacetate
1.76e5 cps
497.01
252.01
296.01
392.01
451.01
511.01
587.01
993.01
m/z, amu


N3
OMs
N
Boc
-0.01
0.48
0.38
2.89
0.45
1.38
2.87
2.16
9.00
7.5
7.0
6.5
6.0
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5.0
4.5
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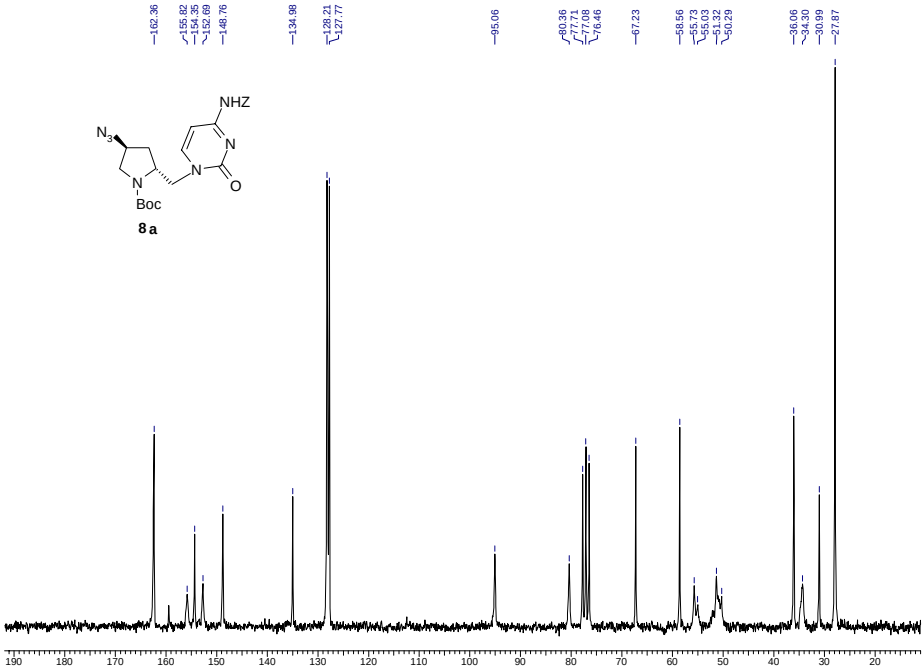
3.0
2.5
2.0
1.5
1.0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155.82
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134.98
128.21
127.77
95.06
80.36
77.71
77.08
76.46
67.23
58.56
55.73
55.03
51.22
50.29
36.06
34.30
30.99
27.87
NHZ
N3
N
N
N
O
Boc
8a
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170
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130
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90
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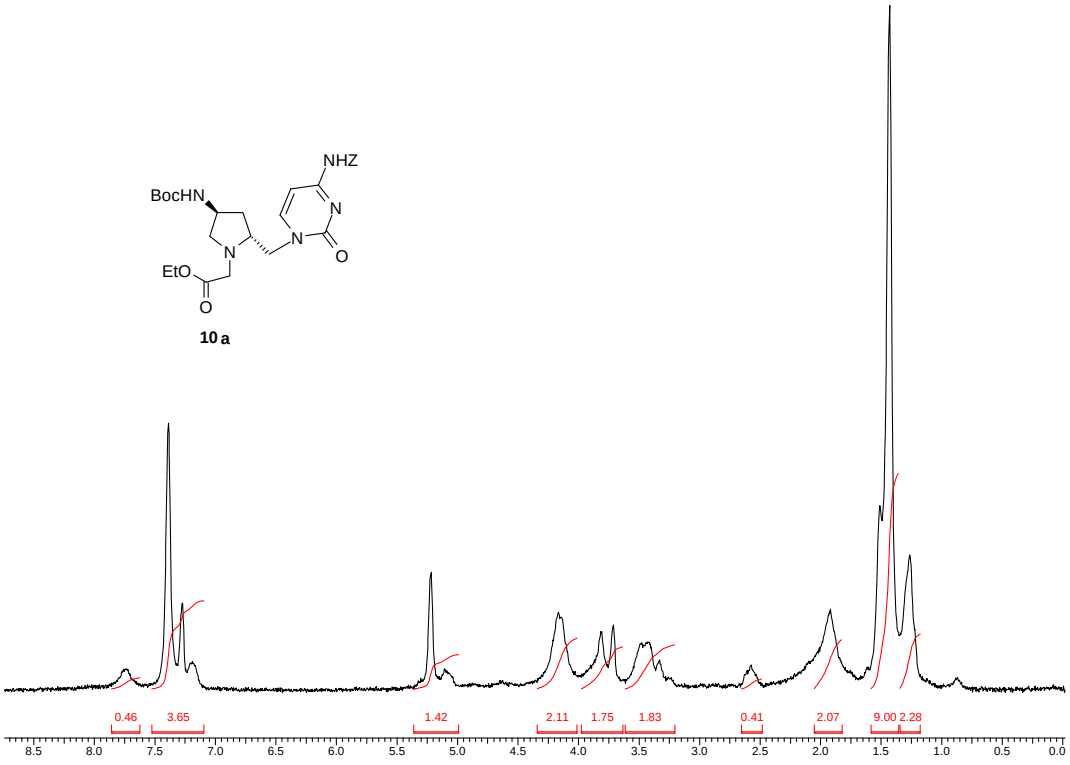
20


NHZ
BocHN
N
N
N
O
EtO
O
O
10 a
0.46
3.65
1.42
2.11
1.75
1.83
0.41
2.07
9.00
2.28
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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4.0
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3.0
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2.0
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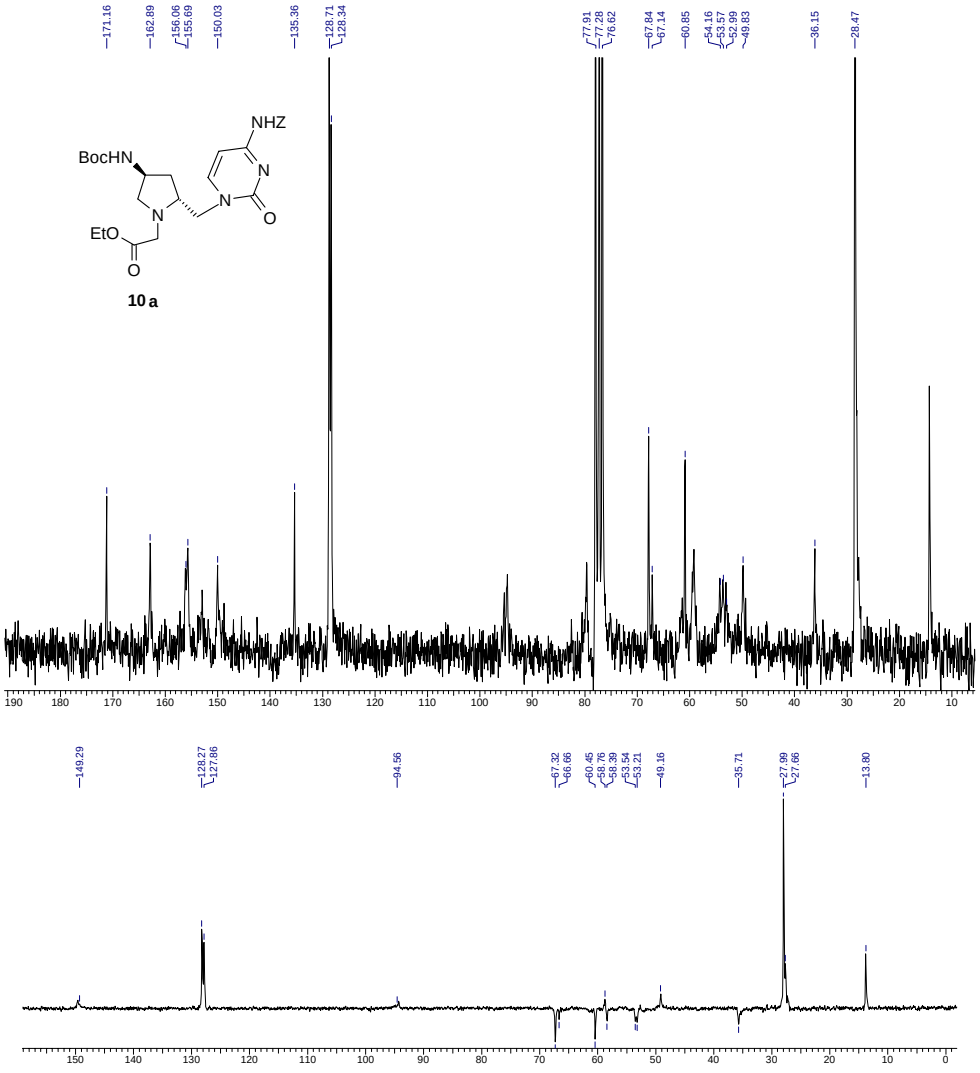
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156.06
155.69
150.03
135.36
128.71
128.34
77.91
77.28
76.62
67.84
67.14
60.85
54.16
53.57
52.99
49.83
36.15
28.47
NHZ
BocHN
N
N
N
O
EtO
O
10 a
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
149.29
128.27
127.86
94.56
67.32
66.66
60.45
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53.21
49.16
35.71
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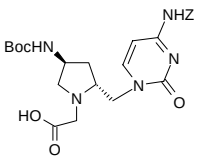
27.66
13.80
150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0


NHZ
BocHN
N
N
N
HO
O
O

(2R,4S) **11 a**

0.26 0.34 0.21 1.95 0.60 0.22 0.46 0.23 0.36 19.25 1.01 1.45 0.01 9.00
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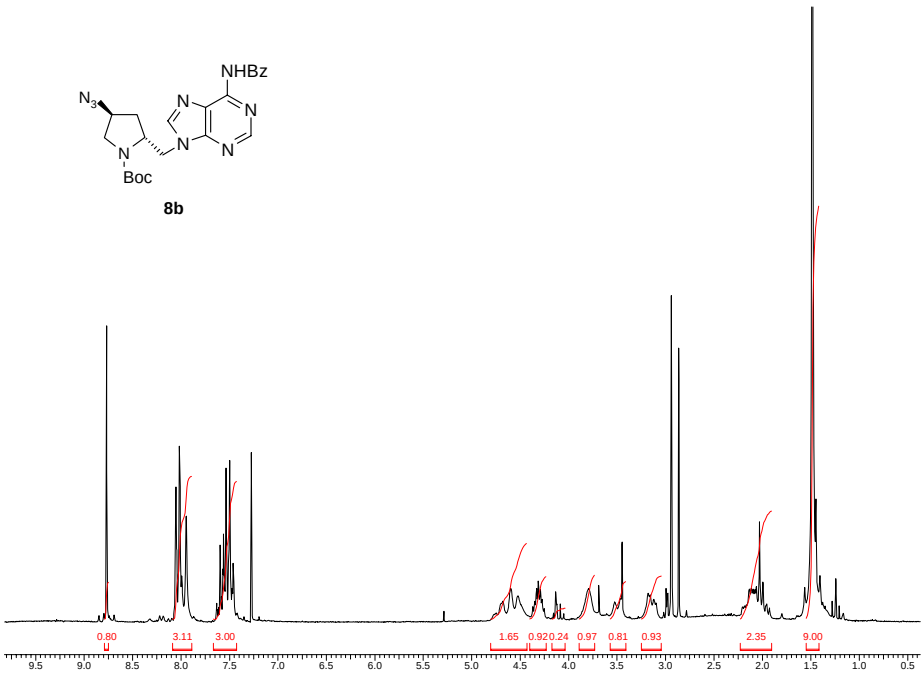
NHBz
N3
N
N
N
N
N
Boc
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3.11
3.00
1.65
0.92
0.24
0.97
0.81
0.93
2.35
9.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
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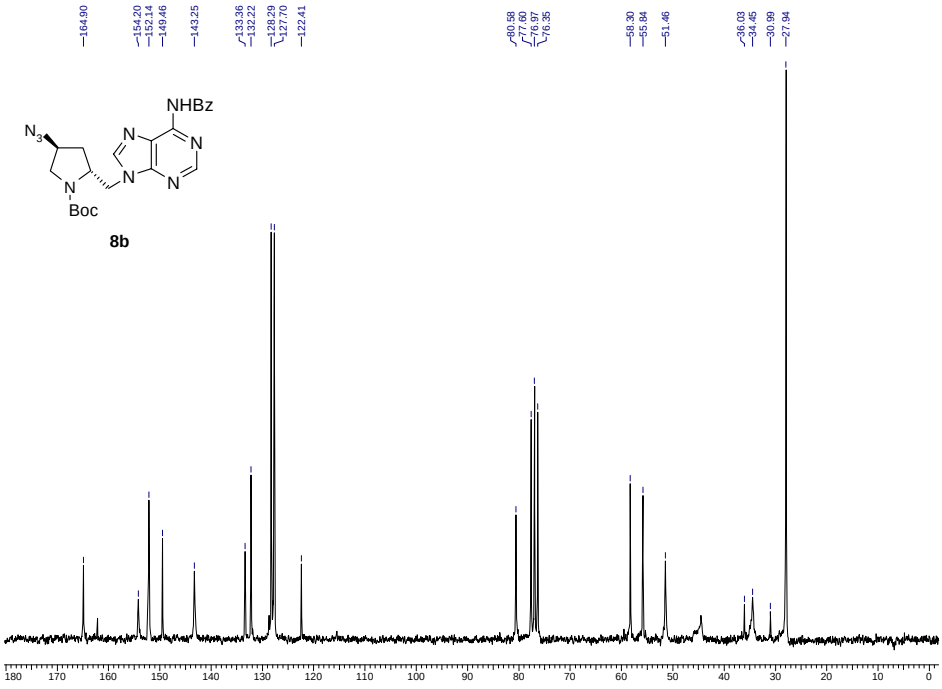
2.0
1.5
1.0
0.5

8b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128.29
127.70
122.41
80.58
77.60
76.97
76.35
58.30
55.84
51.46
36.03
34.45
30.99
27.94
NHBz
N3
N
N
N
N
N
Boc
8b
180
170
160
150
140
130
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110
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10
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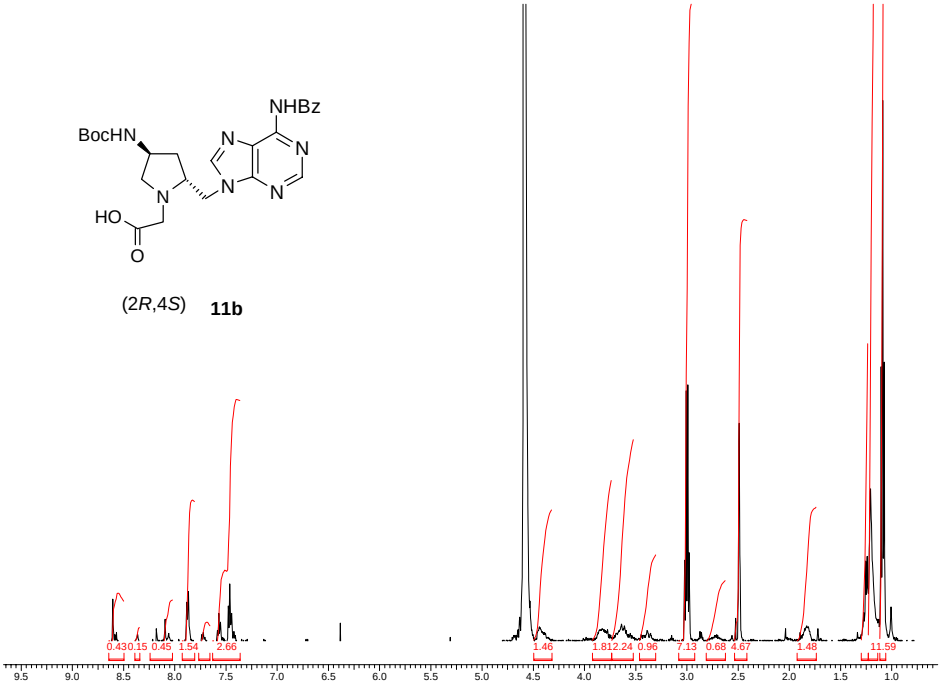
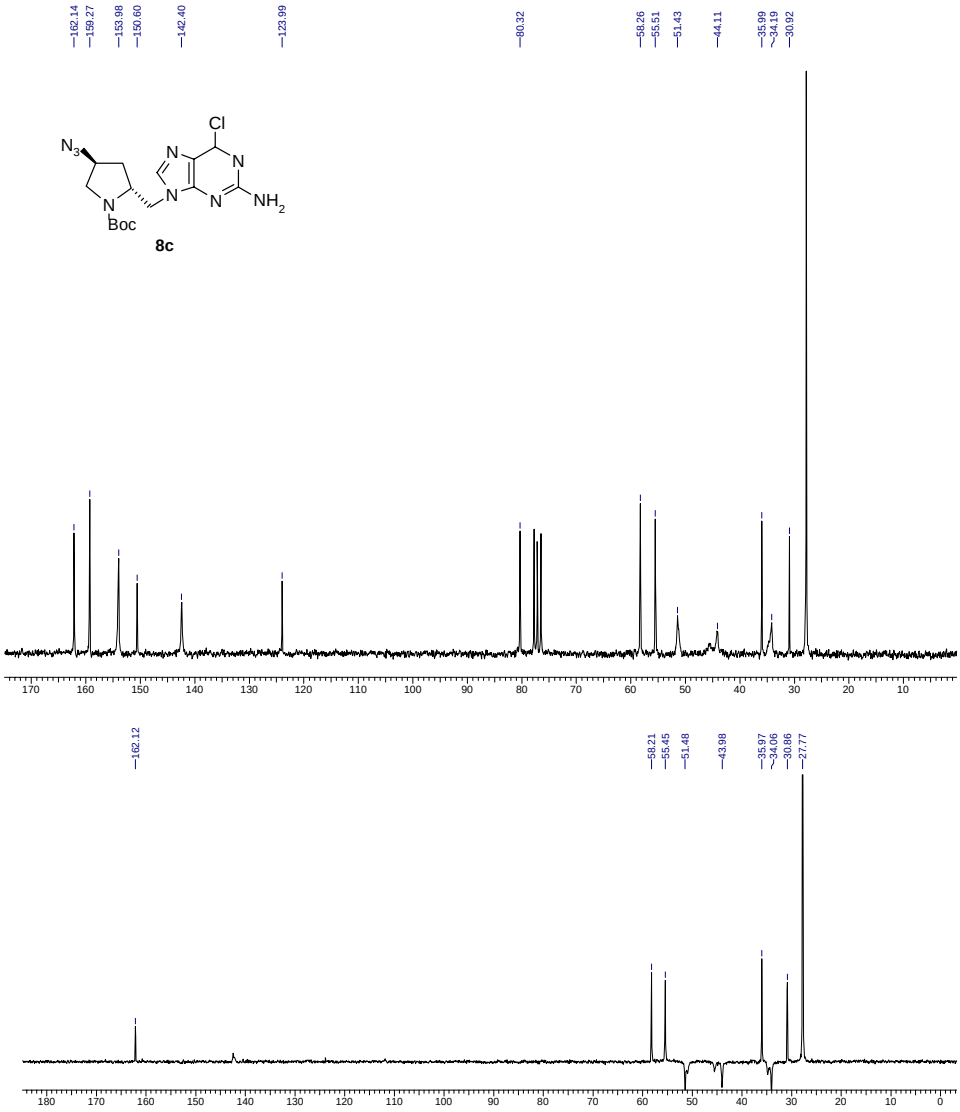

NHBz
BocHN
N
N
N
N
N
HO
O
0.43 0.15 0.45 1.54 2.66
1.46
1.81 2.24 0.96 7.13 0.68 4.67
1.48
11.59
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0

(2*R*,4*S*) **11b**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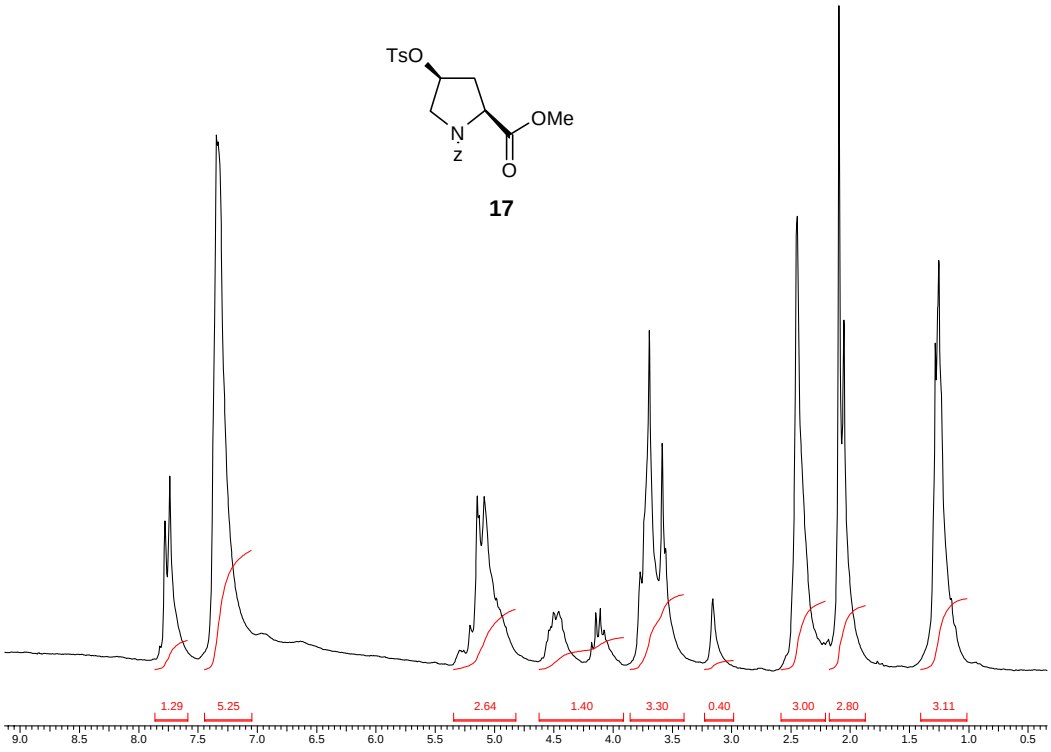
159.27
153.98
150.60
142.40
123.99
80.32
58.26
55.51
51.43
44.11
35.99
34.19
30.92
Cl
N3
N
N
N
N
NH2
N
Boc
8c
162.12
58.21
55.45
51.48
43.98
35.97
34.06
30.86
27.77


TsO
OMe
N
z
O
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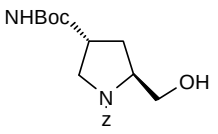
5.25
2.64
1.40
3.30
0.40
3.00
2.80
3.11
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

17


NHBoc
OH
N
z

20

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0.32
1.62
1.52
3.40
0.92
0.91
2.97
0.95
1.12
0.80
9.00
7.5
7.0
6.5
6.0
5.5
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4.5
4.0
3.5
3.0
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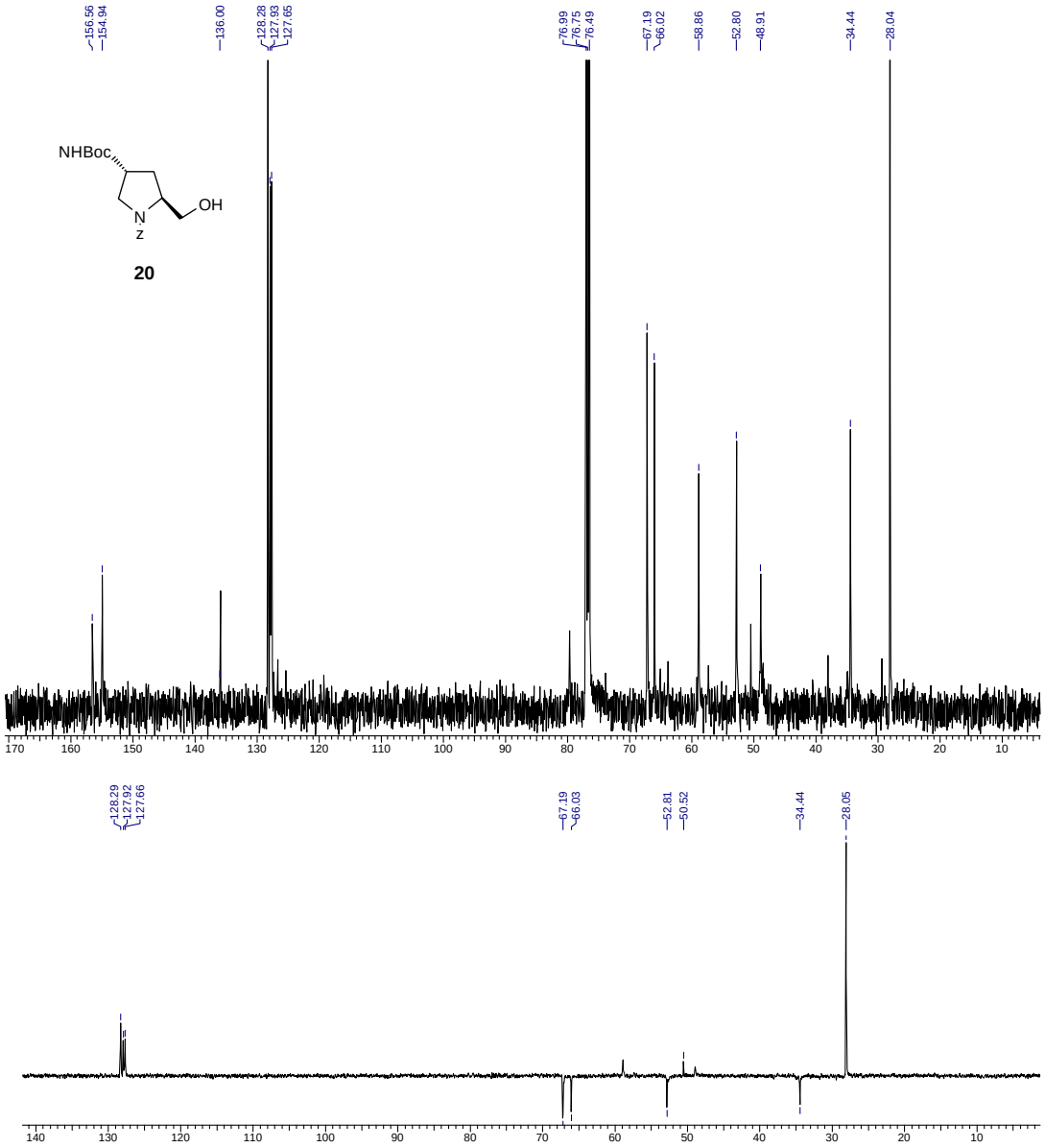
2.0
1.5
1.0
0.5
0.0


156.56
154.94
136.00
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127.93
127.65
76.99
76.75
76.49
67.19
66.02
58.86
52.80
48.91
34.44
28.04
NHBoc
OH
N
z
20
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
128.29
127.92
127.66
67.19
66.03
52.81
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34.44
28.05
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110
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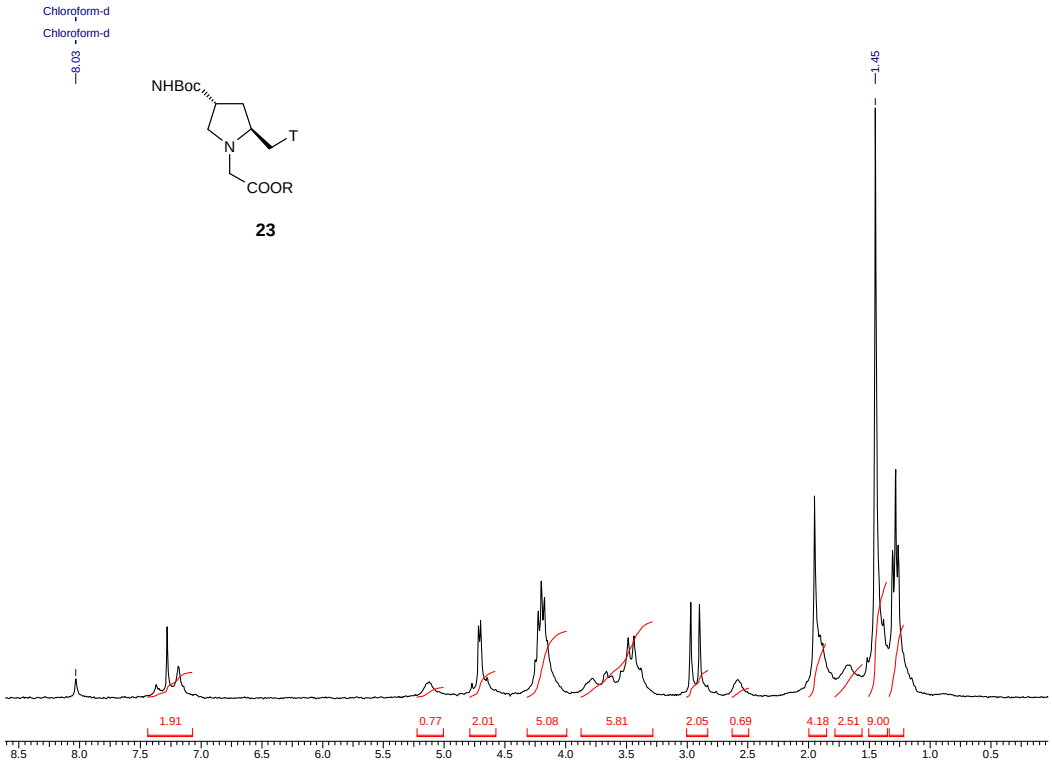
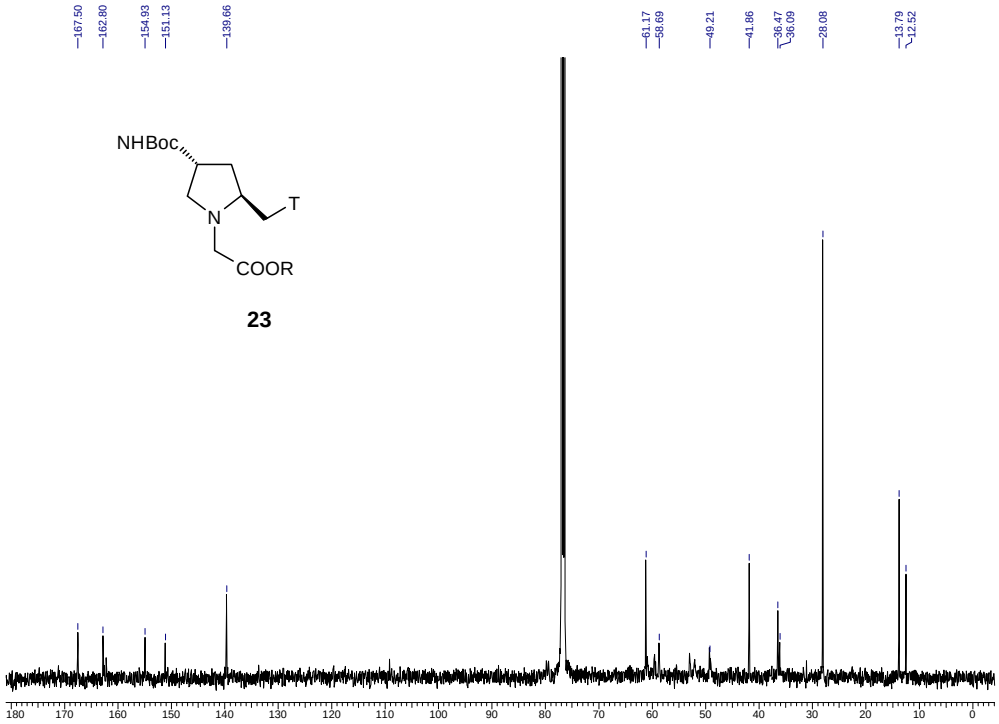
10


Chloroform-d
Chloroform-d
8.03
1.45
NHBoc
T
N
COOR
23
1.91
0.77
2.01
5.08
5.81
2.05
0.69
4.18
2.51
9.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5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151.13
139.66
61.17
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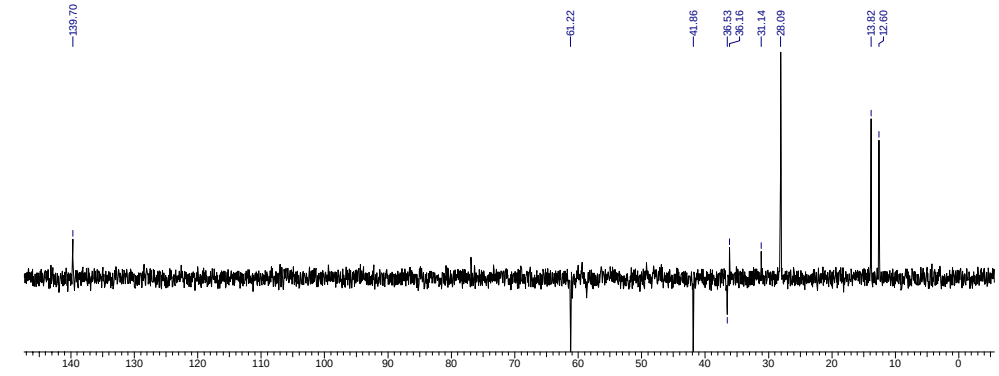
36.47
36.09
28.08
13.79
12.52
NHBoc
N
T
COOR

23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41.86
36.53
36.16
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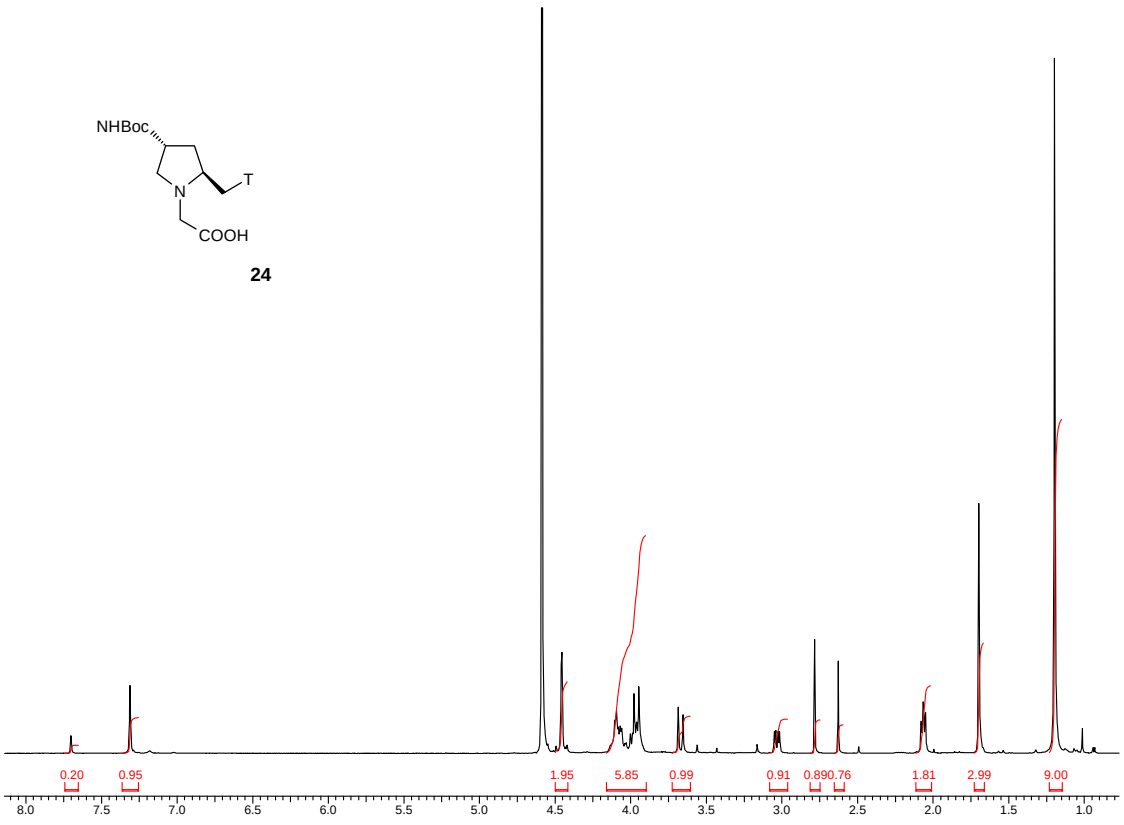
13.82
12.60


NHBoc
T
N
COOH
0.20
0.95
1.95
5.85
0.99
0.91
0.89
0.76
1.81
2.99
9.00
8.0
7.5
7.0
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5.0
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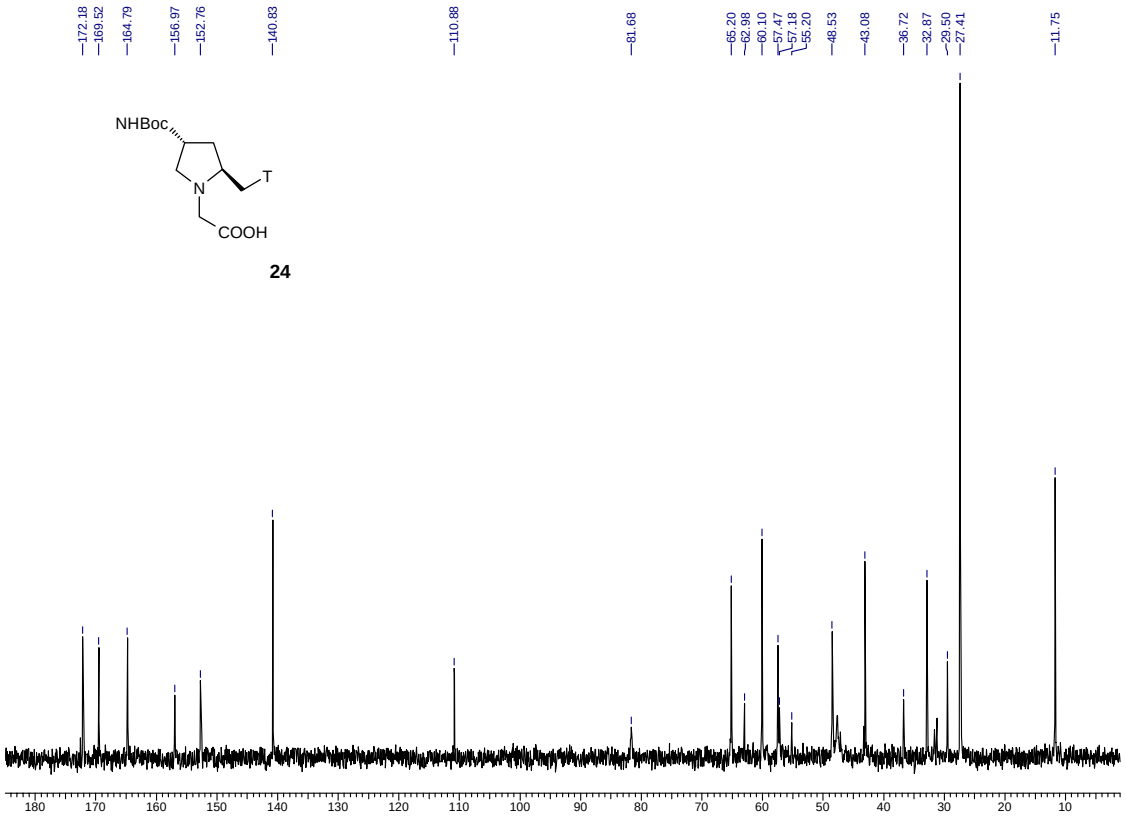
1.0

24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169.52
164.79
156.97
152.76
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110.88
81.68
65.20
62.98
60.10
57.47
57.18
55.20
48.53
43.08
36.72
32.87
29.50
27.41
11.75
NHBoc
N
T
COOH
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170
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150
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120
110
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90
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70
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50
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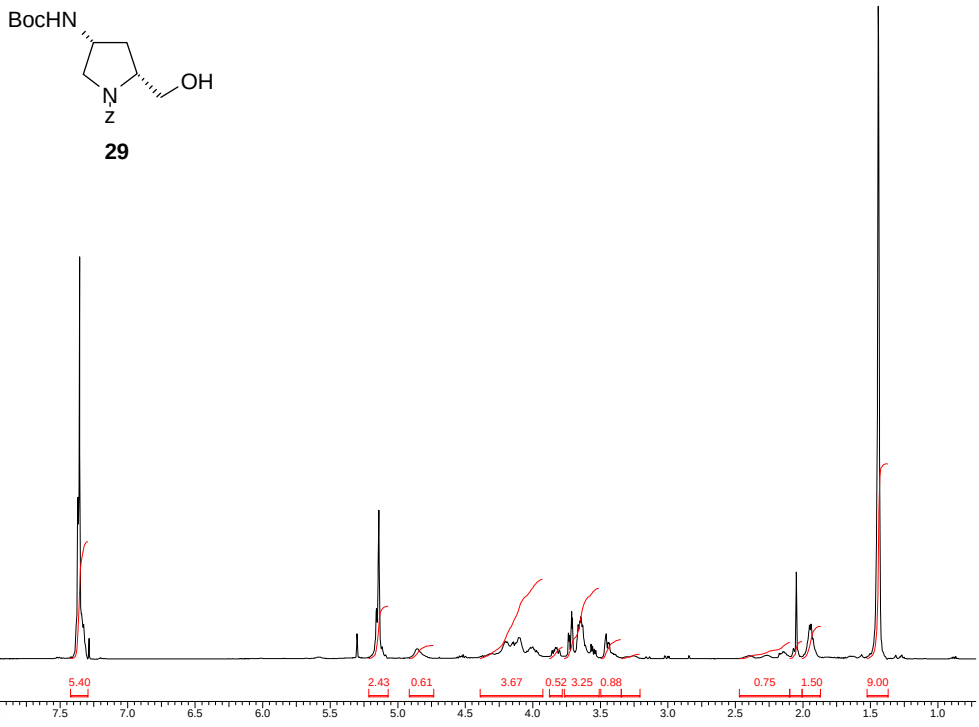
30
20
10

24


BocHN
OH
N
Z
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0.52
3.25
0.88
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9.00
8.0
7.5
7.0
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2.5
2.0
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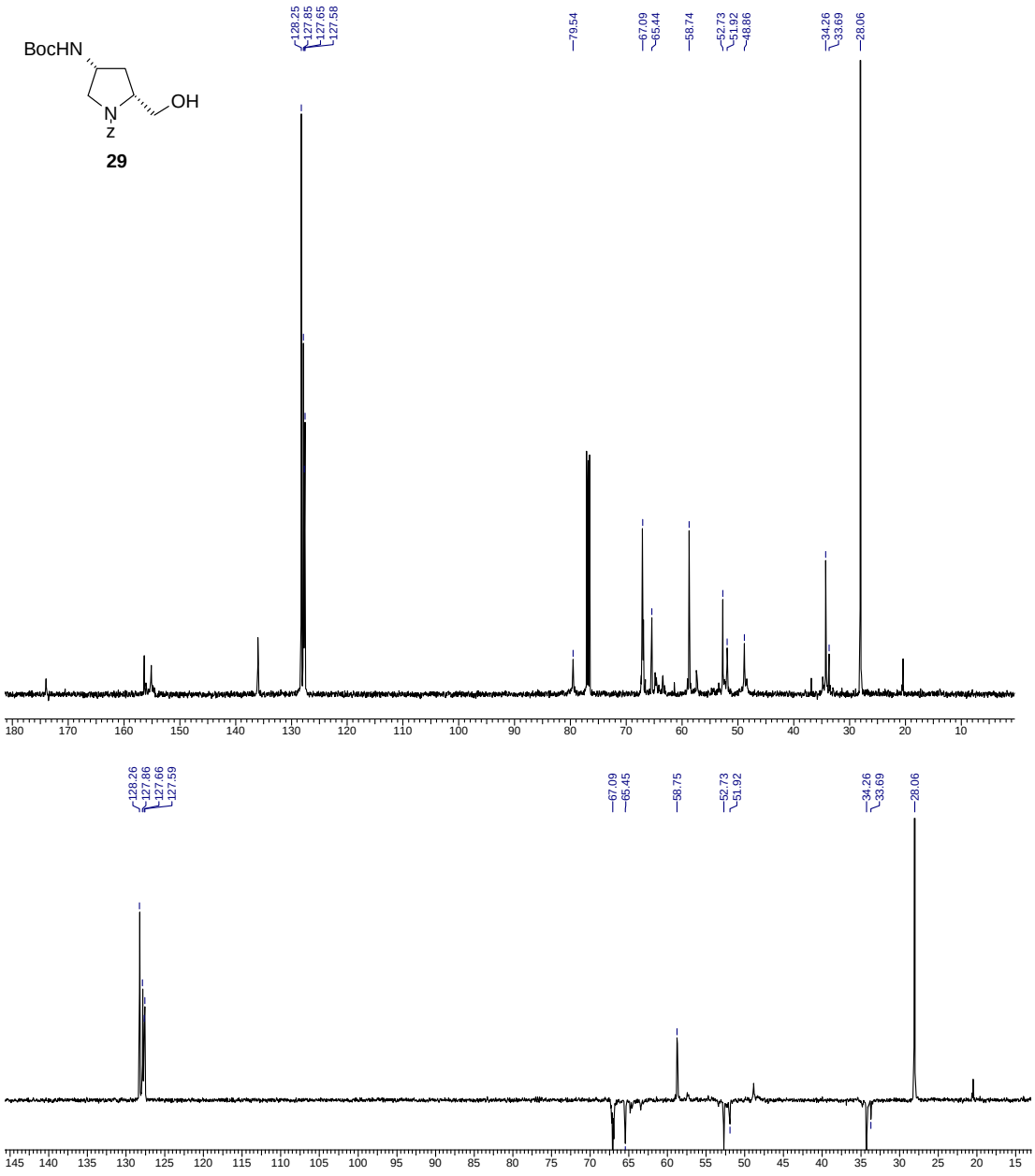
1.0

29


BocHN
OH
N
Z
29
128.25
127.85
127.65
127.58
79.54
67.09
65.44
58.74
52.73
51.92
48.86
34.26
33.69
28.06
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58.75
52.73
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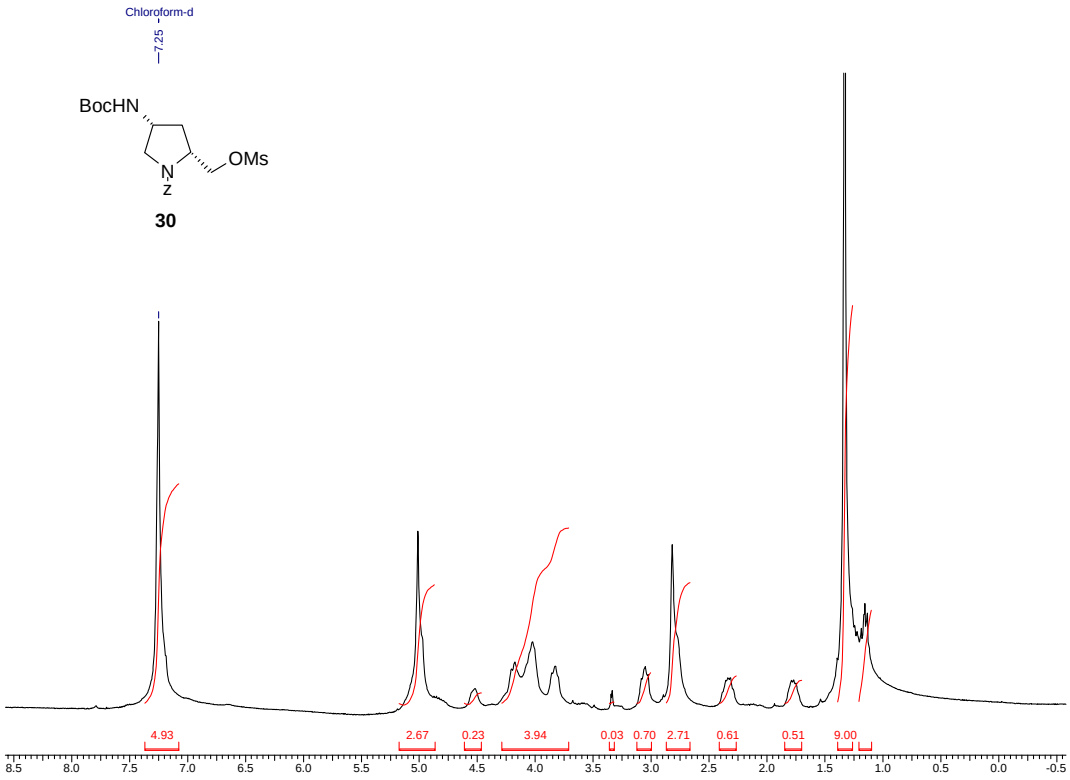
34.26
33.69
28.06


Chloroform-d
7.25
BocHN
OMs
N
Z
30
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2.67
0.23
3.94
0.03
0.70
2.71
0.61
0.51
9.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
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3.5
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2.5
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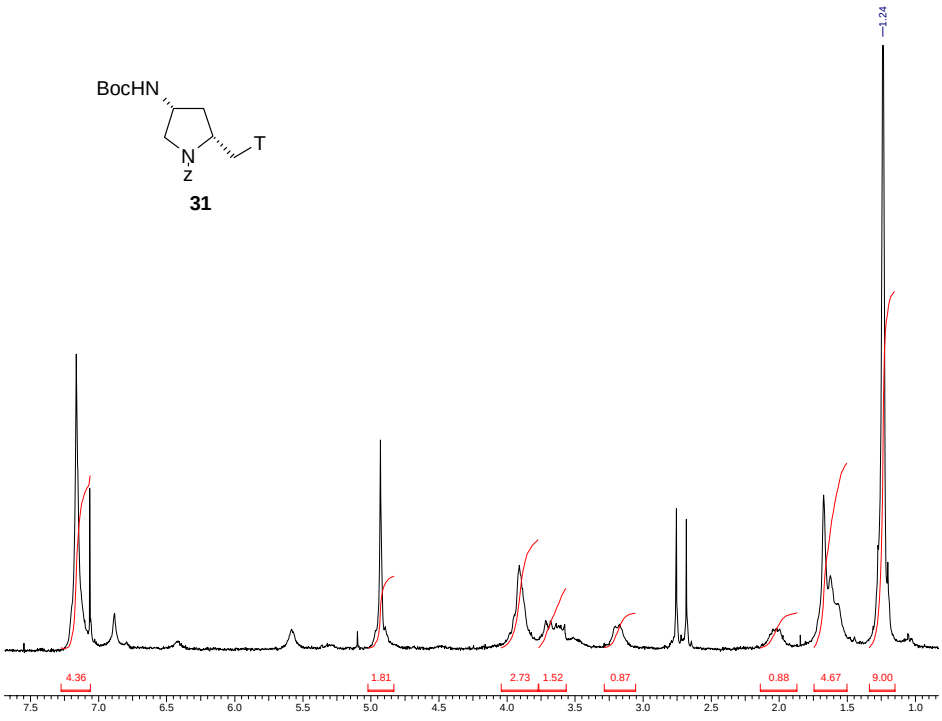
BocHN
T
N
Z
31
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4.36
1.81
2.73
1.52
0.87
0.88
4.67
9.00
7.5
7.0
6.5
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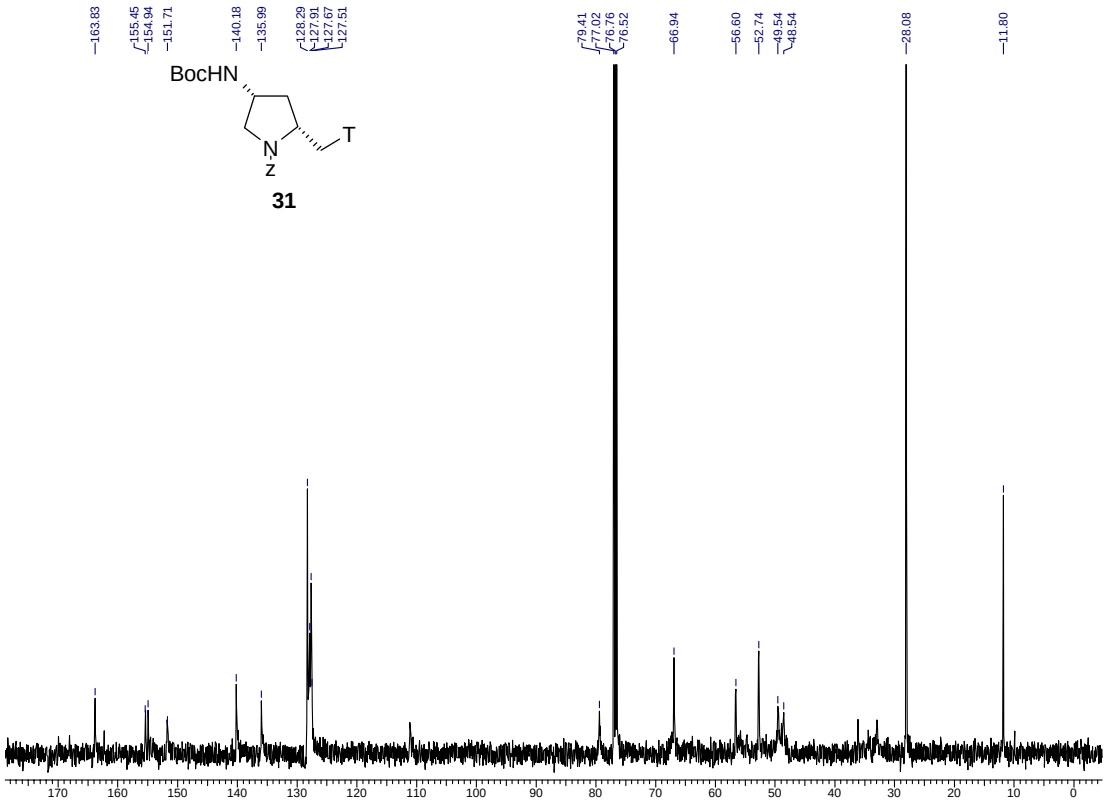
1.5
1.0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77.02
76.76
76.52
66.94
56.60
52.74
49.54
48.54
28.08
11.80
BocHN
N
Z
T
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60
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40
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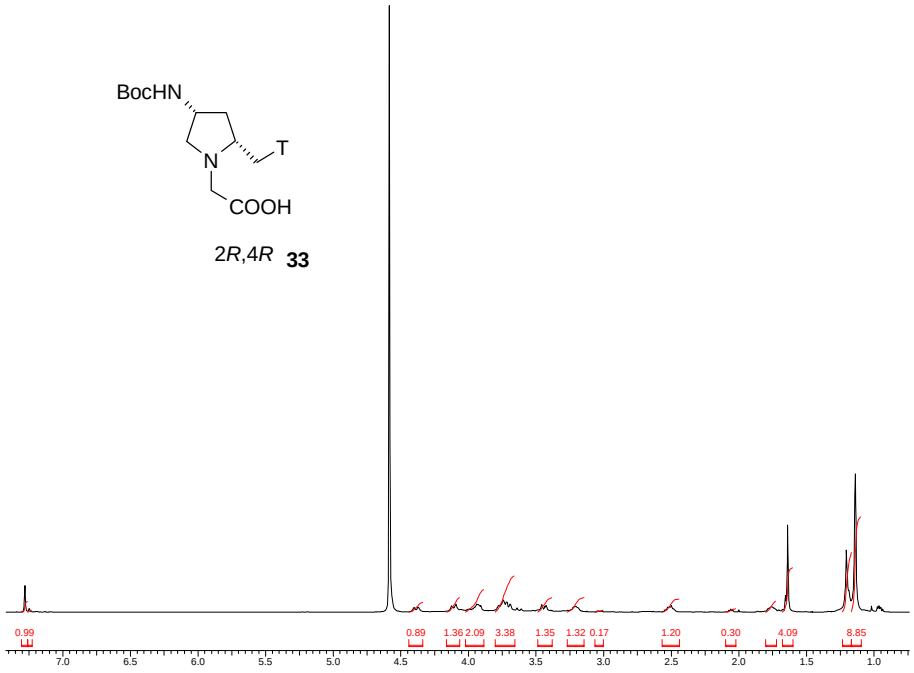
10
0

31


BocHN
T
N
COOH
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0.89
1.36
2.09
3.38
1.35
1.32
0.17
1.20
0.30
4.09
8.85
7.0
6.5
6.0
5.5
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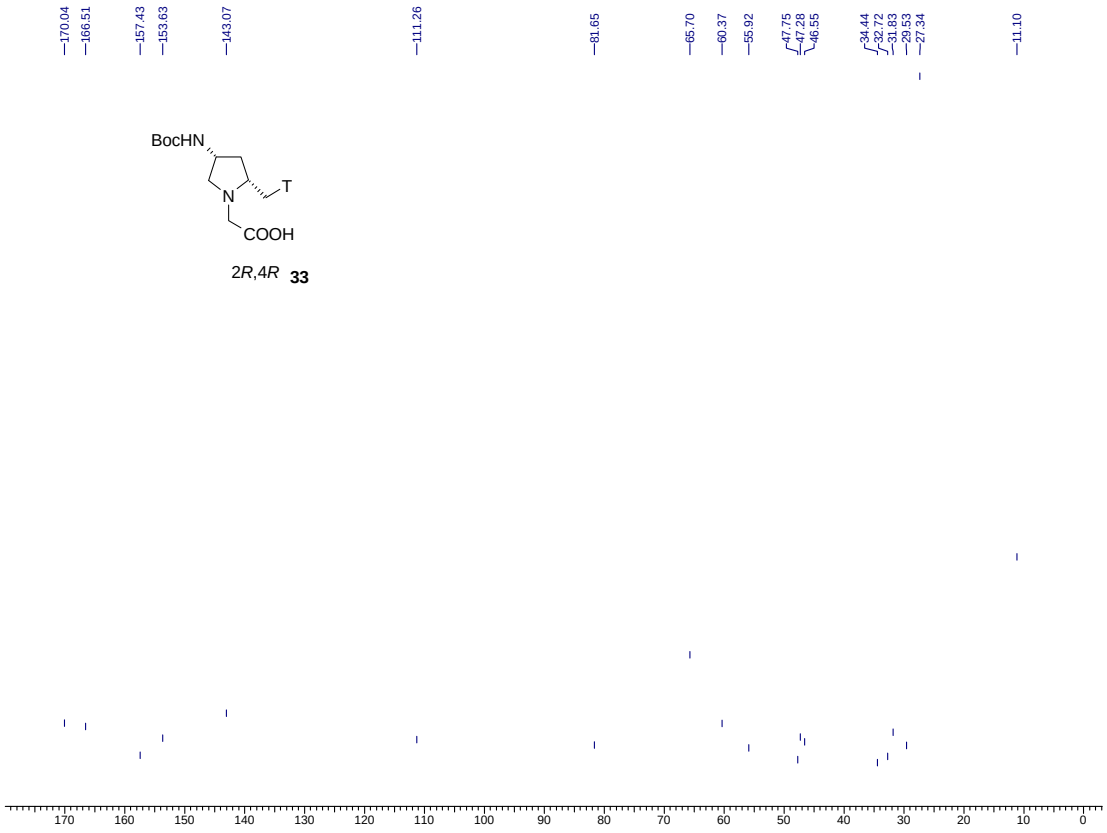
4.0
3.5
3.0
2.5
2.0
1.5
1.0

2*R*,4*R* **33**


170.04
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157.43
153.63
143.07
111.26
81.65
65.70
60.37
55.92
47.75
47.28
46.55
34.44
32.72
31.83
29.53
27.34
11.10
BocHN
N
T
COOH
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

2*R*,4*R* **33**

2.17. REFERENCES

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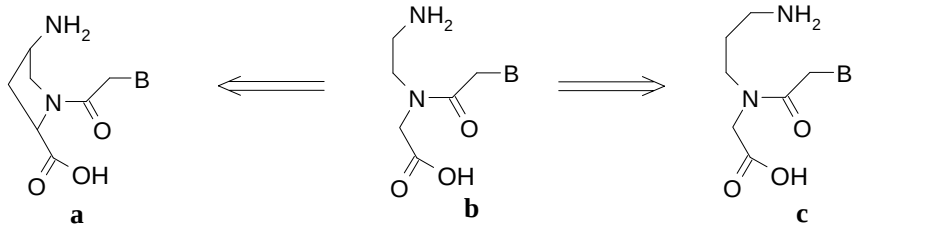
Chapter 3

Trans 4/5-aminopipecolyl-*aeg*PNA chimera: Design, synthesis and binding studies with DNA /RNA in duplex / triplex modes.

3.1. INTRODUCTION

The unique open chain chemical structure of *aeg*PNA has large potential for refinement considering several available modes for providing charge, chirality and structural pre-organization *via* chemical bridges between aminoethyl, glycyl and the nucleobase linker segments of the monomer unit. Most efforts in this direction have been aimed at furthering the potential of PNAs as therapeutic agents with additional requisite properties such as improved water solubility, cellular uptake[1] and discrimination between parallel and antiparallel binding modes.[2] In the previous chapter we discussed the introduction of conformational constraint in *aeg*PNA that also provides a positive charge to the backbone. In this chapter we discuss our efforts that address the effect of five- and six- membered ring structures and their substitution pattern on DNA/RNA recognition selectivity.

In an earlier study, to understand the structural obligations of *aeg*PNA, structural variations were effected by extending the backbone in either 2-aminoethyl segments of *aeg*PNA (figure 1, **I**).[3] AminopropylglycylPNA (*apg*PNA) was thus synthesized by inserting a methylene group in 2-aminoethyl segment (Figure 1,**II**). It was found from UV-Tm studies that PNA:DNA duplexes or triplexes incorporating a single

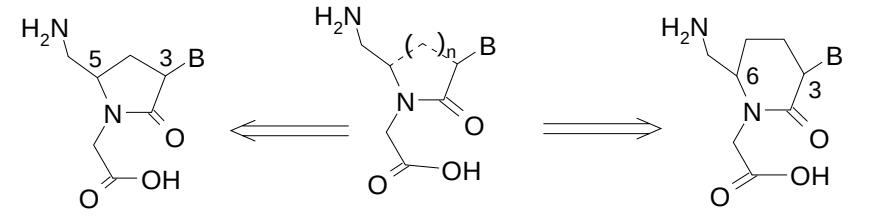


aminoprolyl PNA *pr*PNA **III** | aminoethylglycyl PNA *aeg*PNA **I** | aminopropylglycyl PNA *apg*PNA **II**

Figure 1 *aeg*PNA, backbone extended *apg*PNA and backbone constrained *pr*PNA

*apg*PNA monomer were less stable compared to unmodified *aeg*PNA. This decrease in stability for the chimeric *apg-aeg*PNA was ascribed to the increase in degree of conformational freedom in the backbone and consequent large entropy loss during complex formation. Conversely, such entropy loss could be reduced by using rigid PNA analogs with conformationally constrained backbones.[4] A favorable structural pre-organization of PNA may lead to the desired selective complex formation with the target DNA or RNA, provided that the enthalpic contributions are conserved with effective natural nucleobase recognition ability. In this context one of the very first examples was 4-aminoprolyl PNA (*pr*PNA, Figure 1a, **III**)[5] that evolved by simple introduction of a chemical bridge in *aeg*PNA between aminoethyl and glycyl segments. The chimeric *aeg-pr*PNAs could effect orientation-selective binding to DNA. But the homogeneous *pr*PNAs in any stereochemistry resulted in the structural pre-organization of backbone that was not capable of binding to either DNA or RNA.[4b]

In this class of conformationally constrained PNAs, Nielsen *et al* have reported pyrrolidinone PNA[6] in which PNA backbone is constrained by the introduction of the methylene bridge between aminoethyl and nucleobase linker segment of



b, n = 1 pyrrolidinonePNA

a *aeg*PNA **I**

c, n = 2 piperidinonePNA

Figure 2 a) *aeg*PNA b) PyrrolidinonePNA c) Piperidinone PNA

*aeg*PNA (Figure 2 a). The results from pyrrolidinone PNA (Figure 2 b) showed that PNA oligomers containing (3*S*,5*R*) isomer recognized RNA but its affinity was lower as compared to unmodified *aeg*PNA. This decrease in affinity could be because of unfavorable conformational constraints on the *aeg*PNA. Study of the six-membered analog of pyrrolidinone PNA resulted in the synthesis of piperidinone PNA (Figure 2 c) that introduced an ethylene bridge between the aminoethyl and nucleobase linker segment instead of methylene bridge.[7] Two (3*S*,6*R*) and (3*R*,6*R*) diastereomers with adenine as a nuclobase were synthesized. Incorporation of these modified units in *aeg*PNA resulted in chimeric PNAs that effected large decrease in duplex stability with RNA as well as DNA.

Another example in such efforts led to five-membered aminoethylprolylPNA (*aep*PNA) that was the result of introduction of a methylene bridge between nucleobase linker and glycyl segment in *aeg*PNA (Figure 3 b).[8] The *aep*-*aeg*PNA chimera with different stereochemistry and nucleobases were generally found to stabilize the complexes with DNA but the conferred stability varied with the individual nucleobase.[9] Recently, a six-membered *aep*PNA homolog,

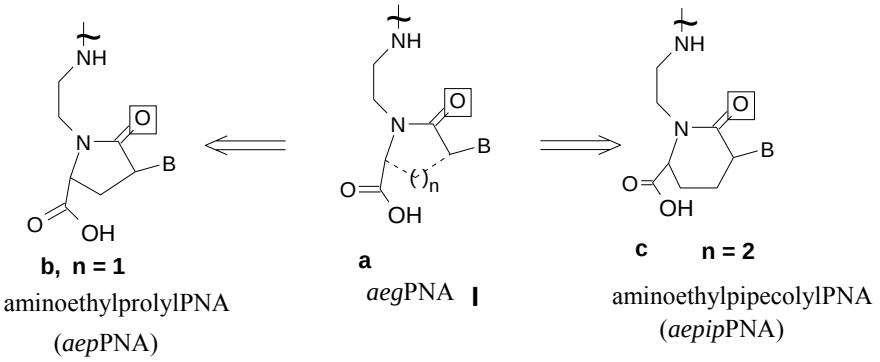


Figure 3: Bridged *aeg*PNAs b. *aep*PNA c. *aepip*PNA

aminoethylpipecolyl PNA (*aepip*PNA) has been reported[10] that replaces the methylene bridge in *aep*PNA by ethylene bridge (Figure 3 c). Both these modified PNAs could be positively charged at physiological pH. The chimera *aeg-aepip*PNA also form stable complexes with DNA. Further work is needed to understand the full potential of these modifications with sequence and nucleobase context.

3.1.1. Rationale for present work

The five-membered ring in the *pr*PNA (Figure 1, **III**) homooligomer seems to exert a conformational constraint and also could adversely affect inter-nucleobase distance-complementarity with target nucleic acids. The effective *pr*PNA conformation is probably incompetent for binding to the target DNA sequence. We envisaged the synthesis of six-membered homologs of *pr*PNA to release the structural strain in a five membered *pr*PNA as in the case of pyrrolidinone PNA and *aep*PNA discussed above. Two positional isomers 4-amino and 5-amino pipecolylPNA can be derived from *aeg*PNA or aminopropylglycyl PNA. The design of proposed monomers is such that the nucleobase attachment to the backbone is through acetyl linker. 5-aminopipecolyl PNA (5-*ampip*PNA) is a result of introduction of ethylene bridge between the β carbon atom of ethylene diamine and α'carbon atom of the glycine unit (Figure 4, **IV**). In case of 4-aminopipecolyl PNA (4-*ampip*PNA, Figure 4,**V**) methylene bridge is introduced between the γ carbon atom of the aminopropyl segment and α' carbon atom of the glycyl segment (Figure 4, **II**). The ethylene bridge in **IV** instead of methylene bridge in *pr*PNA (Figure 4, **III**) could be helpful in releasing the structural strain and the oligomers may be in a position to reorganize to bind to target nucleic acids. The methylene bridge in **V** could be useful in exerting requisite structural strain to the over-flexible

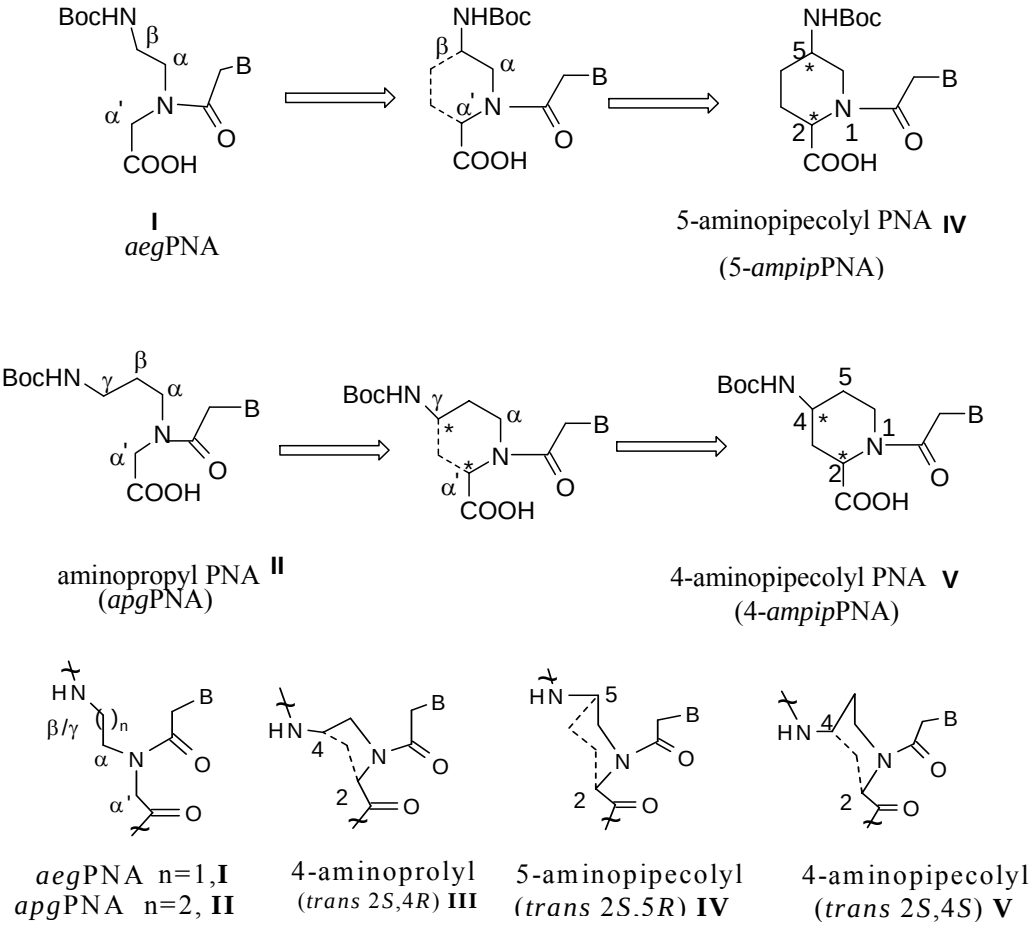


Figure 4 Derivatization of pipecolic acid monomer from *aeg*/*apg*PNA

aminopropyl segment in **II** that caused destabilization in aminoethylpropyl-*aeg*PNA:DNA complexes.

The primary knowledge of the stability of six membered *cis/trans* conformations suggested that a *trans*-5-aminopipecolyl six-membered monomer (Figure 4, **IV**) could be a better choice due to stable, favored diequatorial disposition of the backbone compared to *trans* axial-equatorial disposition in either 4-aminopipecolyl PNA (Figure 4, **V**) or 4-aminoprolyl (Figure 4, **III**) studied earlier.

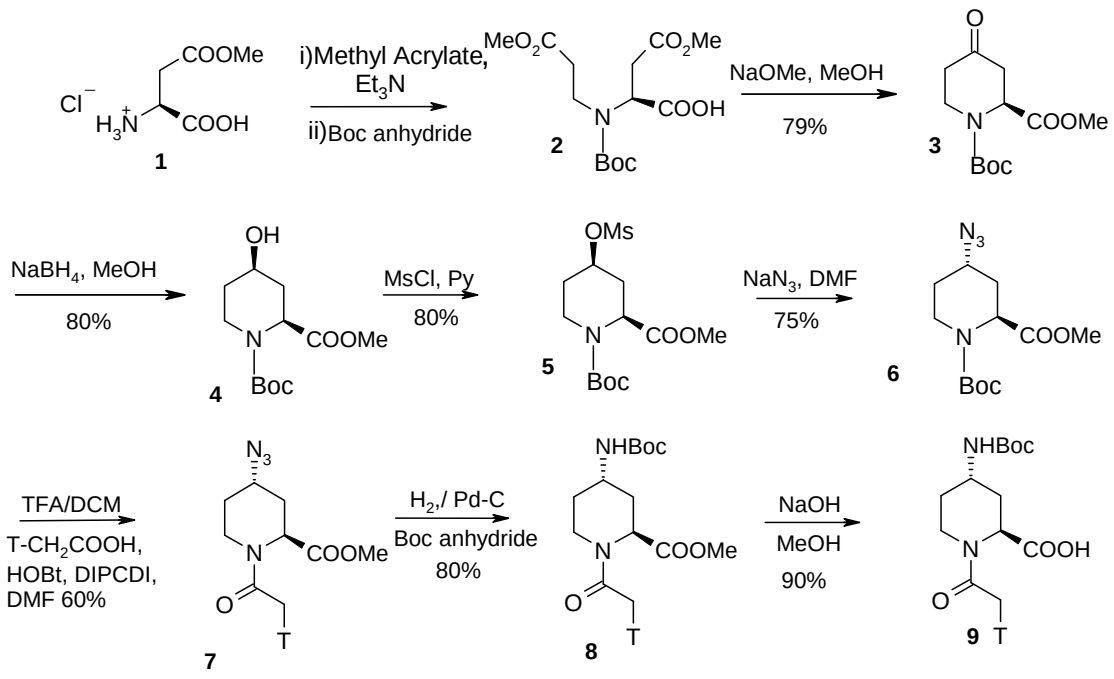
3.1.2. Objectives of the chapter are

1. Synthesis of *trans*–(2*S*,4*S*)-4-(*tert*.butyloxycarbonylamino)-*N*-1-(thymin-1-ylacetyl)-2-pipecolic acid for use in PNA synthesis.
2. Synthesis of *trans*–(2*S*,5*R*)-5-(*tert*.butyloxycarbonylamino)-*N*-1-(thymin-1-ylacetyl)-2-pipecolic acid for use in PNA synthesis.
3. Synthesis of the PNA oligomers using the aminoethyl glycyl (*aeg*) monomers and the modified pipecolyl PNA monomers by Merrifield Solid Phase Peptide Synthesis (SPPS) protocol.
4. Cleavage of the oligomers from the solid support, purification and characterization of the modified PNA oliogmers.
5. Complex formation with DNA/RNA using biophysical techniques such as UV-Tm measurements, CD and gel electrophoresis.

3.2. Synthesis of aminopipecolyl monomers

3.2.1. Synthesis of trans (2S,4S)-4-(tert.butyloxycarbonyl-amino)-N-(thymin-1-ylacetyl)-2-pipecolic acid ***9***

The chiral trans 4-aminopipecolyl PNA thymine monomer was synthesised from *cis* 4-hydroxy L-pipecolic acid, which in turn was synthesised from β-methyl-L-aspartate hydrochloride according to the reported procedure[11](Scheme1). Thus β-methyl-L-aspartate hydrochloride was treated with excess of triethyl amine and methyl acrylate followed by N-protection as its t-butylcarbamate derivative **2**. Formation of piperidine ring was accomplished by treating diester **2** with sodium methoxide. The β keto ester obtained was decarboxylated *in situ* by addition of H_2O and heating to get compound **3**.



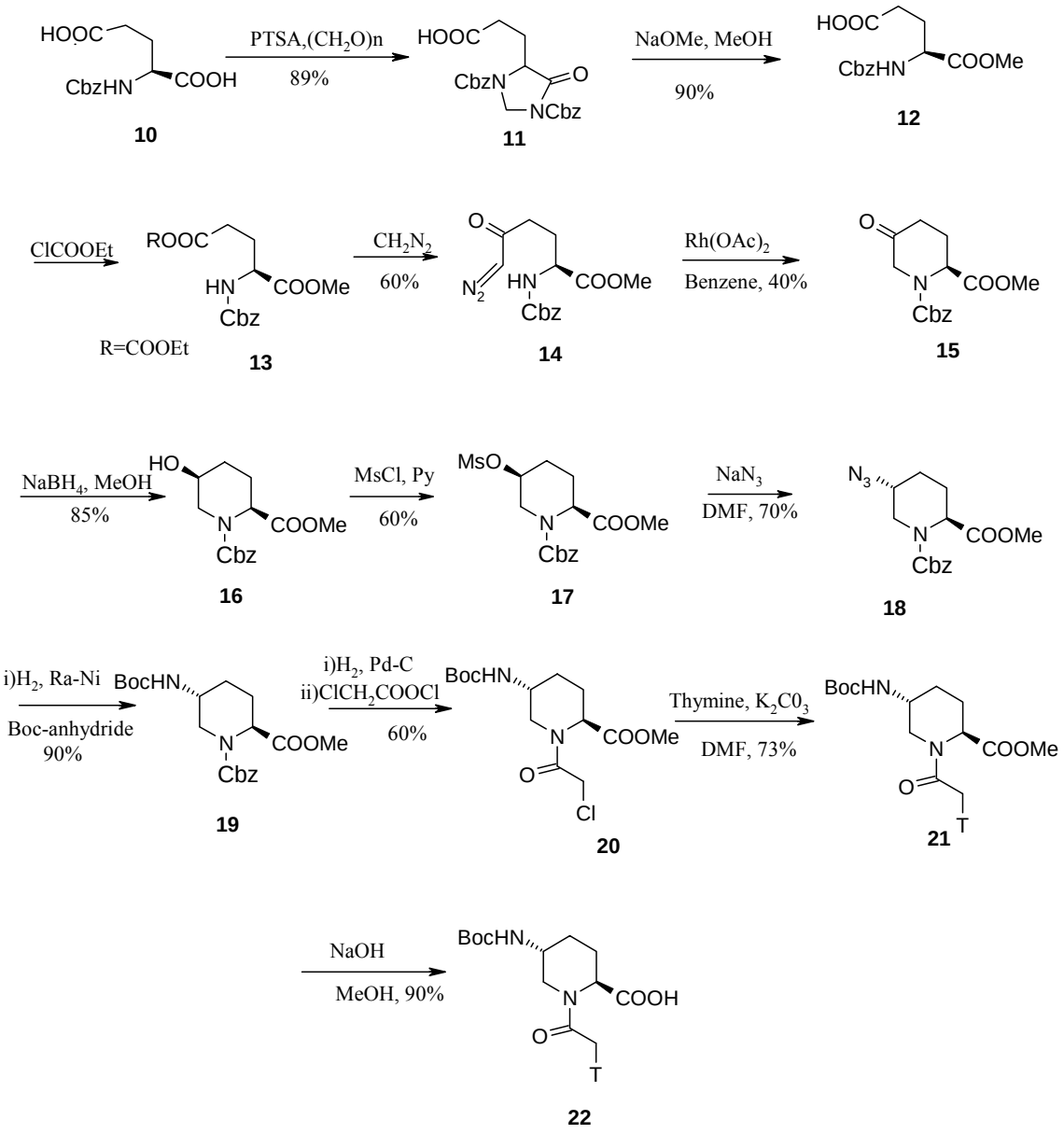
Scheme 1 Synthesis of *trans* (2*S*,4*S*)-4-(*tert.*butyloxycarbonylamino)-*N*-(thymin-1-ylacetyl)-2-pipecolic acid

The keto group in **3** was stereoselectively reduced to alcohol by sodium borohydride to get *cis* alcohol **4**. Complete conversion of keto group to alcohol was confirmed by ^{1}H NMR and IR spectroscopy. The 4-hydroxy group was converted to its mesyl derivative **5** in presence of mesyl chloride in pyridine. SN2 inversion at C4 stereocentre was achieved by treating compound **5** with excess of sodium azide. The piperidine ring nitrogen in **6** was deprotected with TFA/DCM (1:1) and the resulting free amine was coupled with thyminyl acetic acid in presence of HOBt and DIPCDI to get compound **7**. The structure was confirmed by the appearance of characteristic thymine peaks in the ^{1}H NMR spectrum. viz. 7.8 ppm (T-H_6), 1.9 ppm (T-CH_3). The azide group in **7** was reduced to amine by catalyatic hydrogenation, which was *in situ* converted to its carbamate derivative with di-*tert*-butyl dicarbonate. The ester

group in **8** was hydrolyzed with methanolic NaOH to get free acid **9**, which was used to build the PNA oligomers.

*3.2.2. Synthesis of trans (2S,5R)-5-N-(tert.butyloxycarbonylamino)-N-1-(thymin-1-ylacetyl)-2-pipecolic acid **22***

The chiral pipecolyl PNA thymine monomer *trans* (2*S*,5*R*)-5-(*tert*.butyloxycarbonylamino)-*N*-(thymin-1-ylacetyl)-2-pipecolic acid **22** was synthesized from *cis*-*N*-benzyloxycarbonyl-5*S*-hydroxy–2*S*-pipecolic acid methyl ester **16** which in turn was synthesized from L-glutamic acid[12] as shown in Scheme 2. *N*1-Cbz protection in **10** was obtained in quantitative yield by reacting glutamic acid with benzyl chloroformate. Selective protection of α carboxylic acid was achieved via the oxazolidinone **11** in presence of paraformaldehyde and catalytic PTSA in 89% yield. Nucleophilic ring opening of oxazolidinone in **11** was achieved by treatment with two equivalents of sodium methoxide in refluxing benzene, which led to the formation of the α ester **12**. Treatment of **12** with ethyl chloroformate, followed by reaction of the mixed anhydride with diazomethane generated the diazoketone **14**. Characteristic peaks appearing at 2300 cm^{-1} in the IR spectrum confirmed the formation of diazo adduct. Direct conversion of compound **14** to protected 5-oxopipecolic acid **15** was achieved by carbene insertion into the N-H bond by using rhodium acetate as a catalyst. The regio and stereoselective reduction of 5-keto group to alcohol **16** was achieved by treatment with sodium borohydride in methanol. The compound **16** was characterized using ^1H NMR and IR spectroscopy. Further the *cis* stereochemistry of compound **16** was confirmed by lactonization which was brought about readily by brief reaction of **16** with acetic anhydride. The hydroxy group in compound **16** was converted to its mesyl derivative **17** by



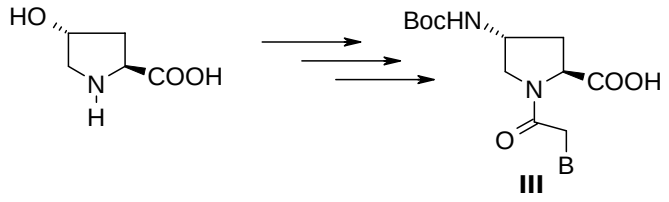
Scheme 2 Synthesis of *trans* (2*S*,5*R*)-5-(*tert*.butyloxycarbonylamino)-*N*-(thymin-1ylacetyl)-2-pipecolic acid

treatment with mesyl chloride in pyridine. Compound **17** on treatment with sodium azide in DMF, gave azide **18** with the inversion of configuration at C-5. Characteristic azide signal at 2110 cm $^{-1}$ was observed in the IR spectrum of **18**. The azide group in **18** was selectively reduced by catalytic hydrogenation to amine using

Ra-Ni in methanol. The resulting amine was converted *in situ* to the corresponding tert.butylcarbamate derivative **19** in presence of Boc-anhydride in 90% yield. The N1-nitrogen benzyloxycarbamate protection in **19** was removed by catalytic hydrogenation using Pd-C. The resulting free amine was subjected to acylation with chloroacetyl chloride to get chloro derivative **20**. *N*-1 alkylation of thymine was effected by treatment with chloro derivative **20** in presence of K_2CO_3 to yield **21**. The methyl ester in **21** was hydrolyzed using sodium hydroxide in aqueous methanol to give *trans* (2*S*,5*R*)-5-(*tert*.butyloxycarbonylamino)-*N*-(thymin-1-ylacetyl)-2-pipecolic acid **22**, which was used to synthesize the PNA oligomers **14**-**17** listed in Table 2.

3.2.3. Synthesis of (2S,4R)-N-(tert. butyloxycarbonyl)-N-1-(thymin-1yl) proline III

Synthesis of (2*S*,4*R*)-*N*-(*tert*. butyloxycarbonyl)-*N*-1-(thymin-1yl) proline was achieved from L-*trans* 4-hydroxy proline as described earlier (scheme 3).[5]



III

Scheme 3 Synthesis of (2*S*,4*R*)-*N*-(*tert*. butyloxycarbonyl)-*N*-1-(thymin-1yl) proline from L-*trans* hydroxy proline.

3.3. Solid phase PNA synthesis

The *aeg*PNA oligomers and the chimeric oligomers comprising *aeg*PNA and modified 4/5*ampip*PNAs (**IV** and **V**) and *pr*PNA **II** were synthesized by standard solid phase peptide synthesis protocols as described in chapter 2 (Section 2.3).[13] PNA oligomer synthesis was carried out from the 'C' to the 'N' terminus using

monomers with free carboxylic acid functions and amino functions protected as Boc derivatives that are cleavable with TFA at the beginning of every cycle. The solid support used was Merrifield resin[14] that was derivatized with β-alanine using the cesium salt method[15] (please see chapter 2). (2*S*,4*R*)-*trans*-4-aminoprolylPNA unit was also introduced in the PNA oligomers for comparison study. The effect of these monomers on triplex-forming ability was tested by synthesizing polypyrimidine decamers. The homothymine PNA oligomers are known to complex with the complementary DNA A_{10} oligomer in a 2:1 PNA:DNA stoichiometry.[16] The control *aeg*PNA T_{10} oligomer **31** was synthesized following the Boc protection strategy. The chiral modified PNA thymine monomer units (**III**, **IV** and **V**) were incorporated into the *aeg*PNA oligomers at predetermined positions and the decamer sequences synthesized are described in Table 1. The capping step at the end of each coupling

Table-1 Polypyrimidine PNA chimera and complementary DNA sequences**.**

Entry	PNA/DNA	
23	H- T T T T T T T T T **t** $_{(2S,4R)}$–β-ala-OH	One prolyl PNA unit at C terminus
24	H- T T T T T T T T T **t** $_{(2S,4S)}$–β-ala-OH	One 4-ampipPNA unit at C terminus
25	H- T T T T T T T T T **t** $_{(2S,5R)}$–β-ala-OH	One 5-ampipPNA unit at C terminus
26	H -**t** $_{(2S,4S)}$ T T T T T T T T T –β-ala-OH	One 4-ampipPNA unit at N terminus
27	H -**t** $_{(2S,5R)}$ T T T T T T T T T –β-ala-OH	One 5-ampipPNA unit at N terminus
28	H- T T T **t** $_{(2S,4R)}$ T T T T T **t** $_{(2S,4R)}$ –β-ala-OH	Two prPNA units
29	H- T T T **t** $_{(2S,4S)}$ T T T T T **t** $_{(2S,4S)}$ –β-ala-OH	Two 4–ampipPNA units
30	H- T T T **t** $_{(2S,5R)}$ T T T T T **t** $_{(2S,5R)}$ –β-ala-OH	Two 5–ampipPNA units
31	H- T T T T T T T T T T –β-ala-OH	Control *aeg*PNA sequence
32	5'- G C A A A A A A A A A A C G – 3'	Complementary DNA sequence
33	5'- G C A A A **T** A A A A A A C G – 3'	Mismatch DNA sequence

t= *pr*PNA or 4/5-*ampip*PNA**,** T = *aeg*PNA **T** =mismatched base in DNA

cycle was not seemed necessary, as the coupling reaction was monitored manually to be complete with a very high coupling efficiency. Homopyrimidine PNA oligomers were prepared in order to reveal the binding of the modified PNA units in a triple helix mode. With a view to explore the induced effects of pipecolyl PNA and prolyl PNA monomeric unit, a single unit was introduced at either N or C terminus (Table 1, PNA **23-27**). To study the effective additivity of chiral monomers on the stability and selectivity of DNA complexation, two units of pipecolyl and prolyl PNAs were introduced within the sequence (Table 1, PNA **28-30**).

To find out the effect of chirality and conformational rigidity on duplex stability, mix purine-pyrimidine sequences with chiral 4-*ampip* and 5-*ampip* units were synthesized (Table 2, PNA **35-36**). The homogeneous backbone modified PNAs were not synthesized as such rigid structured backbone PNAs reported earlier were unable to reorganize in a way that is conducive for binding to nucleic acids.[4b] Sequence **34** is the unmodified *aeg*PNA and was used as a control.

Table 2 Mixed base purine-pyrimidine PNA sequences and complementary DNA in *p* and *ap* orientation.

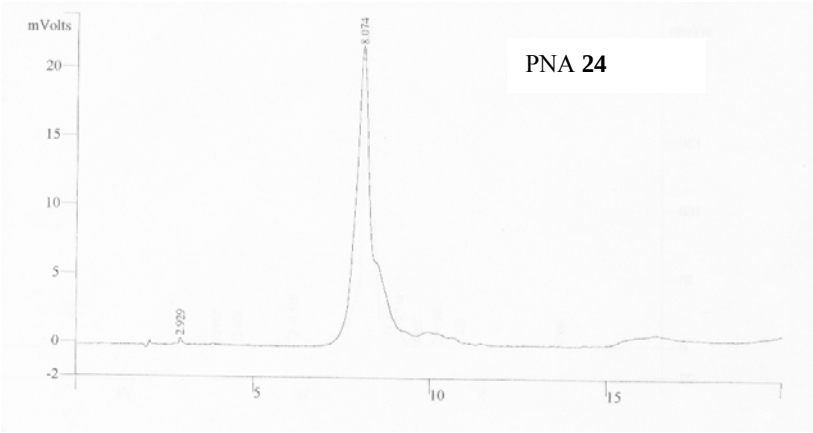
Sequences	PNA	
PNA-**34**	H-GTA GAT CAC T-–β-ala-OH	*aeg*PNA control sequence
PNA-**35**	H- GTA GA **t** $_{(2S,4S)}$CAC T–β-ala-OH	One 4-ampipPNA unit
PNA-**36**	H- GTA GA **t** $_{(2S,5R)}$CAC T–β-ala-OH	One 5-ampipPNA unit
DNA- **37**	5’- AGT GAT CTA C-3’ (*ap*)	*ap*DNA to PNAs **34-36**
DNA- **38**	5’ – CAT CTA GTG A-3’(*p*)	*p*DNA to PNAs **34-36**
RNA- **39**	5’- AGU GAU CUA C-3’ (*ap*)	*ap*RNA to PNAs **34-36**

3.4. Cleavage of the oligomers from the solid support

The synthesized PNA oligomers were cleaved from the solid support using the TFMSA-TFA cleavage procedure.[20] This yielded oligomers bearing β-alanine with free carboxylic acids at their 'C' termini.

3.5. Purification of the PNA Oligomers

Preliminary purification of the PNA oligomers to remove shorter sequences and low molecular weight impurities was achieved by gel filtration through Sephadex G25. These were subsequently purified by FPLC on a semi-preparative C8 reverse-phase column using an ascending gradient of acetonitrile in water containing 0.1% TFA. The purity of the oligomers was rechecked by HPLC on a C18 reverse-phase column and confirmed by MALDI-TOF mass spectroscopy. Some representative HPLC profiles and mass spectra are shown in Figures 5 and 6 and respectively.



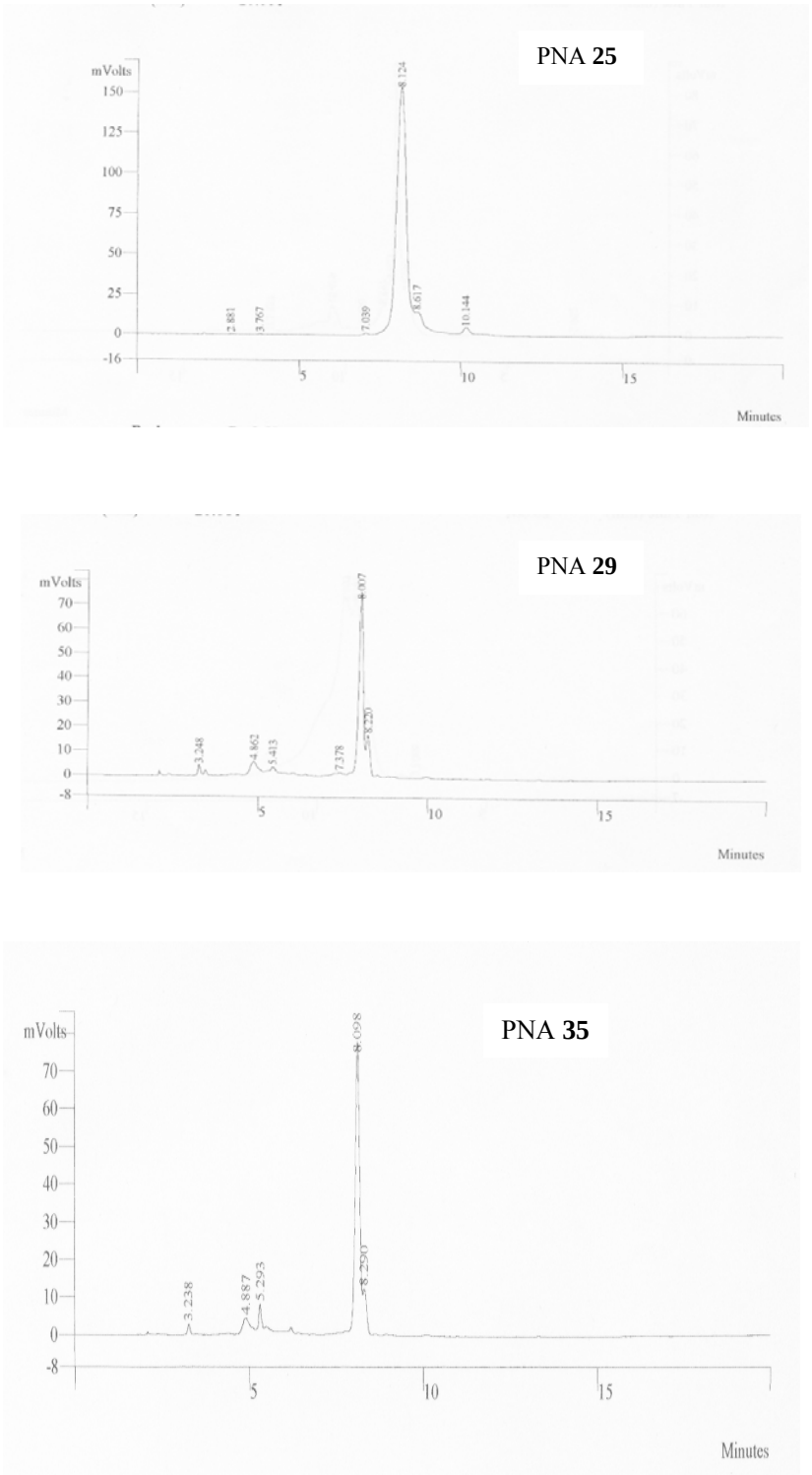
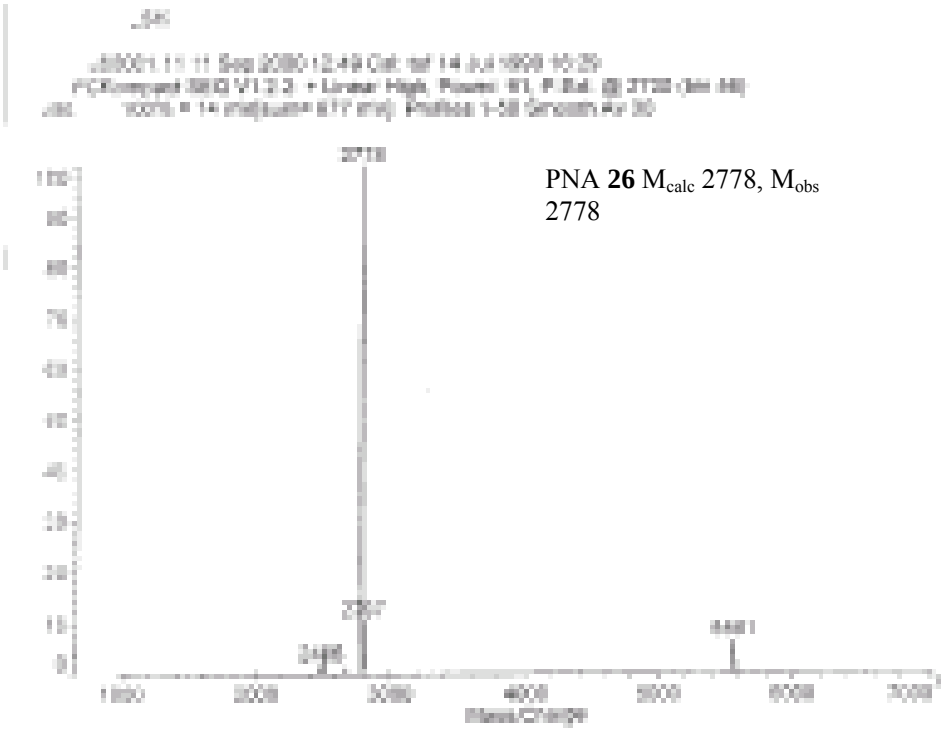


Figure 5 HPLC profiles of oligomers



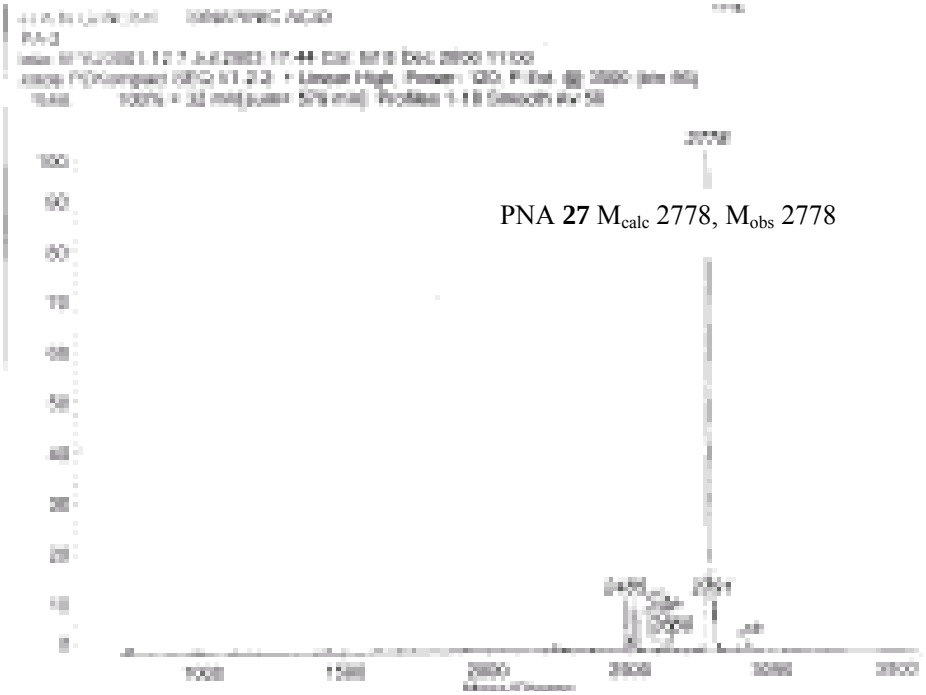


Figure 6 MALDI-TOF spectra for PNA oligomers **26** and **27**

3.6. Synthesis of oligonucleotides

The oligonucleotides were synthesized on a ABI gene synthesizer using the standard β-cyanoethyl phosphoramidite chemistry on a CPG solid support, followed by ammonia deprotection and cleavage.[17] The purity of the oligonucleotides was verified by reverse phase HPLC on a C18 column and was found to be >96%. Consequently, these oligonucleotides were used for the biophysical studies as such, without further purification.

3.7. Results and Discussion

3.7.1. 4/5-aminopip-aegPNA$_2$:DNA triplexes

CD studies

The achiral PNA backbone does not show any significant CD spectrum. However, single-stranded (*ss*) PNAs with modified chiral backbone when complexed with complementary DNA sequences are capable of exhibiting characteristic CD signals. A preferred handedness in the complex may be induced

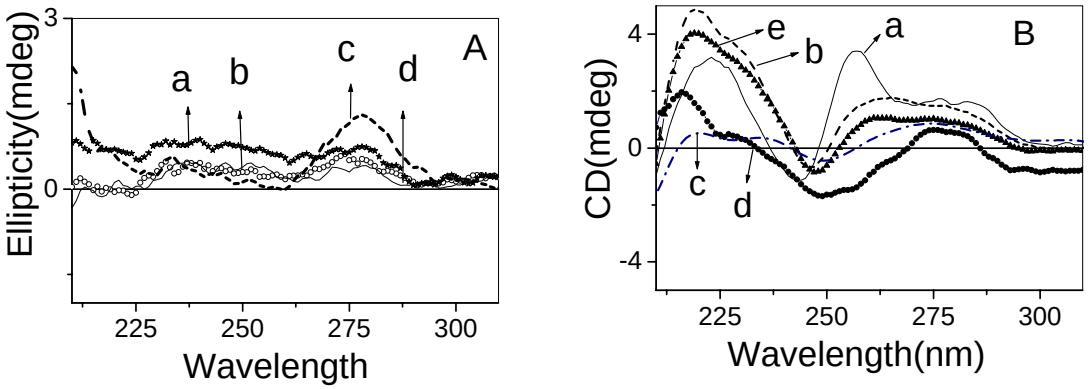


Figure 7 CD profile of **A,** *ss*PNA a) **28**, b) **26**, c) **27 d) 31 B,** PNA$_2$:DNA triplexes a) **27:32**, b) **26:32**, c) **31:32**, d) **30:32**, e) **29:32**

by introducing chiral centers within the PNA strand. The process was described as a "seeding of chirality, beginning from the terminal base pair and migrating through the stack of the bases".[18] The presence of chiral monomers reorganizes the single stranded PNAs and also enhance the helical preferences of the PNA:DNA complexes.

As PNA without pipecolyl units is CD transparent, the observed bands at 260-280 nm in single stranded pipecolyl-PNA arise due to asymmetry induced in base stacking by the pipecolyl units (figure 7A). Upon complex formation with complementary DNA, CD positive maxima at 220-230, 260-280 nm and negative minima at 246 nm, crossover points at 240-250 nm were observed. The positive bands in the region of 260-280 nm seen in the CD spectra of complexes, are characteristic of PNA_2:DNA triplex (Figure 7B).

The circular dichroism spectroscopy was used to study the binding stoichiometry of 4/5aminopipecolyl PNA:DNA complexes. Various stoichiometric mixtures of

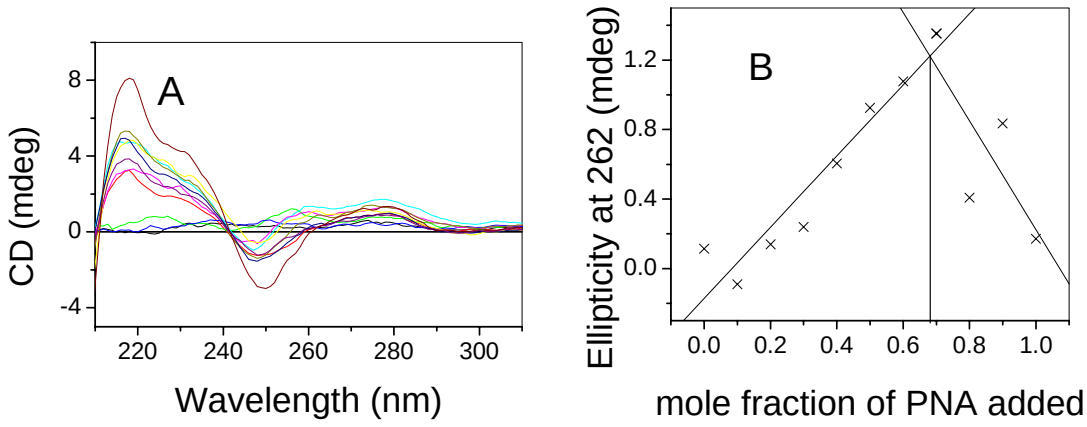


Figure 8 A) CD spectra of molar mixtures of PNA **30** and DNA **32** showing isodichroic points. B) CD mixing curve for PNA **30** and DNA **32** mixtures in the various molar proportions.

PNA **27** and DNA **32** were made with relative molar ratios of PNA:DNA strands of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 10:0 all at same strand concentration of 2μM in sodium cacodylate (10mM, pH 7.3). The samples with the individual strands were annealed and the Figure 8A shows the CD spectra for these mixtures. The plot of ellipticity at 262 nm versus mole fraction of PNA added gave a inflection point at 0.67 indicating PNA_2:DNA binding stoichiometry (Figure 8B). These spectra exhibit positive maxima at 257, 277 and 285 nm, a negative minimum at 244 nm and crossover points at 232 and 247 nm. A positive band in the region of 255 to 260 nm as seen in the CD spectra of the complexes which is characteristic of the poly(dA)$(PNA\text{-}T_8)_2$ complex further confirms that 5-*ampip*-*aeg*PNA binds to DNA in 2:1 stoichiometry. Similar set of experiment was carried out with PNA **29** and DNA **32**, which also showed 2:1 binding stoichiometry.

UV-Tm studies

The UV-temperature studies on prolyl, 4-*ampip* and 5-*ampip*-*aeg* PNA_2:DNA triplexes to understand the effect on thermal stability in terms of these different chiral modifications were undertaken. The normalized absorbance or % hyperchromicity versus temperature plots derived from the UV melting data indicated a single transition indicative of the simultaneous dissociation of the two PNA strands from the DNA in the PNA_2:DNA complex (Figure 9). Table 3 and Figure 9 summarizes the Tm data obtained for UV melting experiments of various PNA_2:DNA hybrids. A single (2*S*,4*S*) prolyl unit when present at C terminus is less tolerated in the sequence (Table 3, Figure 9A, **23:32**, ΔTm= -3°C) but much better than (2*S*,4*S*) 4-*ampip*PNA (Table 3, figure 9B, **24:32**, ΔTm –12°C) and (2*S*,5*R*) 5-*ampip*PNA (Table 3, Figure 9C, **25:32,** ΔTm= -6°C) compared to the unmodified

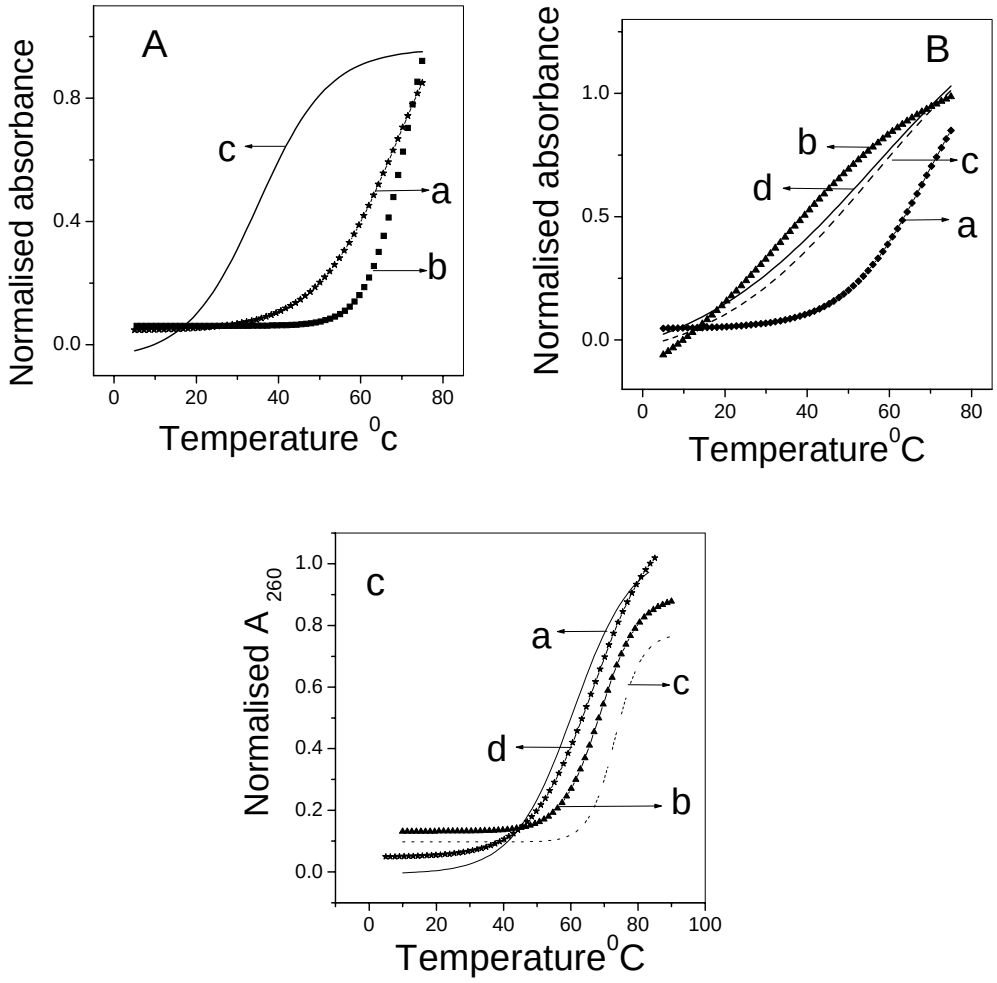


Figure 9 Uv-Tm profile of **A,** a) **31:32**, b) **23:32**, c) **28:32 B**, a) **31:32**, b) **29:32**, c) **24:32**, d) **26:32 C**, a) **25:32,** b) **27:32**, c) **30:32**, d) **31:32**
Buffer: 10mM Sodium cacodylate, 100Mm NaCl, 0.0Mm EDTA. pH 7.3 Melting experiments were performed with a PNA:DNA strand concentration of 1μM each. The concentration were calculated on the basis of the absorbance at 260nm using molar extinction coefficient of A, G, C and T for DNA.

*aeg*PNA (**31:32**). Interestingly, when the aminopipecolyl units were present at N-terminus, (2*S*,4*S*) unit in PNA **26** caused a destabilization of the complex (Table 3, **26:32**, ΔTm= -11°C) while the (2*S*,5*R*) unit had only a minimal effect in increasing the stability of the complex with DNA (Table 3, **27:32**, ΔTm= + 1°C). Increasing the number of modified units to two led to favorable complexation with DNA for the

Table 3 UV-Tm data for *pr*, 4/5-*ampip*-*aeg* PNA_2:DNA complexes.

PNA sequences with Modified monomer unit **t**	(2*S*,4*R*) **t** = *pr*PNA		(2*S*,4*S*) **t** = 4-*ampip*PNA		(2*S*,5*R*) **t** = 5-*ampip*PNA	
	Triplex	Tm°C	Triplex	Tm°C	Triplex	Tm°C
H-TTTTTTTTT**t**-β-ala-OH	**23:32**	64	**24:32**	55	**25:32**	61
H-**t**TTTTTTTTT-β-ala-OH	-	-	**26:32**	56	**27:32**	68
H-TTT**t**TTTTT**t**-β-ala-OH	**28:32**	35	**29:32**	34	**30:32**	78
H-TTTTTTTTTT-β-ala-OH	67 (**31:32**)					
mismatch	-	-	**29:33**	30	**30:33**	69

Experiments were repeated at least thrice and the Tm values were obtained from the peaks in the first derivative plots. Tm for mismatch control **31:33** = 61°C

(2*S*,5*R*) 5-*ampip*PNA (Table 3, **30:32,** ΔTm = +11°C) was observed, but highly unfavoured complexation for the 4-*ampip*PNA (Table 3, **29:32,** ΔTm = - 33°C) and *pr*PNA (**28:32,** ΔTm = - 34°C). These results indicate that 5-*ampip* unit seems to be assisting *aeg*PNA in binding to complementary DNA in triplex mode. In comparison, both 4-*ampip*PNA and *pr*PNA units in the oligomer were detrimental to the PNA_2:DNA complex stability.

Mismatch studies

PNA oligomers **29** and **30** with mismatched DNA **33** at the site complementary to the pipecolyl unit led to the anticipated decrease in Tm values of the corresponding triplexes as compared to the fully complementary triplex by 4°C and 9°C respectively. These decreases in Tm are similar to those observed when a single mismatch is introduced in an unmodified PNA-DNA hybrid (**31:33**, ΔTm -6°C). This indicates that the modified units in fact recognize the complementary DNA base and strongly support that the 4 and 5-ampipecolyl monomers participate in

Watson-Crick/HG hydrogen bonding interactions and contribute to the overall sequence-specific recognition and binding to the complementary DNA.

Gel shift assay

The formation of stable 4/5-*ampip-aeg*PNA complexes with complementary DNA was also established by gel shift assay. The PNA's modified with single unit of 4/5-aminopipecolyl PNA and the control PNA were individually treated with complementary DNA and complexation was monitored by non-denaturing gel

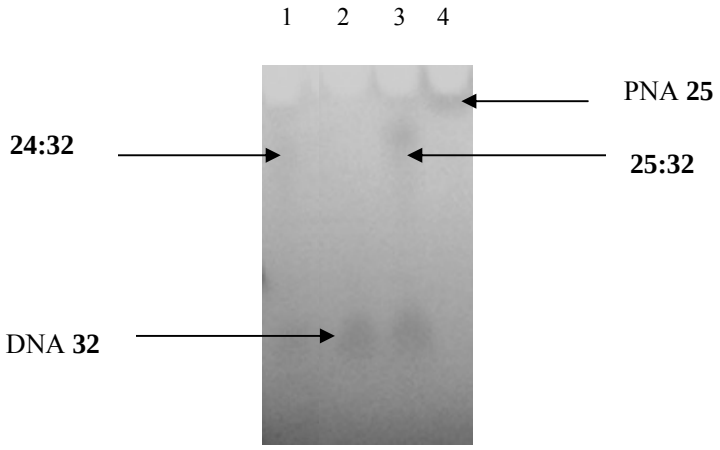


Figure 10 15% polyacrlamide Gel Electrophoresis of pipecolylPNA:DNA complexes. Lane1: triplex, **24:32** Lane 2: DNA *ss***32** Lane3: Triplex **25:32** Lane 4 : *ss* PNA**25**

electrophoresis at 10°C. The single stranded DNA, PNA and PNA:DNA complexes were visualized on a fluorescent TLC background and the results are shown in figure 10 The PNA:DNA complexes derived from 4/5-*ampip-aeg*PNA with single modification at C terminus were significantly retarded , the single stranded PNA did not move out of the well (Figure10, lanes 4, *ss*PNA **25**). These results were in accordance with the data obtained from UV–thermal-melting studies with these

oligomers which also underlined the specificity of the hydrogen bonding between the complementary base pairs.

3.7 2. 4/5-ampip-aegPNA:DNA/RNA Duplexes

UV-Tm studies

The introduction of chiral pipecolyl PNA monomers into PNA oligomer was envisaged to impart specific directionality in DNA/PNA binding and hence differentiate between parallel and antiparallel modes of binding. Table 4 summarizes the Tm data obtained for UV melting experiments of PNA-DNA hybrids designed to form either parallel or antiparallel duplexes. The establishment of correct duplexes were indicated by sigmoidal transitions and confirmed by peaks in first derivative plots. The unmodified PNA **34** with achiral β-alanine at C-terminus formed duplex with complementary DNA in both parallel and antiparallel orientations, with parallel mode slightly destabilizing than the antiparallel duplex (Table 4, Figure 11, **34:38**, ΔTm = -1°C). The results in table indicate that all the 4/5-aminopipecolyl-*aeg*PNA

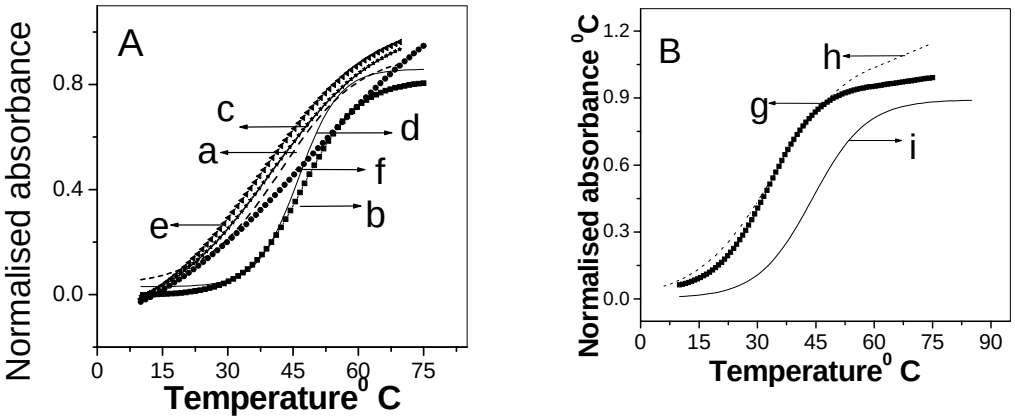


Figure 11 UV-melting profiles of **A**: PNA:DNA a) **36:37**, b) **34:37**, c) **36:38**, d) **34:38**, e) **35:38**, f) **35:37 B**: PNA:RNA, g) **35:39**, h) **36:39**, i) **34:39.**

Table 4 UV-Tm data for the pipecolyl PNA mixed base PNA:DNA duplexes.

PNA	DNA(*ap*)		DNA(*p*)	
	Complex	Tm°C	Complex	Tm°C
H-GTAGATCAC T-–β-ala-OH	**34:37**	47	**34:38**	46
H- GTAGA **t** $_{(2S,4S)}$ CAC T–β-ala-OH	**35:37**	48	**35:38**	37
H- GTAGA **t** $_{(2S,5R)}$ CAC T–β-ala-OH	**36:37**	43	**36:38**	40

oligomers bind preferentially in *ap* mode with complementary DNA. 4-aminoprolyl unit in the internal position of mixed purine-pyrimidine sequences was found to stabilize complexes in *ap* DNA orientation.[4b] This trend is also observed with *aeg*PNAs tethered with lysine[19] at the C-terminus. A single (2*S*,4*S*) pipecolyl unit (**35:38**), is seen to destabilize the parallel duplex by 9°C whereas antiparallel duplex is slightly stabilized (**35:37,** ΔTm =1°C, Figure 11A, Table 4). Introduction of single 5-aminopipecolyl unit in the middle of an *ap*PNA:DNA duplex, (**36:37**) effected greater than that observed for the 4-aminopipecolyl PNA.

Binding properties of the 4/5-aminopipecolyl-*aeg*PNAs with (*ap*) RNA **39** was also studied considering the differential structural requirement for PNA:RNA compared to PNA:DNA complexes. Both 4/5-aminopipecolyl-*aeg*PNA decamers **35** and **36** hybridized to RNA, but with a large decrease in Tm compared to the

Table 5 UV-Tm data for the pipecolyl PNA mixed base PNA:RNA duplexes.

PNA	RNA (*ap*)	
	Complex	Tm°C
H-GTAGAT CAC T-–β-ala-OH	**34:39**	43
H- GTAGA **t** $_{(2S,4S)}$ CAC T–β-ala-OH	**35:39**	38
H- GTAGA **t** $_{(2S,5R)}$ CAC T–β-ala-OH	**36:39**	32

unmodified PNA **34** (Table5, Figure 11B). So in duplex both the modifications are showing preferential binding to DNA over RNA.

Gel shift assay

The formation of PNA:*ap*DNA duplexes was also confirmed by the nondenaturing gel-shift assay (Figure 12). Lane 2 shows the significant retardation in gel by duplex form between PNA **36** and DNA **37**.The mobility retardation shifts in triplex as compared to duplex is caused due to increased molecular weight, resulting in a higher retardation of the PNA_2: DNA triplex.

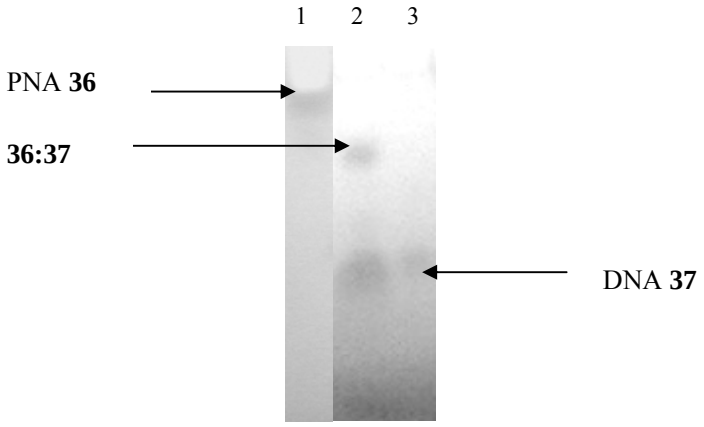


Figure 12 15% polyacrlamide Gel Electrophoresis of pipecolylPNA:DNA complexes. Lane 1: *ss* PNA **36** Lane 2 Duplex **36:37** Lane 3: DNA **37**

3.8. Summary

New conformationally restricted 4-*ampip*PNA and 5-*ampip*PNA thymine monomers have been synthesized. These monomers were introduced in the *aeg*PNA backbone using solid phase peptide synthesis protocol. The % hyperchromicity vs temperature plots derived from the UV melting data indicated a single transition characteristic of PNA_2:DNA melting, wherein both PNA strands dissociate from the DNA strands

simultaneously, in a single step. UV-Tm experiments indicated that (2*S*,5*R*) pipecolyl unit to be quite efficient in binding to complementary DNA in triplex mode. The differences in the stabilization of duplex versus triplex modes with 4-amino and 5-amino pipecolyl units could arise from the difference in their structural features that fine tune the internucleobase geometries. It is interesting to note that the constrained *apg*PNA analogue, 4-aminopipecolyl (2*S*,4*S*) in **35** reverts the destabilization caused by the open chain *apg*PNA unit in the same sequence.

These results lead us to believe that a biased pre-organized conformation capable of maintaining the correct internucleobase-distance could be particularly important in order to preserve specific base pairing. Structural differences in PNA:DNA, PNA:RNA and PNA_2:DNA reflected in the control of *aeg*PNA complex formation with DNA/RNA sequences through elegant use of substitution pattern of six membered pipecolic acid derivatives.

The further work requires studies of these modifications in *aeg*PNA in sequence context, with other stereochemistry of the backbone and also other natural nucleobases. The synthesis of homogeneous pip backbone for duplex/triplex recognition would also be interesting.

3.9. Experimental

(2*S*,4*R*)-Hydroxy-*N*-1-(*tert.*butyloxycarbonylamino)-pipecolic acid methyl ester 4

^{1}H NMR $CHCl_3$-d δ : 5.5-4.5 (m, 1H), 4.0 (m, 1H, H4), 3.88 (s, 3H, COOMe), 3.11

(m, 1H), 2.21 (broad d, J= 14.3 Hz, 1H), 1.79 (ddd, J_1= 3.6Hz, J_2= 6.8Hz, J_3= 14.3 Hz, 1H), 1.50-1.60 (m, 1H,), 1.47 (s, 9H,$C(CH_3)_3$)

^{13}C NMR $CDCl_3$ δ = 172.7, 156.3, 80.9, 61.3, 53.4, 51.3, 35.8, 32.4, 28.5

(2*S*,4*R*)-4-*O*-methylsulphonyl-*N*-1-(*tert.*butyloxycarbonylamino)-pipecolic acid methyl ester 5

To a stirred, cooled solution of compound **4** (1.5g, 5.1mmol) in dry pyridine (10ml) was added methane sulphonyl chloride (0.7ml, 6.11mmol) drop wise. Stirring was continued for 2 h when TLC indicated complete conversion of starting material. The solvent was removed under vacuum, and residue was taken in water and extracted with ethyl acetate (2x15ml), pooled the organic layers, washed with water (2x10ml) followed by brine (1x10ml) and dried over sodium sulphate. Evaporation of organic layer yielded a crude product, which was then purified by silica gel column chromatography. (Yield, 70%)

H^1 NMR $CHCl_3$-d δ : 5.08-4.2 (m, 2H, H4, H2), 4.12-3.8 (m, 1H, H6), 3.79 (s, 3H, OCH_3), 3.0 (s, 3H, Oms), 2.5-2 (m, 2H, H3), 2-1.62, (m, 4H, H5), 1.44, (s, 9H, $C(CH_3)_3$)

^{13}C NMR $CHCl_3$-d δ : 171.2, 151.3, 80.6, 74.6, 51.4, 51.2, 38.5, 31, 29.8, 28.3

ESIMS calculated for $C_{13}H_{23}NO_7S$ (M+) 337, Obs. 338 (M+1)

(2*S*,4*S*)-4-azido-*N*-1-(*tert.*butyloxycarbonylamino)-pipecolic acid methyl ester 6

To the compound **5** (0.74g, 1.99mmol) in dry DMF was added NaN_3 (0.76g, 11.6mmol) The mixture was stirred at 60°C for 12 h. When TLC indicated the complete conversion of starting material, DMF was removed under vacuum, water

(10ml) was added to mixture and extracted with EtoAc (3x15ml). Organic layers were pooled, washed with water (1x10ml) and brine (1x10ml), concentrated to give crude product which was then purified by silica gel column chromatography (Yield 0.56g, 90%)

$[\alpha]^{24}_{D}$ -20 (c .008,MeOH)

H^1 NMR $CHCl_3$-d δ : 5.02-4.9 (m, 2H, H2, H6), 3.72 (s, 3H, COOMe), 2.85 (s, 2H), 2.48-1.58 (m, 4H, H3, H5), 1.44 (s, 9H, $C(CH_3)_3$)

^{13}C NMR $CHCl_3$-d δ : 173.5, 151.2, 80.7, 55.4, 54.3, 40.5, 31.9, 30, 27.9.

ESIMS calculated for $C_{15}H_{18}N_4O_4$ calc 318, Obs. 319(M+1)

(2*S*,4*S*)-4-azido-*N*-1-(thymin-ylacetyl)-pipecolic acid methyl ester 7

Compound **6** (1g, 2.2mmol) was taken in 50%TFA/DCM (5mL), and stirred at ambient temperature for 30 min. The solvent was removed under reduced pressure and residue was taken in water and neutralized with 10% aq. $NaHCO_3$ followed by repeated extraction with ethyl acetate (10mlx 2) The combined organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed to obtain the free amine. The residual oil was desiccated over KOH for 12h. The free amine (4.3mmol), HOBt (0.54g, 4.3mmol) and thymine acetic acid (0.86g, 4.8mmol) were dissolved in dry DMF(10ml). The solution was cooled in an ice bath, DCC (0.9g, 4.5 mmol) was added and the reaction mixture was stirred for 1h in an ice bath and then for 3 h at room temperature.[20] The reaction mixture was then concentrated to half its volume, water was added before extracting with ether (10ml x 2). The pale yellow residue after removal of ether was purified on a silica gel column using pet ether-ethylacetate gradient as eluent to obtain as a white solid.

^{1}H NMR $CHCl_3$-d δ : 9.5 (s, 1H, Thy), 5.3 (s, 1H, NHBOC), 4.3-4.2 (m, 2H, H2), 3.7 (m, 3H, COOMe), 3.4-3.2 (m, 2H, H6), 2.45-2.38 (m, 1H, H3), 2.0-1.8 (m, 1H, H5),1.8 (s, 3H, Thy–CH3), 1.7-1.4, (m, 2H, H3, H5)

^{13}C NMR $CHCl_3$-d δ : 171.9, 169.1, 162.6, 156.2, 137.9, 134.7, 155.6, 155.2, 80.2, 54.6, 53.5, 52, 45.3, 41.0, 36.1, 34.2, 28.1, 12.5

(2*S*,4*S*)-4-(*tert.*butyloxycarbonyl)-*N*-1-(thymin-ylacetyl)-pipecolic acid methyl ester 8

Compound **7** (1g, 3.4mmol) in metahnol was hydrogenated under pressure (40psi) using 10% Pd-C (100mg) as a catalyst for 2h which was converted *insitu* to the corresponding tert.butylcarbamate by Boc-anhydride. The product was purified by silica gel column chromatography to obtain as a gum.

H^{1} NMR $CHCl_3$-d δ : 7.8 (s, 1H, Thy-H6), 5.22 (bs, 1H, BOC-NH), 3.9 (m, 1H, H6), 3.68 (s, 3H, COOMe), 3.4-3.2 (m, 1H, CH_2-Thy), 3.2-2.91 (m, 1H, CH_2-Thy), 2.5-2.38 (m, 1H, H3), 2.0-1.8 (m, 1H, H5),1.9 (s, 3H, Thy-CH_3), 2.10-1.7, (m, 2H, H3, H5), 1.41 (s, 9H, $C(CH_3)_3$)

^{13}C NMR $CHCl_3$-d δ : 171.9 (COOMe), 169.1(NCO), 161.1 (BOCCO), 160.2 (Thy-C4), 156.9 (Thy-C2), 137.7 (Thy-C6),1 34.6, 155.2, 80.2 ($C(CH3)_3$, 60.7, 54.6, 53.5 (C4), 52 (CH_2-Thy), 45.3 (C2), 41.0, 36.1(C5), 34.2 (C3), 28.1 ($C(CH_3)_3$, 13.9,12.5 (Thy-CH_3)

(2*S*,4*S*)-4-(*tert*-butyloxycarbonyl)-*N*-1-(thymin-ylacetyl)-pipecolic acid 9

Compound **8** (0.2g, 0.15mmol) was dissolved in 2N NaOH in methanol:water (1ml) and stirred for 20min. The pH of the solution was adjusted to 2 by addition of cation

exchange resin, which was then filtered off. The filtrate was then evaporated to get the product as white foam (190mg, 95%).

^{1}H NMR, D2O δ (200MZ): 7.3 (s, 1H), 7.0 (s, 1H), 5.4(m, 1H), 5.0 (m, 1H), 4.7 (m, 1H), 4.3 (m, 1H), 3.8 (s, 3H), 3.5 (m, 2H), 2.6 (m, 1H), 2.1(m, 1H), 2.0 (s, 3H), 1.5 (s, 9H)

ESIMS calc 410, M obs 411

_N_- benzyloxycarbonyl L-glutamic acid 10

Glutamic acid (10g, 14.7mmol) was dissolved in water (50ml), and to it $NaHCO_3$ (37g, 47mmol) was added slowly while stirring the reaction mixture. Benzyl choloroformate (30ml, 50% solution in toluene, 21mmol) was added in one portion and reaction mixture was stirred for 16 h when TLC indicated complete disappearance of starting material. Toluene was removed under vacuum and mixture was diluted with water (20ml) and then extracted with ether (3x10ml). Ethereal layers were discarded and the aqueous layer was brought to pH 2 with conc. HCl. Product was extracted from aq. layer with ethyl acetate (5x15ml), pooled the organic layers, washed with water (2x10ml) followed by brine (1x10ml) and dried over sodium sulphate. Evaporation of organic layer yielded compound (Yield, 95%)

_N_-benzyloxycarbonyl-4-oxazolidine L-glutamic acid 11

A mixture of *N*-benzyloxycarbonyl-L-glutamic acid **10** (17g, 281), paraformaldehyde (3g), PTSA (0.6g) in benzene (500ml) was refluxed for 5h liberated water being removed azeotropically by means of a Dean-Stark distilling apparatus. The benzene solution was washed with water and extracted with 5%

$NaHCO_3$. pH of the aqueous layer was adjusted to 2 with conc. HCl on ice cooling and extracted with ethyl acetate (2x15ml), pooled the organic layers, washed with water (2x10ml) followed by brine (1x10ml) and dried over sodium sulphate. Evaporation of organic layer yielded a crude product, which was then purified by silica gel column chromatography. (Yield, 90%)

^{1}H NMR $CHCl_3$-d δ : 10.84 (bs, 1H, COOH), 7.32 (s, 5H, Ar), 5.52 (m, 1H, 2H), 5.2-5.11 (dd, 2H, CH2-Ar), 2.49-2.45 (m, 2H, H3), 2.32-2.18 (m, 2H, H4)

***N*-benzyloxycarbonyl-L-glutamic acid-2-methyl ester 12**

Compound **11** (8g, 27mmol) was taken in dry methanol and stirred at 0C for 20min. To this sodium metal (1.9g, 81mmol) was added slowly while maintaining the temperature. The reaction mixture was further stirred for 1 h when TLC indicated complete conversion of starting material. Solvent was evaporated to dryness and residue was taken in water (20ml). pH of this aqueous layer was adjusted to 2 with $KHSO_4$, extracted with ethyl acetate (2x15ml), pooled the organic layers, washed with water (2x10ml) followed by brine (1x10ml) and dried over sodium sulphate. Evaporation of organic layer yielded a crude product, which was then purified by silica gel column chromatography (Yield, 90%).

^{1}H NMR $CHCl_3$-d δ : 7.4 (s, 5H, Ar), 5.2 (s, 2H, CH_2-Ar), 4.5-4.3 (m, 1H, H2), 3.49(s, 3H, COOMe), 2.5 (m, 2H, H3), 2-2.3 (m, 2H, H4)

***N*-benzyloxycarbonylamino-6-diazo-5-oxoL-glutamic acid-2-methyl ester 14**

Compound 12 (3.42g, 10.9mmol) was taken in dry THF and stirred at 5°C for 30 min. To this triethyl amine (1.22ml, 12mmol) and ethylchloroformate (1.31ml,

12mmol) were added drop wise. After 30 min. excess of diazomethane was added carefully to the reaction mixture till effervescence siezes. When TLC indicated complete consumption of starting material, evaporation of organic layer yielded a crude product, which was then purified by silica gel column chromatography. (Yield, 60%)

$[\alpha]^{24}_{D}$ -9.5 (c .004,MeOH)

^{1}H NMR $CHCl_3$-d δ : 7.4 (s, 5H, Ar), 5.24 (s, 4H, CH_2-Ar, CHN_2), 4.5-4.3 (m, 1H, H2), 3.7 (m, 3H, COOMe), 2.7-1.7 (m, 4H, H3, H4)

5-oxo-*N*-benzyloxycarbonyl-L-pipecolic acid methyl ester 15

A concentrated solution of diazoketone **14** (1.85g, 5.79mmol) in benzene (15ml) was added dropwise to a solution of rhodium diacetate (24mg, 5.44mmol) at reflux in benzene (10ml). The solution was heated for a further 30 min. Evaporation of solvent followed by column chromatography gave as a oil.(Yield 70%)

$[\alpha]^{24}_{D}$ +11.8 (c=1.62, $CHCl_3$)

^{1}H NMR $CHCl_3$-d δ : 7.33 (s, 5H, Ar), 5.4-5.0 (m, 2H, CH_2-Ar), 4.7 (m, 1H, H2), 4.3-4.0 (m,3H, H6,H5), 3.4 (s, 3H, COOMe), 2.6-2.3 (m, 2H, H4)

^{13}C NMR $CHCl_3$-d δ : for the major rotamer δ 198.3 (C5), 170.4 (COOMe), 155.8, 135.6, 128.3, 128.0 (Ar) 68.0 (CH_2-Ar), 62.4 (C6), 54.9(C3), 33.2 (C4), 21.8 (OCH_3)

IR (neat) cm^{-1} 1737 and others.

***cis*(2*S*,5*S*)-5-Hydroxy-*N*-1-(benzyloxycarbonyl)-L-pipecolic acid-2-methyl ester 16**

A cooled (0 °C) solution of ketone **15** (160 mg, 0.48 mmol) in MeOH (20 mL) was treated with $NaBH_4$ (18 mg, 0.48 mmol). After being stirred for 1 h, the reaction mixture was concentrated *in vacuo* and the residue was dissolved in ethyl acetate (20 mL). The organic solution was washed with dil HCl and brine, dried over Na_2SO_4 and concentrated. The resulting residue was purified by column chromatography to give **16** as an oil: (150 mg, 93% yield)

$[\alpha]^{24}_D$ +25.5 (c .02,MeOH)

ESIMS calculated for $C_{15}H_{19}O_5N$ 293, Found 294(M+1)

^{1}HNMR $CHCl_3$-d δ : 7.29 (s, 6H, Ar), 5.67 (m,1H), 5.04 (s, 2H,CH_2-Ar), 4.27 (m, 3H,H6,H5), 4.5-3.86 (m, 1H, H2), 3.59 (s, 3H, COOMe), 2.34 (m, 2H, H3), 1.84-1.63 (m, 2H. H4)

^{13}C NMR $CHCl_3$-d δ : for the major rotamer δ 169.6, 155.7, 136.0, 128.2, 127.9, 81.4, 67.7, 60.8, 55.4, 52.6, 27.7, 24.8, 13.7

IR (neat) cm^{-1} 1737, 1716

(2*S*,5*S*)-5-*O*-methylsulphonyl-*N*-1-(benzyloxycarbonyl)-pipecolic acid methyl ester 17

To a stirred, cooled solution of compound **16** (1.5g, 5.1mmol) in dry pyridine (10ml) was added methane sulphonyl chloride (0.7ml, 6.11mmol) drop wise. Stirring was continued for 2 h when TLC indicated complete conversion of starting material. The solvent was removed under vacuum, and residue was taken in water and extracted with ethyl acetate (2x15ml), pooled the organic layers, washed with water (2x10ml) followed by brine (1x10ml) and dried over sodium sulphate. Evaporation of organic

layer yielded a crude product, which was then purified by silica gel column chromatography. (Yield, 70%)

$[\alpha]^{24}_{D}$ -27.5 (c .004,MeOH)

ESIMS calculated for $C_{16}H_{21}NO_7S$ (M+) 371, Obs. 372(M+1)

H^1 NMR $CHCl_3$-d δ : 7.35 (s, 5H, Ar), 5.2 (d, 2H, C$\underline{H_2}$-Ar), 5-4.75 (m, 1H, H5), 4.7-4.3 (m, H6), 3.7 (m, 3H,COOMe), 3.1 (m, 3H, oms), 2.5-1.4 (m, 5H, H3,H4,H2)

^{13}C NMR $CHCl_3$-d δ : 170 ($\underline{C}$OOMe), 158 (N-$\underline{CO}$O), 137.1 136.0, 128.4, 128.1, 127.8 (Ar), 67.8 (CH_2-Ar), 52.3 (C5), 44.9 (C6), 38.6 (C2), 27.9 (C4), 20 (C3)

(2*S*,5*R*)-5-azido-*N*-1-(benzyloxycarbonyl)-pipecolic acid methyl ester 18

To the compound **17** (0.74g, 1.99mmkol) in dry DMF was added NaN_3 (0.76g, 11.6mmol). The mixture was stirred at 60°C for 12 h. When TLC indicated the complete conversion of starting material, DMF was removed under vacuum, water (10ml) was added to mixture and extracted with EtOAc (3x15ml). Organic layers were pooled, washed with water (1x10ml) and brine (1x10ml), concentrated to give crude product which was then purified by silica gel column chromatogralhy (Yield 0.56g, 90%).

$[\alpha]^{24}_{D}$ -20 (c .008,MeOH)

ESIMS calculated for $C_{15}H_{18}N_4O_4$ calc 318, Obs. 319(M+1)

H^1 NMR $CHCl_3$-d δ : 7.35 (s, 5H, Ar), 5.2 (s, 2H, CH_2-Ar), 5.1-4.8 (m, 1H, H5), 4.35-4.1 (m, 1H, H6), 3.9-3.6 (m, 4H, COOMe, H2), 3.49-3.2 (m, 1H, H6), 2.2-2 (m, 1H, H3), 1.9-1.5 (m, 2H, H4)

(2*S*,5*R*)-*5*-*N*-(*tert.*butyloxycarbonyl)-*N*-1-(benzyloxycarbonyl)-pipecolic acid methyl ester 19

Compound **18** (0 .74g, 2.32 mmol) was taken in ethyl acetate. To this Ra/Ni (0.5 ml) and ditert. butyl dicarbonate (0.55ml, 2.52mmol) was added. This mixture was subjected to hydrogenation for 2h at pressure of 40 psi in a Parr- hydrogenation apparatus. Ra/Ni suspension was removed by filtration through celite. The filtrate was concentrated to give crude product, which was then purified by column chromatography. (Yield 0.9 g, 90%)

^{1}H NMR $CHCl_3$-d δ :7.3 (s, 5H, Ar), 5.15 (s, 2H, CH_2-Ar), 5.0-4.75 (s, 1H, H5), 4.2-4 (m, 1H, H6), 3.7 (s, 4H, COOMe, H6'), 3.35-2.9 (m, 1H, H2), 2.49-1.6 (m, 4H, H3, H4), 1.4 (s, 9H, $C(CH3)_3$)

^{13}C NMR $CHCl_3$-d δ : 171.5 (COOCH3), 159.8 (N-COO), 155.2 (BOCCO), 136.5, 128.6, 128.1, 127.7(Ar), 79.4 ($C(CH_3)_3$, 67.6 CH_2-Ar), 52.3 (C5), 46.0 (C6), 44.2 (C2), 28.4 ($C(CH_3)_3$, 25.9 (C4), 21.1(C3).

ESIMS calculated for $C_{20}H_{28}N_2O_6$ (M+) 392, Obs. 419(M+17)

(2*S*,5*R*)-5-*N*-(*tert.*butoxycarbonyl)-*N*-1-(chloroacetyl)-pipecolic acid methyl ester 20

Compound **20** (0.61g, 1.55mmol) was dissolved in methanol and 10%Pd-C (90mg) was added in to it. This mixture was subjected to hydrogenation at 60 Psi for 8h. The catalyst was filtered off over celite and filtrate was evaporated under vacuum to obtain free amine. Free amine (0.19g, 0.73mmol) was taken in 10% Na_2CO_3 solution in dioxane:water (1:1) and cooled in an ice bath. Chloroacetyl chloride (0.16ml, 1.4mmol) was added with vigorous stirring. pH was maintained around 9

during reaction with addition of more Na_2CO_3. After 1 h, reaction mixture was concentrated to remove the dioxane. The product was extracted with EtOAc and was purified by column chromatography. (Yield 0.11g, 60%)

ESIMS calculated for $C_{14}H_{23}N_5O_3Cl$ (M+) 334, Obs. 335(M+1)

^{1}HNMR $CHCl_3$-d δ :4.9-4.8 (d, 1H, H5), 4.2-3.8 (m, 2H, $NCOCH_2$), 3.7 (s, 3H, COOMe), 3 (s, 1H, H2), 2.4-2 (m, 2H, H6), 1.9-1.51 (m, 3H, H3, H4), 1.4 (s, 10H, $(C(CH_3)_3$, H4)

^{13}C NMR CHCl3-d δ :170.9 ($COOCH_3$), 170.4 (COONH), 167.0 (Boc<u>C</u>O), 155.08, 80.34, 79.5 ($\underline{C}(CH_3)_3$, 74.9 (CH_2), 74.6, 52.2 (C5), 46.7 (C6), 45.3, 44.1 (C2), 40.6, 38.6, 28, 27.8 ($C(\underline{C}H_3)_3$), 24.8 (C4), 20.5 (C3)

(2*S*,5*R*)-*5*-(*tert*.butyloxycarbonyl)-*N*-1-(thymin-ylacetyl)-pipecolic acid methyl ester 21

Compound **20** (0.15g, 0.49mmol) was stirred with anhydrous K_2CO_3 (0.186 g, 1.34mmol), thymine (0.17g, 1.34mmol) in dry DMF at 60^{o}C for 5 h. When TLC indicated complete disappearance of starting material, DMF was evaporated and crude product was loaded on column (Yield 110mg, 73%)

H^1 NMR $CHCl_3$-d δ : 9.2 (s, 1H), 7.3 (s, 1H, Thy-H6), 5.5 (d, 1H, Boc-NH), 4.9-4.8 (m, 1H, CH2-thy), 4.3 –4.2 (m, 1H, CH2-Thy), 4.1-3.8 (m, 2H, H6), 3.75 (s, 3H, COOMe), 3.6-3.49 (m, 1H, H2), 2-1.6 (m, 8H, Thy-CH_3, H3, H4), 1.45 (s, 9H, $C(CH_3)_3$)

^{13}C NMR $CHCl_3$-d δ:170.85 (COOCH3), 167.13 (NCOO), 164.71 ($ThyC_4$), 155.74 ($ThyC_2$), 140.94 ($ThyC_6$), 52.50, 54.14 (C5), 48.18 (CH_2-Thy), 46.12 (C6), 44.64 (C_2), 28.33 ($C(CH_3)_3$, 25.32 (C4), 21 (C3), 12.22 (Thy-CH_3)

ESIMS calculated for $C_{19}H_{28}N_4O_7$ (M+) 424, Obs. 425(M+1)

(2*S*,5*R*)-*5*-(*N-tert.*butyloxycarbonyl)-*N*-(thymin-ylacetyl)-pipecolic acid 22

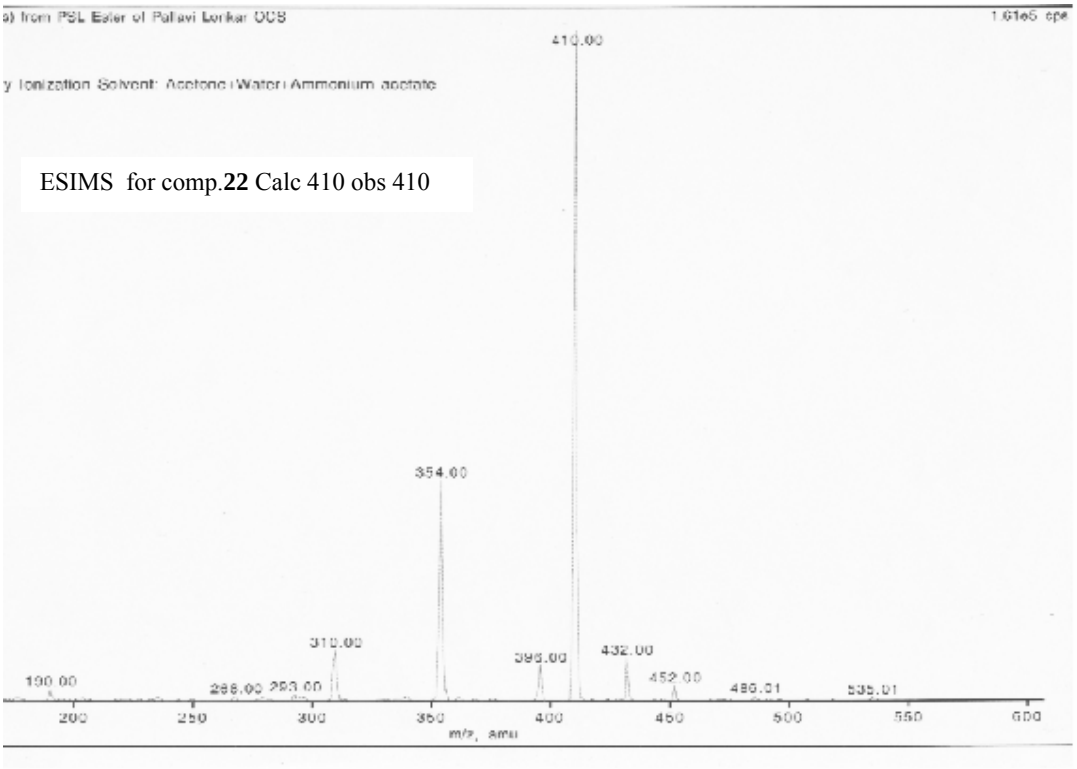
Compound **21** (120mg, 0.15mmol) was dissolved in 2N NaOH in methanol:water (1ml) and stirred for 20min. The pH of the solution was adjusted to 2 by addition of cation exchange resin, which was then filtered off. The filtrate was then evaporated to get the product as white foam (100mg, 95%)

H^1 NMR D_2O (200MZ): 7.29 (s, 1H, Thy-H6), 5.13 (bs, 1H, Boc-NH), 4.9-4.78 (m, 1H, CH_2-Thy), 4.64 (s, 1H, CH_2-Thy), 4.61-4.3 (m, 1H, H6), 3.85-3.73 (m, 1H, H4), 3.70-3.4 (m, 2H,H2), 2.46-1.88 (m, 2H,H5), 1.82 (s, 3H, H3), 1.79-1.65 (m, 2H, H4), 1.38 (s, 9H, $C(CH_3)_3$),

^{13}C NMR D_2O :170.62 (COOH), 169.99 (NCOO), 157.43 (Thy-C4), 153.63 (Thy-C2), 143 (Thy-C6), 47.75 (C6), 47.28, 46.55 (C2), 29.53 ($C(CH_3)_3$, 27.34 (C3),11.10 (Thy-CH_3)

3.10. Appendix

♦



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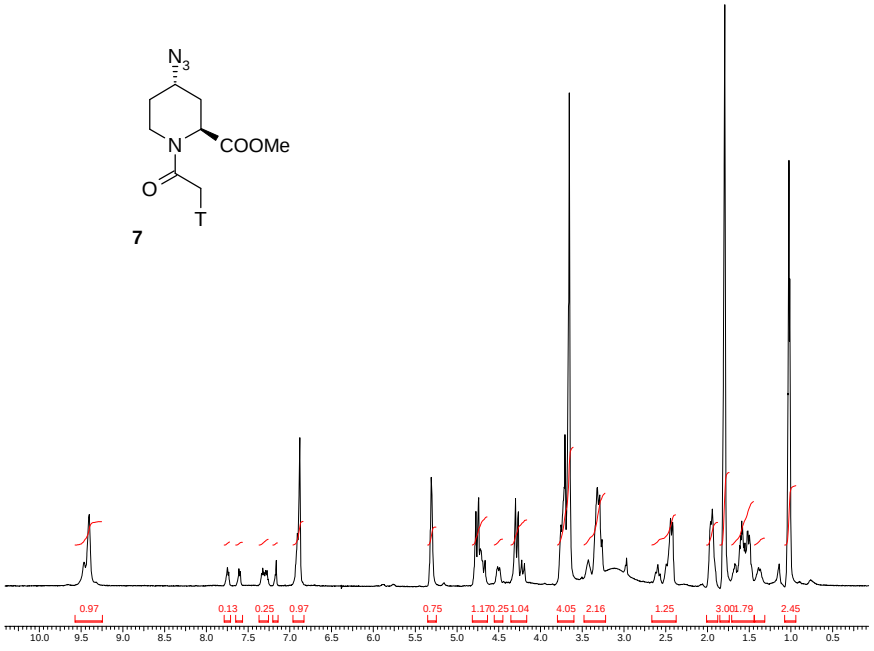
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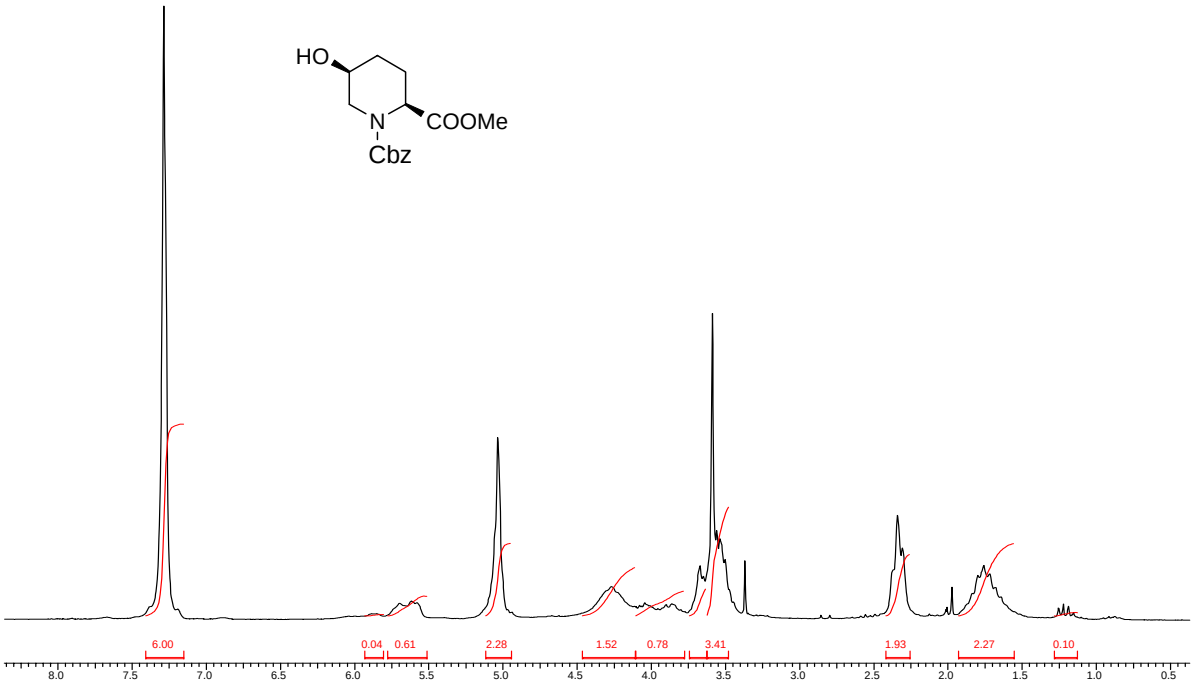
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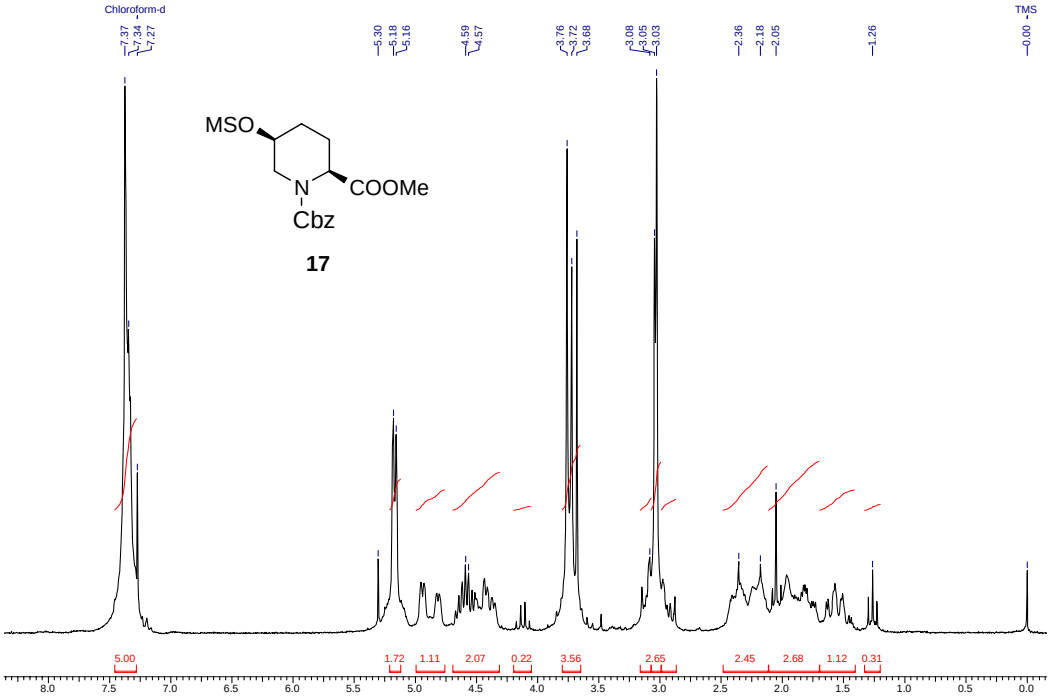
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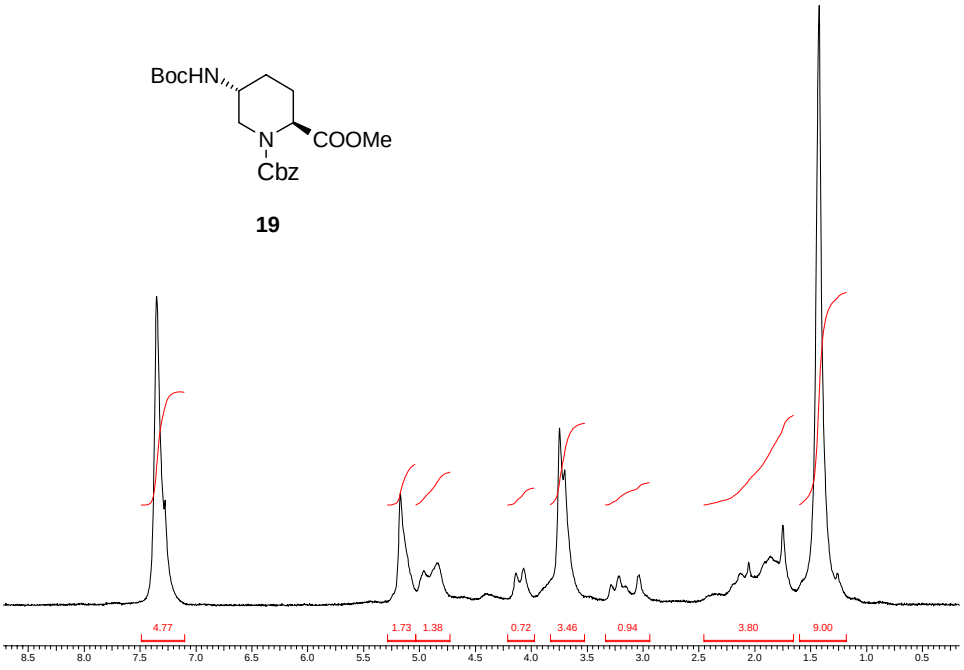
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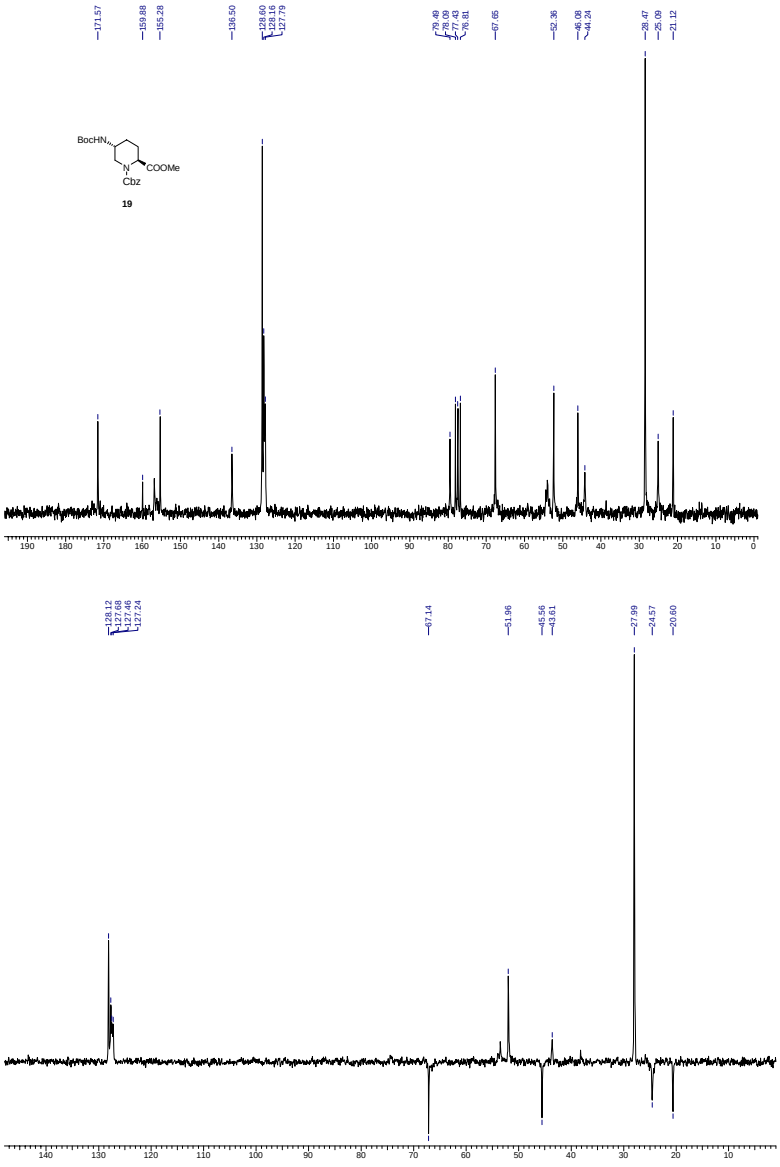
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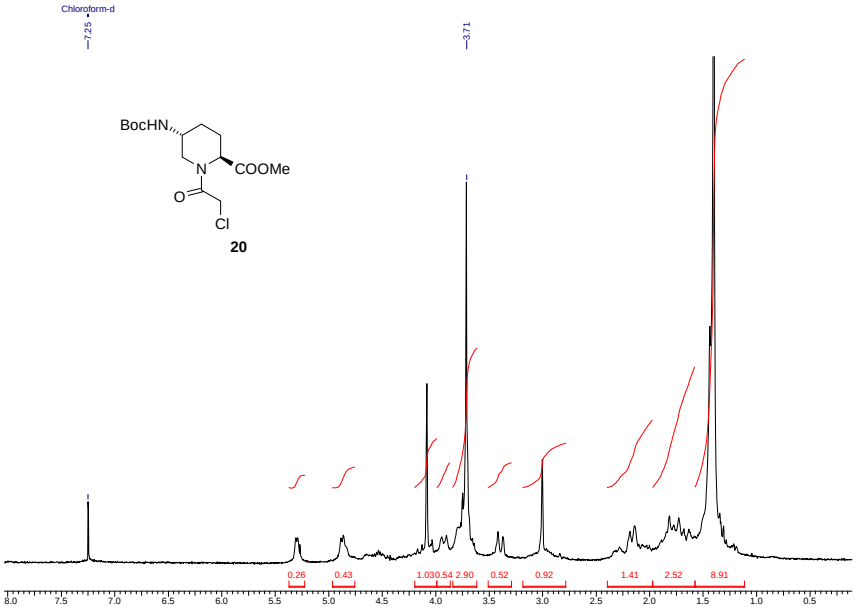
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0.52
0.92
1.41
2.52
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8.0
7.5
7.0
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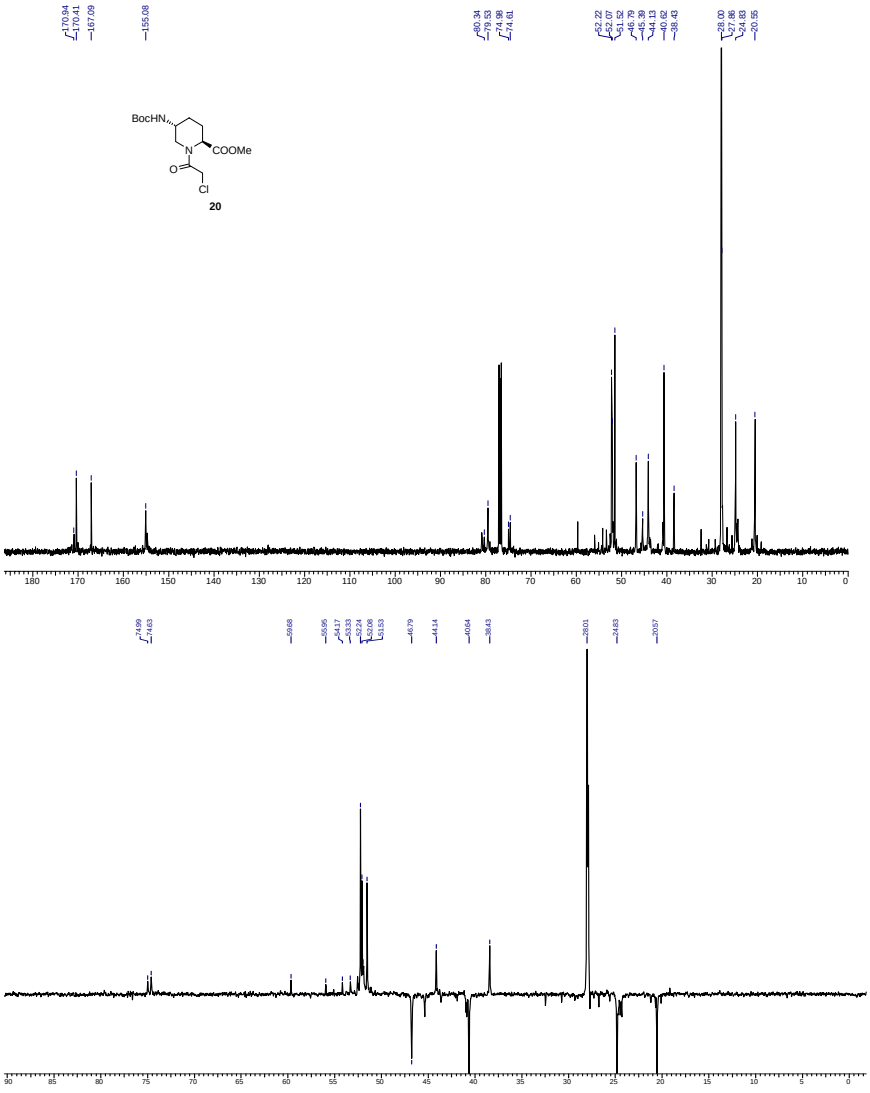
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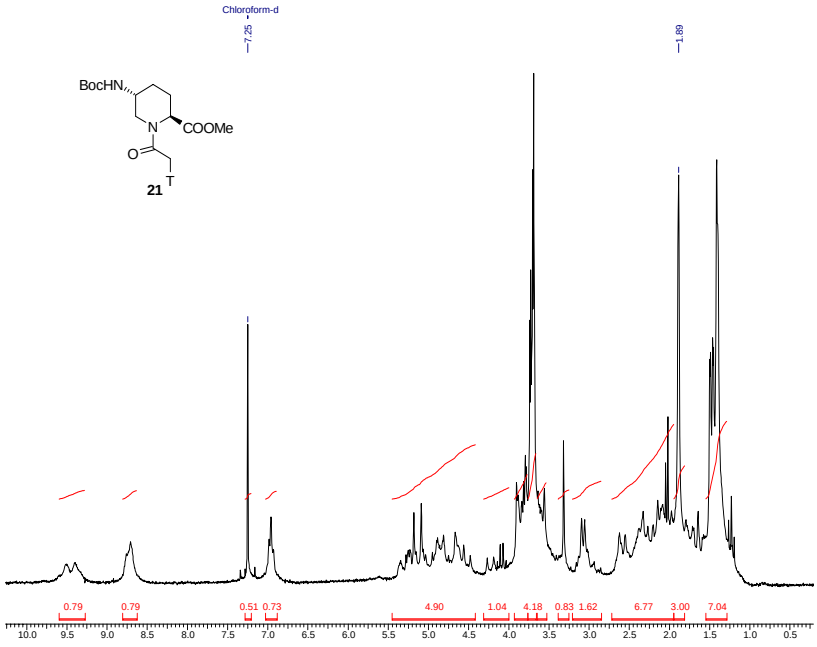
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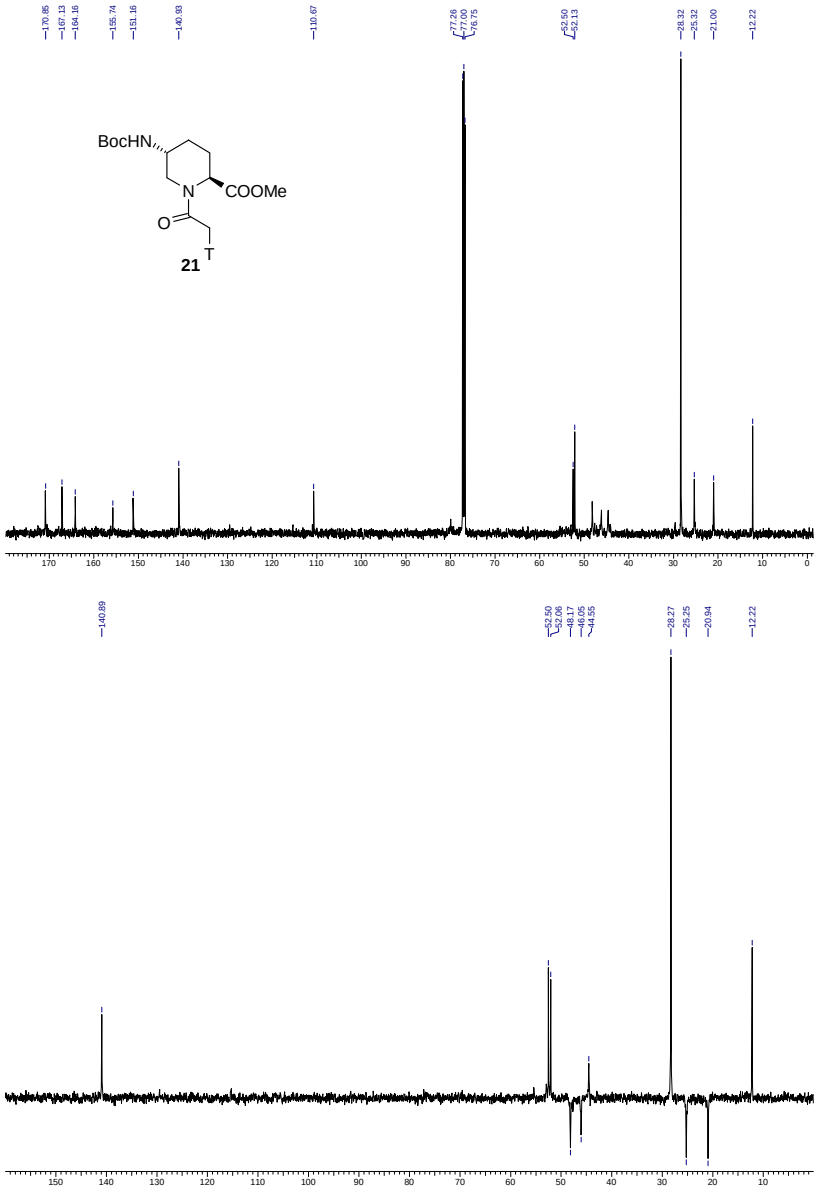
BocHN
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Chapter 4

Chiral Piperidine-*aeg*PNA: Design, Synthesis and hybridization studies with DNA.

4.1. INTRODUCTION

The remarkable medicinal importance of *aeg*PNAs as DNA/RNA mimics in antisense therapeutics has challenged chemists to circumvent the limitations of their *in vivo* efficacy.[1] The conformational freedom in the nucleobase linker arm and the backbone aminoethyl and glycyl segments in *aeg*PNA were found to be a cause of unfavorable entropic loss during complex formation with complementary DNA/RNA.[2] In conjunction with introducing conformational constraint to address this problem, the efforts are also directed towards refining the *aeg*PNA properties such as water solubility, cellular uptake and discrimination between parallel versus antiparallel binding modes.[3] In the case of modified oligonucleotides with modified sugar-phosphate backbone, improved complex formation was observed with target

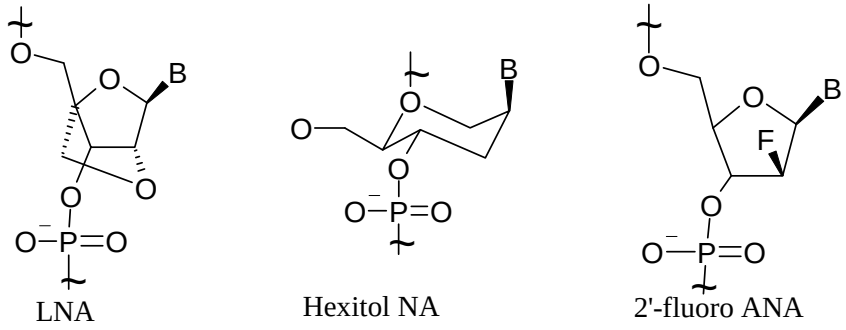


Figure 1 Conformationally constrained nucleic acids.

complementary RNA/DNA by the DNA analogues that assume A-type of sugar conformation such as LNA[4], Hexitol nucleic acids[5] or 2' modified nucleic acids.[6] The main reason for this improved stability of the complexes is attributed to the

conformational pre-organization due to locking or freezing the sugar ring conformation that is prevalent in their complexes with RNA. The structural information of the PNA:DNA/RNA complexes is limited with only PNA:DNA crystal structure and PNA_2:DNA and PNA:RNA NMR data.[7] It remains to be exploited the large number of possibilities for modulating the conformational features of PNA in a variety of ways considering acyclic nature of *aeg*PNA with flexibility in aminoethylglycyl backbone and the acetyl nucleobase linker segment. Construction of the five membered pyrrolidine based conformationally restricted chiral PNAs have met with some success in this direction. The introduction of the rigid chair conformation of the six-membered rings that determine the orientation of the ring substituents with respect to each other will further be expected to add to the structural diversity of PNA. Apart from hexitol,[8] cyclohexene[9] and altritol[10] based DNA analogs that have anionic phosphate backbone, there have been only a few uncharged modified DNA mimics having a six membered ring in the backbone. Morpholino nucleic acids (Figure 2a) are by far the best-studied example.[11] Early molecular modeling suggested that morpholinos should effectively bind complementary nucleic acids. The ability of morpholinos for DNA/RNA recognition is well established and their properties may be dictated by the conformational constraints of the six membered ring structures. The other earlier reported six membered glucopyranosyl nucleic acids (GNA)[12] based on sugar nucleoamino acid monomers were more water soluble than PNA and showed better binding to DNA with high sequence discrimination ability (Figure 2b). Thermodynamic parameters have suggested an entropic gain in GNA due to a pre-organized scaffold.

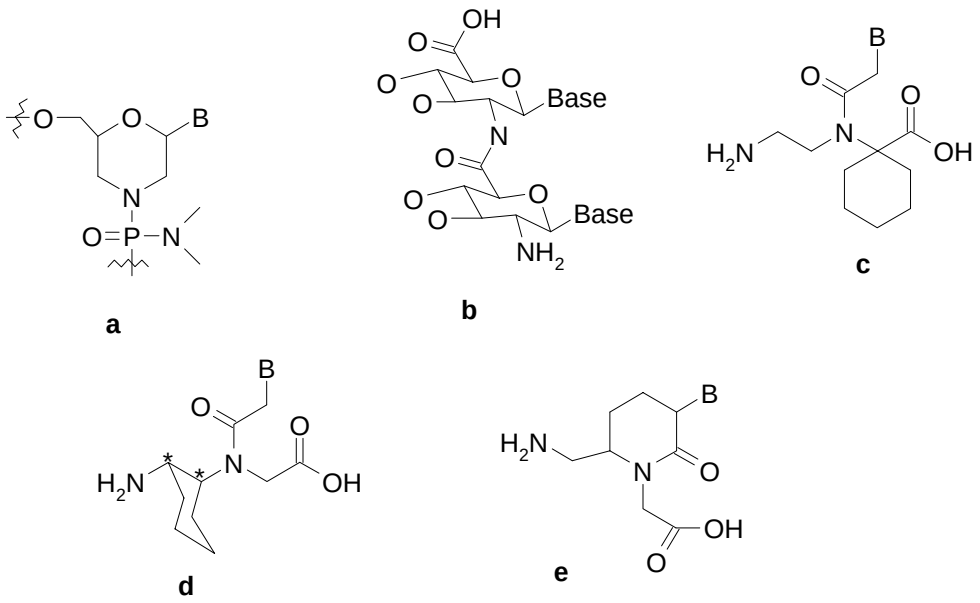


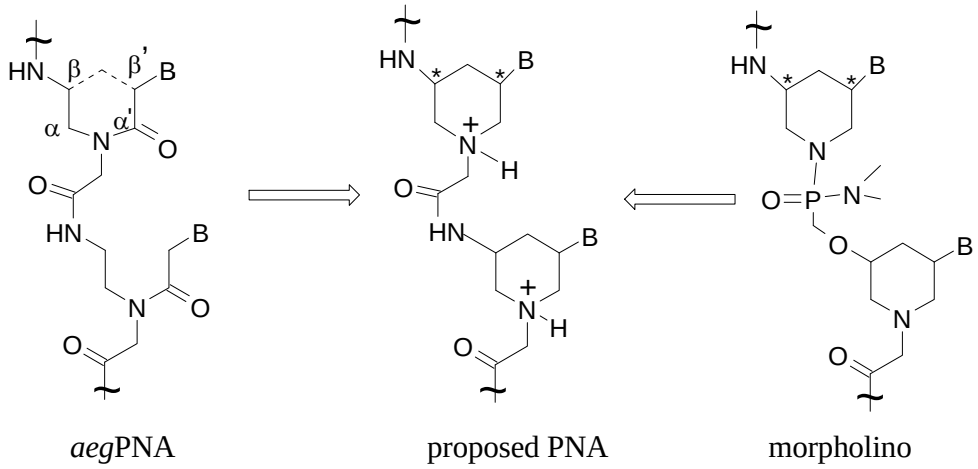
Figure 2 a) morpholino, b) glucosamine PNA, c) spiro PNA, d) cyclohexyl PNA, e) piperidinone PNA

Trans-1,2 cyclohexylamino (Figure 2d) and spirocyclohexyl rings (Figure 2c) have been employed in the monomers by Lagriffoule[13] and Maison[14] respectively. PNAs that bear (*S*,*S*) cyclohexyl ring in the aminoethyl part of the PNA backbone hybridize with complementary DNA similar to *aeg*PNA, while those with an (*R*,*R*) cyclohexanyl ring significantly decrease the binding properties. Recently, it was shown that the other two enantiomers *i.e.* cyclohexanyl PNA (1*S*,2*R* and 1*R*,2*S*) in the *aeg*PNA structure preferred to bind to RNA with higher affinity than to DNA in homothymine sequences leading to stereodiscrimination in recognition of DNA and RNA.[15] Another effort in this direction is the piperidinone PNA, a homologue of the pyrrolidinone PNA as described in chapter 1. The two adenine monomers (3*R*,6*R*) and (3*S*,6*R*) when

incorporated into the oligomer, resulted in a large decrease in the duplex stability (Figure 2e).[16,17]

4.1.1. Rationale for the Present work

The above mentioned literature underlines that the fine-tuning the PNA structure with a six membered ring could lead to achieve maximum advantages for binding to target RNA/DNA sequences. The attributes of positive charge in the backbone with a five-membered heterocyclic ring and the construction of a six-membered pipecolic acid



*aeg*PNA proposed PNA morpholino

Figure 3 Derivation of the piperidinyl PNA structure from the parent *aeg*PNA structure.

based PNA retaining the tertiary-amide group are presented in the preceding chapters. These attributes can be combined together to arrive at a six membered PNA analogue that is chiral and could be positively charged due to the presence of tert. amine group instead of tert. amide linker to the nucleobase. We arrived at a PNA modification that is conceptually derived from *aeg*PNA by introducing a methylene bridge between β carbon atom of aminoethyl segment and β'carbon atom of linker to nucleobase, and

simultaneously removing tert.amide carbonyl group. This resulted in chiral piperidine PNA analogue that is also a piperidine-amide analogue of morpholino oligonucleotides (Figure 3). The structure also may have similarities with the successful hexitol nucleic acid with equatorial (hydroxymethyl), equatorial (hydroxy group) and axial nucleobase substitutions.[18] The design of the monomer is such that to maintain the preferred *N*-1 equatorial alkyl substitution on the piperidine ring[19] and 1,3- *cis* diequatorial disposition

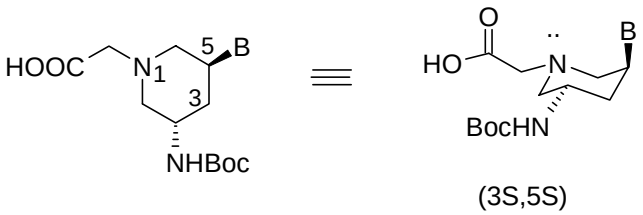


Figure 4 Proposed conformationally frozen peptide nucleic acid analogues

of the backbone, the orientation of nucleobase needs to be axial (Figure 4). As in the case of hexitol nucleic acids, the axial orientation of the nucleobase and equatorial disposition of the backbone may lead to the formation of stable duplexes with natural nucleic acids in contrast to hexose nucleic acids that assume an all equatorial substitution pattern.[20] The synthesis of monomer unit *trans*–(3*S*,5*S*)-3-*N*-(*tert*.butyloxycarbonylamino)-5-(thymin-1-yl)-piperidin-*N*-1-acetic acid was planned from naturally occurring L-*trans*-4-hydroxy proline.

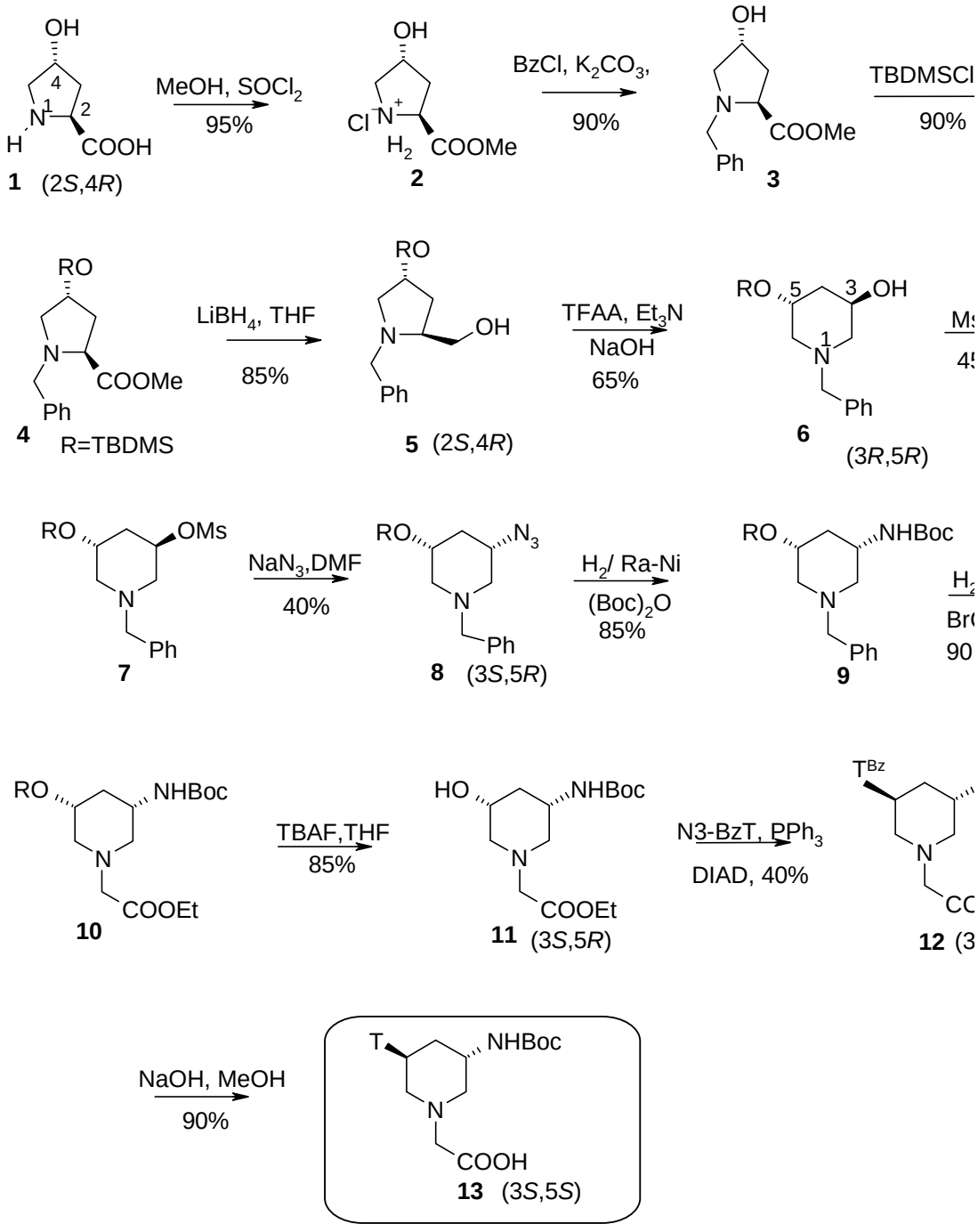
4.1.2. Objectives of the chapter

1. Synthesis of *trans*–(3*S*,5*S*)-3-*N*-(*tert*.butyloxycarbonylamino)-5-(thymin-1-yl)-piperidin-*N*-1-acetic acid for use in PNA synthesis.

2. Synthesis of the PNA oligomers using the aminoethylglycyl PNA monomers and the modified piperidinylPNA monomers by Merrifield solid phase peptide synthesis (SPPS) protocol.
3. Cleavage of the oligomers from the solid support, purification and characterization of the modified PNA oliogmers.
4. Study of PNA:DNA hybridization using biophysical techniques such as UV-spectroscopy, CD spectrophotometry and gel electrophoresis.

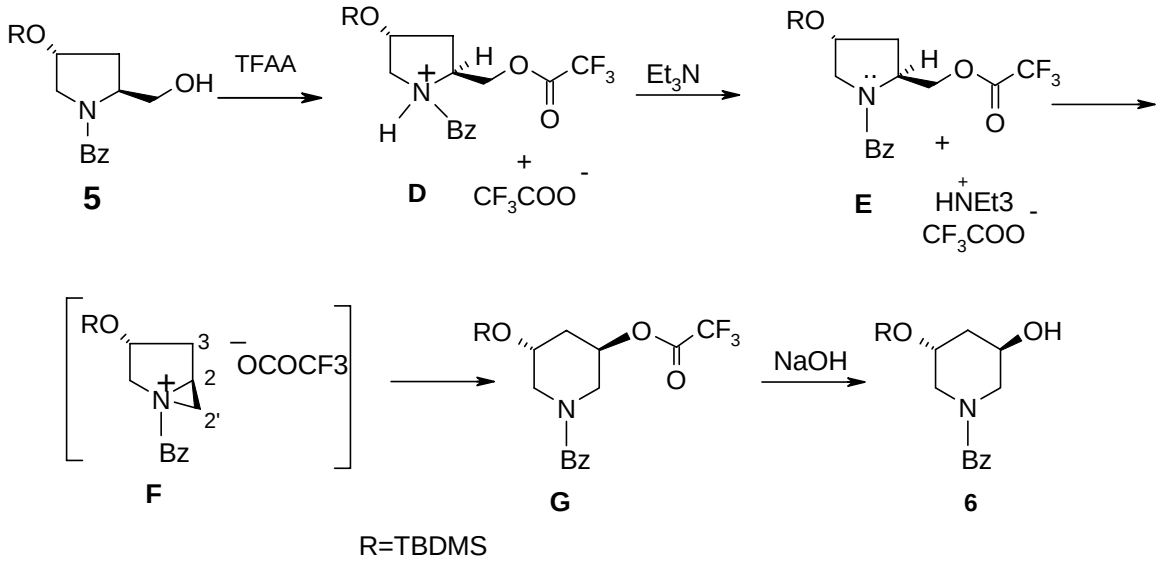
4.2. Synthesis of *trans*–(3*S*,5*S*)-3-(*tert*.butyloxycarbonylamino)-5-(thymin-1-yl)-piperidin-1-yl-acetic acid

Synthesis of the required modified PNA monomer was achieved from naturally occurring *trans* (2*S*,4*R*)-hydroxy L-proline (Scheme 1). The *N*-1 benzyl protected compound **3** was obtained in quantitative yield by reaction of *trans*-4-hydroxy-proline methyl ester **2** and benzyl chloride in presence of K_2CO_3 in DMF. 4-hydroxy group was protected as its tert.butyldimethylsilyl derivative, which showed upfield signals at 0.0 and 1.3 ppm corresponding to the silyl methyl groups in the ^{1}H NMR spectra. Ester function in **4** was reduced to alcohol **5** by $LiBH_4$ in THF. The complete reduction of ester group was confirmed by the disappearance of a peak at 3.5 ppm (methyl ester) and appearance of multiplet at 3.3 ppm corresponding to the CH_2OH group in the ^{1}H NMR spectrum. Compound **5** was treated with trifluroacetic anhydride followed by Et_3N to give the ring expanded six membered rearranged product **6** with retention of configuration.[21] As seen from scheme 2, the first step for the formation of the six



Scheme 2 Synthesis of *trans*–(3*S*,5*S*)-3-(*tert*.butyloxycarbonylamino)-5-(thymin-1-yl)-piperidin-1-yl-acetic acid

membered ring from pyrrolidine methanol derivative **5** implies the trifluroacetylation of

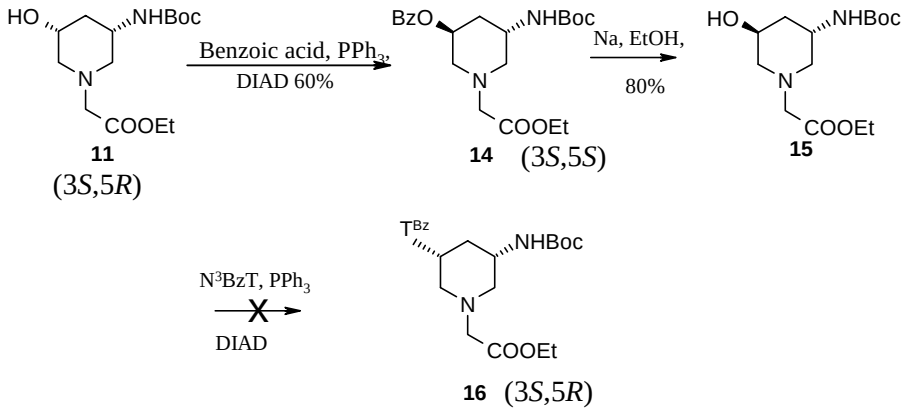


Scheme 2 Mechanism for the formation of six membered ring with retension of configuration from pyrrolidine methanol.

the hydroxy group of the 4-*O*-silyl-pyrrolidine-2-methanol by trifluroacetic anhydride and formation of the corresponding quaternary ammonium salt **D** (Scheme 2). In the absence of base, no rearrangement was observed. With triethyl amine, amino acetate **E** (Scheme 2) is formed which undergo a S_Ni process to give the tight ion pair **F** that generates the stable acetate **G**. Saponification of the acetate by NaOH affords the desired product **6**. Mesylation of the resulting unprotected hydroxyl group in **6** with mesyl chloride (Scheme 1) in pyridine gave the mesylate derivative **7**, which in its ^{1}H NMR spectrum showed signal at 3.3 ppm for 3 proton (OMs). The C3 mesylate **7** was treated with excess of sodium azide in DMF to give (3*S*,5*R*) *cis*-azide **8** with inversion of configuration at C3. A characteristic peak appearing at 2106 cm^{-1} in the IR spectrum of compound **8** and disappearance of mesyl signal in ^{1}H NMR confirmed the formation of azide derivative. Selective hydrogenation of azide to amine in the presence of *N*-1

benzyl group and simultaneous protection with Boc-anhydride was effected by subjecting compound **8** to hydrogenation in the presence of Ra-Ni for 2 h to give compound **9** in 85% yield. Piperidine ring nitrogen in **9** was deprotected by hydrogenation and the resulting free amine was alkylated with ethyl bromoacetate in presence of DIPEA in dry THF to get **10**. ^{1}H NMR spectrum of compound **10** showed a quartet and triplet at 4.3 (CH_2) and 1.37 (CH_3) ppm respectively and disappearance of peaks in the aromatic region. Silyl protecting group of 5-OH in compound **10** was removed using TBAF in THF to get **11**. *Trans*-(3*S*,5*S*)-3-(*tert*.butyloxycarbonylamino)-5-(*N*3-benzoylthymin-1-yl)-piperidine derivative **12** was synthesized by treating compound **11** with *N*3-benzoyl thymine under Mitsunobu conditions. The ester function in **12** was hydrolyzed using aqueous methanolic sodium hydroxide to get the protected thymine monomer **13** that could be used for solid phase synthesis of chimeric *aeg*PNA-piperidine PNA.

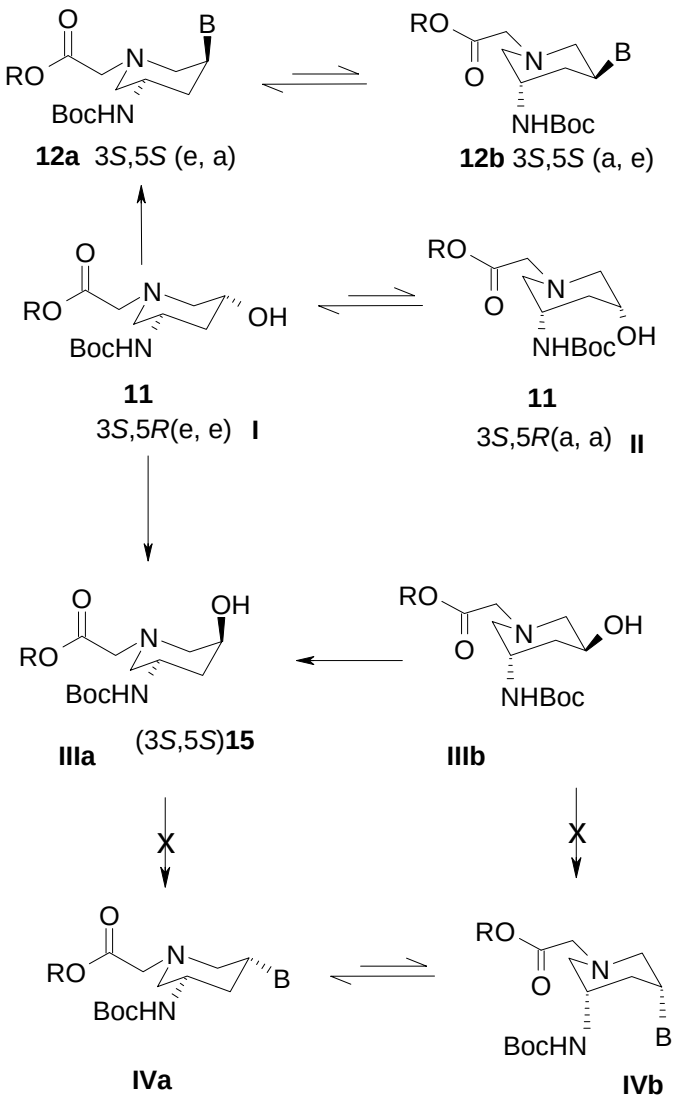
At this point, for comparison we envisaged the synthesis of thymine monomer with



Scheme3: Synthesis of *cis*–(3*S*,5*R*)-3-(*tert*.butyloxycarbonylamino)-5-(thymin-1-yl)-piperidin-1-yl-acetic acid

(3*S*,5*R*) stereochemistry (Scheme 3) in which substitution pattern would be all equatorial. Compound **11** was subjected to Mitsunobu reaction conditions with benzoic acid as a nucleophile to get (3*S*,5*S*) benzoyl derivative **14** with an inversion of configuration at C5. Benzoyl derivative was subjected to solvolysis using Na/EtOH to give free 5*S*-OH in 80% yield. A second Mitsunobu reaction with *N*3-benzoyl thymine as a nucleophile failed to give the desired product (3*S*,5*R*) **16** (Scheme 3).

The chiral piperidine ring in **11** can be in two conformations (Scheme 4, **I** and **II**). The diequatorial conformation **I** will be sterically less demanding as compared to diaxial **II**. The equatorial orientation of OH in **I** is probably more suitable for the Mitsunobu displacement by the *N*3-benzoyl thymine to get **12a**. The equatorial leaving group orientation seems to be sterically viable for this reaction. The (3*S*,5*S*) hydroxy piperidine derivative (Scheme 4, **III)** was found to be unreactive under Mitsunobu alkylation with *N*3-benzoyl thymine. Possibly, the Boc-amino function prefers to remain equatorial as in **IIIa** (Scheme 4) and the nucleoplilic displacement of the axial hydroxy group seems to be sterically unfavoured although the product will be more stable 3,5 diequatorial **IVa**. The equatorial hydroxy leaving group as in **IIIb** might have been suitable for Mitsunobu displacement but that brings Boc-amino functionality and the incoming N3-BzT in axial orientation (**IVb**) and hence sterically unfavorable. We therefore also envisage in compound **12** (Scheme 4) the Boc-NH group to be in eqatorial orientation and the nucleobase in axial. Energy minimization using Sybyl program (IRIX 6.1) of the two possible conformations of **12a** and **12b** has shown that



Scheme 4 Formation of (a,e) *trans* isomer **12**

the conformation with equatorial Boc-amino and axial nucleobase as in **12a** to be a lower energy conformation.

4.3. Solid phase PNA synthesis

The *aeg*PNA oligomers and the modified *aeg*-piperidinyl PNA oligomers with chimeric backbone were synthesized by standard solid phase peptide synthesis

protocols. PNA oligomer synthesis was carried out from the 'C' to the 'N' terminus using monomers with free carboxylic acid functions and amino functions protected as Boc derivatives that are cleavable with TFA at the beginning of every cycle. The solid support used was Merrifield resin[22] that was derivatized with β-alanine using the cesium salt method[23] (See Chapter 2) to achieve a loading value of 0.5 meq/g resin. β-alanine is linked to the resin *via* a benzyl ester linkage enabling cleavage of the synthesized oligomer by TFMSA-TFA to afford the 'C' terminal free acid. The resin, upon attachment of the β-alanine spacer amino acid, was suitably down-loaded to attain a loading capacity of 0.1 to 0.2 meq/g resin (Please refer to Chapter 2), which has been reported to give optimum yields in PNA synthesis. The loading capacity of the resin was determined by picrate assay.[24] The synthesis was carried out by repetitive coupling cycles, each cycle comprising (i) deprotection of the Boc-amino group using TFA in dichloromethane to generate the amine group as TFA salt, (ii) neutralization of the resulting TFA salt by DIPEA to liberate the amine, (iii) coupling of the resin-bound amino groups with the incoming amino acid using 1-hydroxy benzotriazole and HBTU as the coupling agents. The deprotection and coupling steps were monitored by the ninhydrin method using Kaiser's test.[25]

4.4. Synthesis of *aeg*PNA and *aeg*-piperidinylPNA oligomers

4.4.1. Homopyrimidine sequences

PNA polypyrimidine sequences are known to form triplexes with complementary DNA in PNA_2: DNA binding stoichiometry. To study the effect of the chiral, positively charged piperidinyl PNA thymine monomer on triplex forming ability of the oligomers, thymine octamers were synthesized by incorporating this modified unit at predetermined positions. The capping step at the end of each coupling cycle was not necessary, as the completion of the coupling reaction with a very high coupling efficiency was monitored manually using Kaiser test. The series of octamer sequences synthesized are listed in Table 1. The control *aeg*PNA T8 oligomer **17** was synthesized

Table 1 Polypyrimidine PNA and complementary polypurine DNA sequences.

Entry	Resin-linked PNA Oligomer	
17	H-T T T T T T T T -β-ala–OH	Control *aeg*PNA
18	H-**t** T T T T T T T -β-ala–OH	One (3*S*,5*S*) unit at N terminus
19	H-T T T T T T T **t** -β-ala–OH	One (3*S*,5*S*) unit at C terminus
20	H-T T T **t** T T T T -β-ala–OH	One internal (3*S*,5*S*) piperidinyl unit
21	H-T T T **t** T T T **t** -β-ala–OH	Two (3*S*,5*S*) piperidinyl unit
22	H-C T C **t** T T T **t** -β-ala–OH	Two (3S,5S) piperidinyl unit mixmer
23	H-C T C T T T T T -β-ala–OH	Control *aeg* foe seq. **22**
24	5'GC-A A A A A A A A CG-3'	Complementary DNA to PNA-T_8
25	5'GC-A A A **T**A A A A CG-3'	Mismatch DNA to PNA-T_8
26	5'A A A A A G A G-3'	Complemetary DNA to PNA **22** and **23**

T/C = *aeg*PNA **t** = (3*S*,5*S*) piperidinyl PNA-T

following the Boc protection strategy. With a view to explore the induced effects of piperidinyl PNA monomeric unit, a single unit was introduced at either N or C terminus

(Table 1, Entry, **18** and **19**). Further this modified unit was also introduced in the center of the sequence (Table 1, Entry, **20**) and to study the effect of introduction of increasing number of piperidinyl units on the stability and selectivity of DNA complexation, two units were introduced within the sequence ((Table 1, Entry, **21**). Homopyrimidine mix sequence having both thymine and cytosine was also synthesized (Table 1, Entry, **22**).

4.4.2. Mixed purine-pyrimidine sequences with aeg and aeg-piperidinylPNA backbone

In order to study the effect of duplex formation potential of piperidinyl PNA, it was imperative to synthesize mixed sequences incorporating both, purines and pyrimidines. The piperidinyl PNA -T monomer was therefore incorporated into dodecamer at N terminus of the sequence (Table 2, Entry **28**).

Table 2 Mixed base PNA comprising A and T nucleobases.

Entry	PNA/DNA oligomer	
27	H-TATATTATTATT -β-ala–OH	Control PNA sequence
28	H-**t** ATATTATTATT -β-ala–OH	One piperidinyl unit at N terminus
29	5'A A T A A T A A T A T A3'	*ap* DNA to PNA **27**and **28**
30	5'A T A T A A T A A T A A3'	*P* DNA to PNA **27** and **28**

T = *aeg*PNA, **t** = (3*S*,5*S*) piperidinyl PNA-T

4.5. Cleavage of the oligomers from the solid support

The synthesized PNA oligomers were cleaved from the solid support using the TFMSA-TFA cleavage procedure.[20] This yielded oligomers with free carboxylic acids at their 'C' termini. The mixed pyrimidine with cytosine and thymine oligomers **22** and **23** in which the exocyclic amine of cytosine was protected as its benzyloxycarbamate

derivative needs no additional deprotection as N^4-Cbz group gets deprotected during TFA-TFMSA treatment. Remaining PNA homopyrimidine sequences (**17**-**21**) and a mix purine-pyrimidine sequence *i.e.* adenine-thymine (**27** and **28)** having no exocyclic protecting groups for the nucleobases were cleaved using TFA-TFMSA treatment.

4.6. Purification of the PNA oligomers

Preliminary purification of the full length PNA oligomer that removed truncated sequences and low molecular weight impurities was achieved by gel filtration through Sephadex G25. The oligomers were further purified by FPLC on a semi-preparative C8 reverse-phase column using an ascending gradient of acetonitrile in water containing 0.1% TFA. The purity of the oligomers was checked by HPLC on a C18 reverse-phase column and confirmed by MALDI-TOF mass spectroscopy. Some representative HPLC profiles and mass spectra are shown in Figures 5 and 6 respectively.

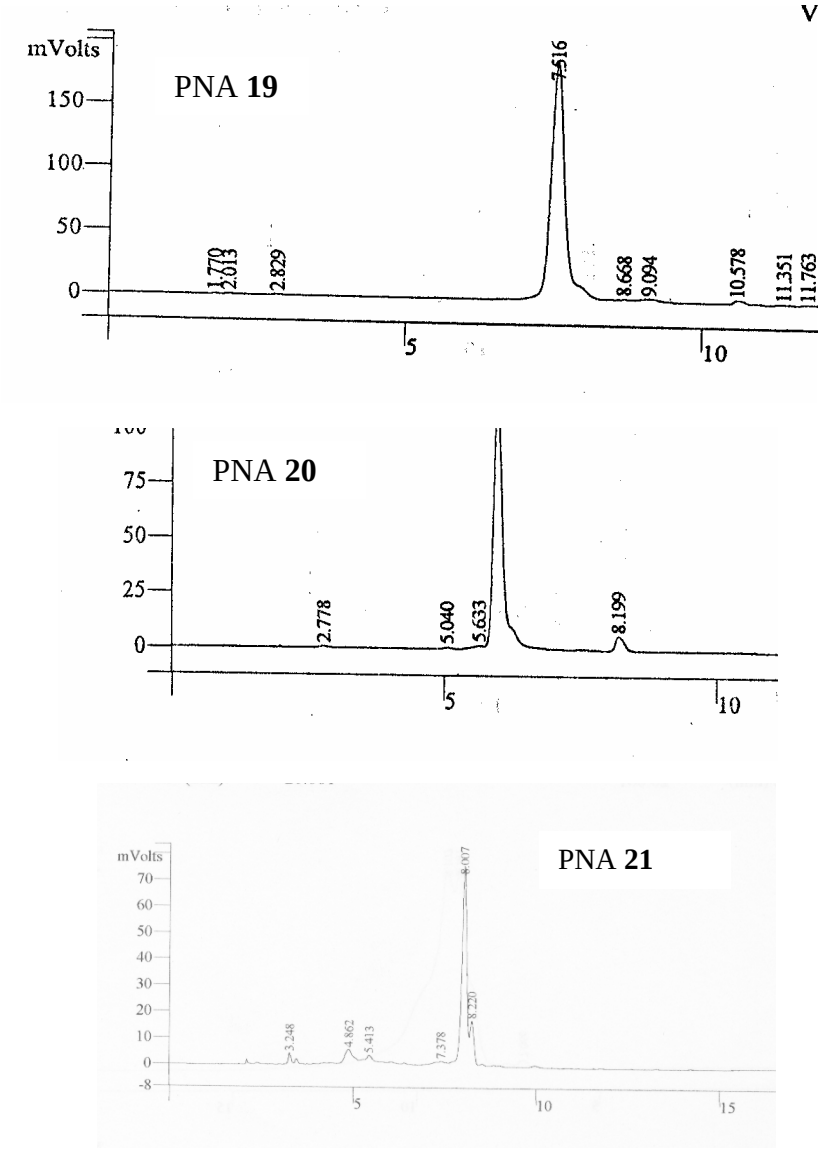
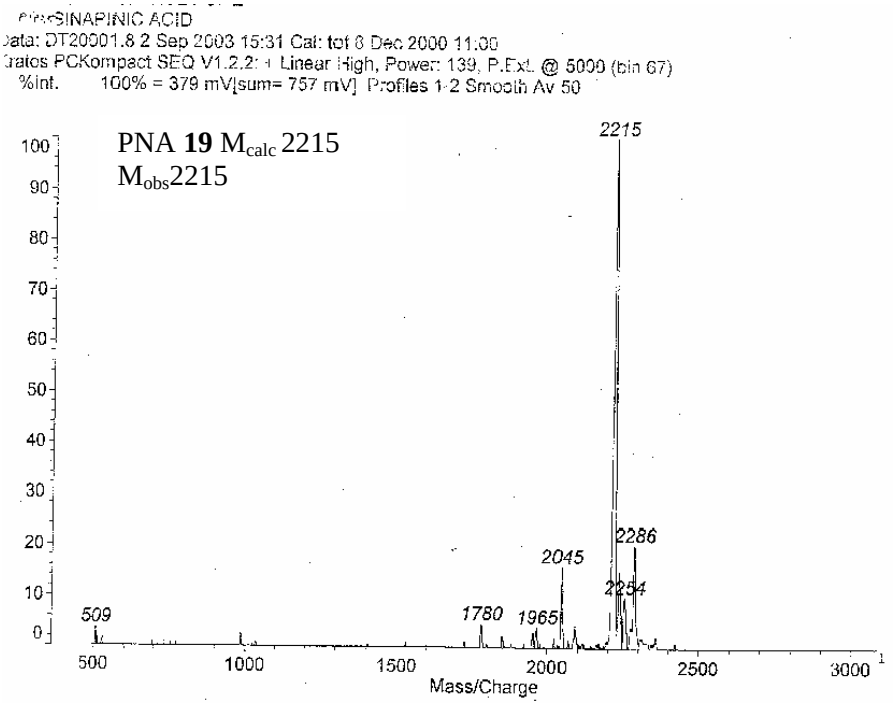


Figure 5 Representative HPLC profiles of Piperidinyl PNA oligomers.



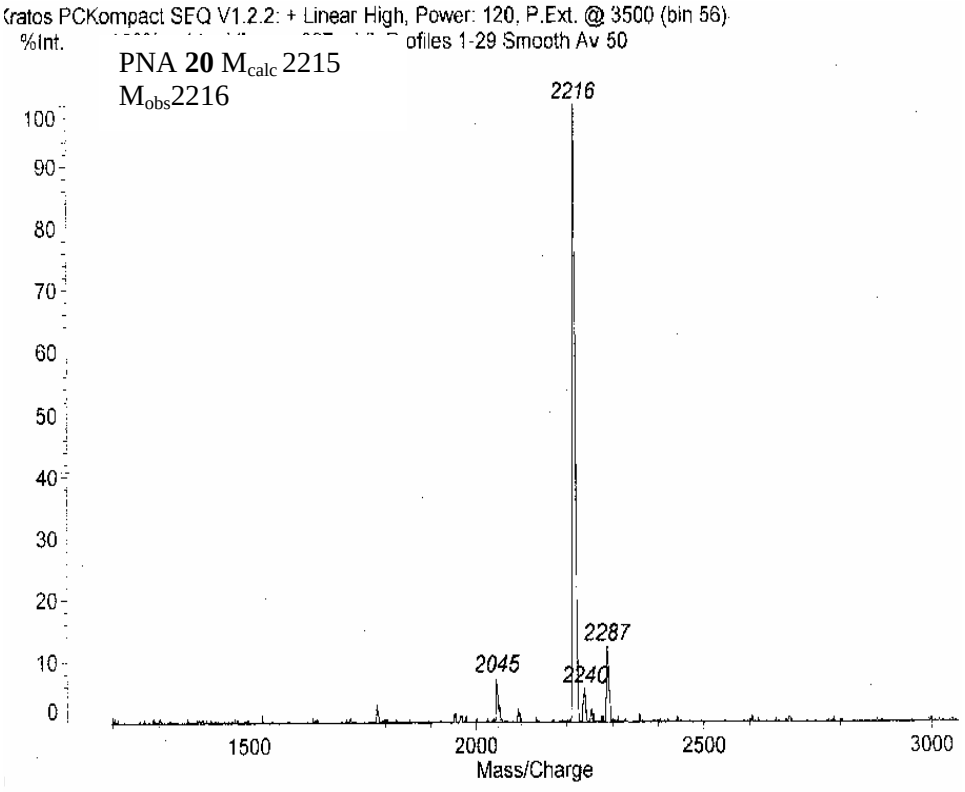


Figure 6 MALDI-TOF spectra for piperidinyl-PNA oligomers **19** and **20**

4.7. Synthesis of oligonucleotides

The oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus automated synthesizer using the standard β-cyanoethyl phosphoramidite chemistry on a CPG solid support, followed by ammonia deprotection and cleavage.[26] The purity of the oligonucleotides was verified by reverse phase HPLC on a C18 column and was found to be >96%. Consequently, these oligonucleotides were used for the biophysical studies as such, without further purification.

4.8. Results and Discussion

4.8.1. PNA_2:DNA triplexes

CDstudies: Binding stoichiometry

CD measurements are useful in the study of PNA:DNA/RNA complexes. This technique can be used to monitor and characterized the resulting structure as described

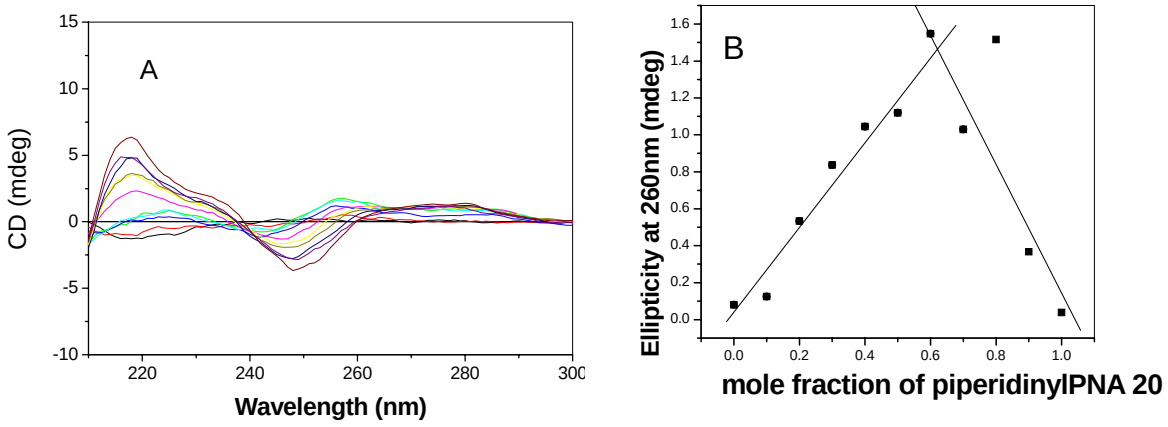


Figure 7 A) CD mixing curve for PNA **20** and DNA **24** mixtures in the various molar proportions. B) CD spectra of molar mixtures of PNA **20** and DNA **24** showing isodichroic points.

in chapter 1. The stoichiometry of strands in the complex was obtained from the mixing curves, in which the optical property at a given wavelength is plotted as a function of the mol fraction of one of the strands.[27] Various stoichiometric mixture of PNA **20** and DNA **24** were made with relative molar ratios of PNA:DNA strands of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 10:0 all at total strand concentration of 2μM in sodium cacodylate buffer (10mM, pH 7.3). The samples with the individual strands were annealed. The CD spectra of the various mixtures are shown in figure 7A. These spectra exhibit positive maxima at 220 and 260-280 nm, a negative minimum at ~250 nm. The plot of ellipticity at 260 nm *vs* mole fraction of *aeg*-piperidinyl PNA showed inflection point at 0.63 indicating PNA_2:DNA binding stoichiometry (Figure 7B). The positive bands in the region of 260 to 280 nm as seen in the CD spectra of the complexes are characteristic of the poly(dA)$(PNA\text{-}T_8)_2$ complexes, confirms the piperidinyl PNA:DNA complexes to be in 2:1 stoichiometry.

UV-Tm studies

Figure 8 shows the Tm profiles for PNA_2:DNA triplexes derived from *aeg*PNA and piperidinyl PNA sequences. Generally, the UV-Tm values obtained for PNA:DNA complexes in 1:1 and 2:1 stoichiometry were almost identical and the plots of absorbance or % hyperchromicity at 260 nm *vs* temperature were sigmoidal, indicative of a two state cooperative transition. These studies indicate that the oligomer **19:24** with one (3*S*,5*S*) piperidinyl PNA unit at its C terminus gave a melting temperature stabilization of 7°C over the control complex **17:24** (Figure 8, Table 3). A synergistic effect was observed with one more unit in the center of the sequence (PNA **21:24,**

Figure 8, Table 3) and the PNA_2:DNA complex was further stabilized by about 4°C (Figure 8, Table 3). A single modified unit in the center of the sequence seems to be accommodated in the uniform *aeg*PNA backbone causing only a minor effect (PNA **20:24**, ΔTm= -2°C). Interestingly, the piperidinyl unit when present at the N terminus (**18**:**24**) exhibited UV-Tm effect that was reverse to that observed when this unit was present at the C terminus although the transition is sharp (Figure 8, Table 3). In this case the complex is destabilized by 8°C. The mixed C/T homopyrimidine PNA_2:DNA

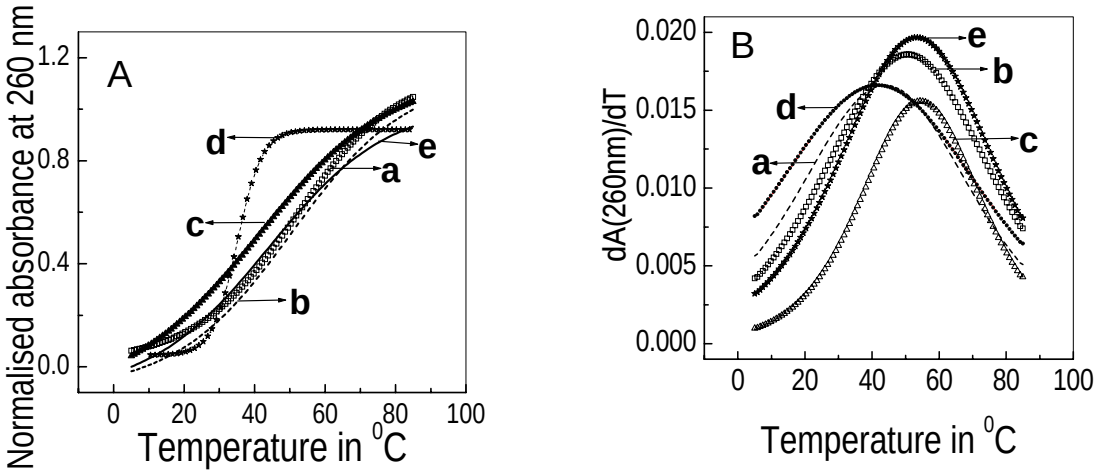


Figure 8 A)UV-Tm profile, **B**) first derivative plots of a) **17:24**, b) **19:24**, c) **21:24**, d) **20:24**, e) **22:26**

(**22:26**) containing two piperidinyl units gave a melting temperature stabilization of 3°C over the control complex (**23:26**) when the orientation of the DNA strand was antiparallel with respect to both PNA strands. The stabilization caused by the piperidine unit on PNA_2:DNA triplexes thus seems to be governed both by the position of the piperidine unit in the sequence and also the DNA sequence itself.

Table 3 Uv-Tm of PNA_2:DNA triplexes

Sequence	PNA_2:DNA	UV-Tm°C
H-T T T T T T T T -β-ala- OH	**17:24**	43 (10)
H- **t** T T T T T T T -β-ala- OH	**18:24**	35 (15)
H-T T T T T T T **t**-β-ala- OH	**19:24**	50.7 (15)
H-T T T **t** T T T T -β-ala- OH	**20:24**	41.4 (6)
H-T T T **t** T T T **t** -β-ala- OH	**21:24**	54.4 (9)
H-C T C **t** T T T **t** -β-ala–OH	**22:26**	54 (11)
H-C T C T T T T T -β-ala–OH	**23:26**	51 (18)

Buffer: 10 mM sodium cacodylate, 100mMNaCl, 0.01mM EDTA, pH 7.3 T= *aeg*PNA, **t =** (3*S*,5*S*) piperidinyl unit. Values in parenthesis indicate % hyperchromicity

Mismatch studies

The complexes of PNAs were constituted with DNA containing a mismatch base. The specificity of PNA:DNA interaction was established by the mismatch studies with the DNA oligomer **25** bearing a T-T mismatch in the center of the sequence. The PNA_2:DNA complexe (**20:25**) comprising the piperidinyl PNA and DNA having a single mismatch gave linear, non-sigmoidal plots and failed to show any peak in the first derivative plots. As a consequence, no melting temperature was detected for these complexes.

Gel shift assay

Electrophoretic gel shift assay was used to establish the binding of different PNAs to the complementary DNA. The binding of piperidinyl PNA to complementary DNA was examined by gel retardation studies as shown in figure 9. The PNAs modified with one or two units of piperidinyl PNA were individually treated with complementary DNA **24** or DNA **26** and the complexations were monitored by non-denaturing gel electrophoresis at 10°C. The complexes were visualized on a fluorescent TLC background and the gel results are shown in figure 9. The PNA:DNA complexes derived from piperidinyl PNAs with single modified unit eg. **18:24** (lane 1), **20:24** (lane 2) were significantly retarded in the gel. The complex involving two piperidinyl units **21:24** and **22:26** (lane 3 and lane 5) was retarded more and remains close to well. The stability of the complexes **18:24** is less than **20:24** and **21:24** as studied by Uv-Tm and accordingly in gel studies presented here show presence of *ss*DNA and unbound

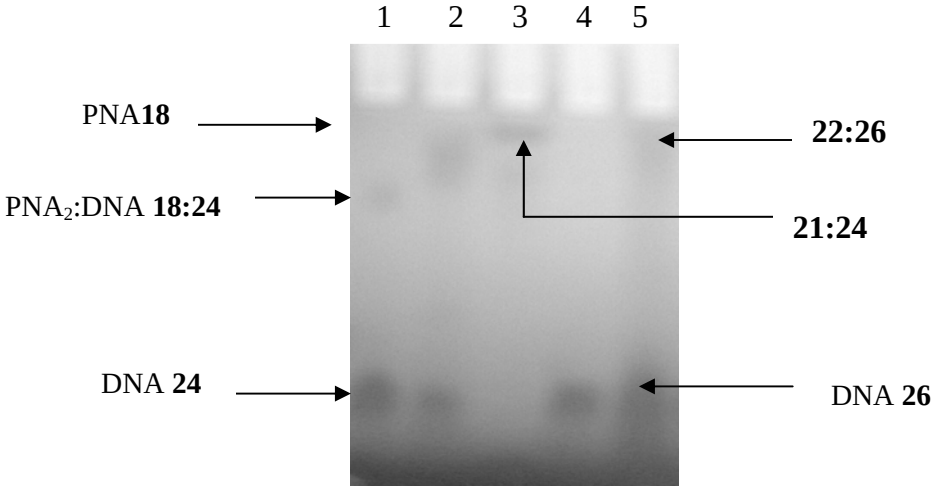


Figure 9 Polyacrylamide Gel Electrophoresis of Piperidinyl PNA$_2$:DNA complexes Lane 1, **18:24**; Lane 2, **20:24**; Lane 3, **21:24**; Lane 4, *ss*DNA **24**; Lane 5, **22:26.**

PNA in lane 1. These results were in accordance with the data obtained from the UV-thermal melting studies with these oligomers, which also underlined the specificity of the hydrogen bonding between the complementary base pairs.

4.8.2 PNA:DNA duplexes

UV-Tm Studies

Based on the above results, it was thought worthwhile to examine the effect of piperidinyl PNA backbone chirality on the binding orientation in duplexes with mixed purine/pyrimidine sequences. So mixed PNA sequence **27** and **28** compromising adenine and thymine were synthesized. The piperidinyl PNA unit was incorporated at the N terminus of the dodecamer (Table 2). The two duplexes were constituted by individually mixing equimolar amount of complementary DNA **29** or **30** and achiral *aeg*PNA **27** or chiral piperidinyl PNA **28**. In the unmodified *aeg*PNA **27** the antiparallel duplex was more stable than the parallel duplex by about 2° C (Figure 10, Table 4). The

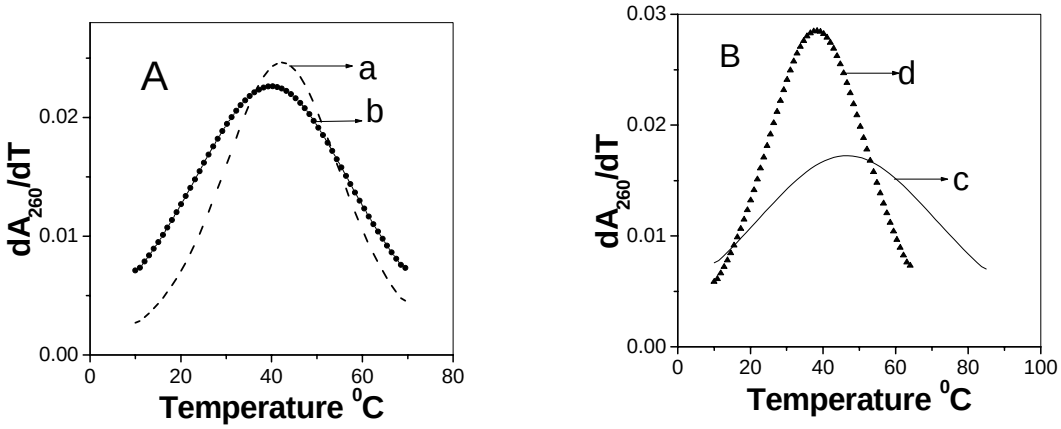


Figure 10 UV-Tm profile of PNA:DNA duplexes.
A, a) **27:29**, b) **27:30 B**, c) **28:29**, d) **28:30**

oligomer **28** having (3*S*,5*S*) piperidinyl PNA unit stabilizes the antiparallel complex by 5°C compared to the control *aeg*PNA while the parallel duplex destabilized by 3°C compared to the control sequence. This discrimination in parallel *vs* antiparallel binding by modified PNA improved *p* and *ap* binding by PNA from 2°C to 10°C (Table 4).

Table 4 UV-Tm (°C) of PNA:DNA duplexes

PNA sequence	PNA:DNA	Tm° C	PNA:DNA	Tm° C
H-T A T A T T A T T A T T -β-ala- OH	**27:29**	41	**27:30**	39
H-**t** A T A T T A T T A T T -β-ala- OH	**28:29**	46	**28:30**	36

4.9. Summary

Synthesis of new six membered, chiral, positively charged piperidine PNA analogue is accomplished. The oligomers having piperidinyl PNA units were shown to possess very interesting DNA binding properties. The % hyperchromicity *vs* temperature plots derived from the UV melting data indicated a single transition, characteristic of triplex melting where both PNA strands dissociate from the DNA strand simultaneously in a single step. The presence of conformationally frozen six membered piperidine unit at C terminus induced favorable pre-organization of PNA to interact with target DNA, leading to more stable triplex. The duplex formed by mix purine-pyrimidine sequences with thymine piperidinyl unit at N terminus enhanced the difference in Uv-Tm effectively indicating discrimination in binding to parallel and antiparallel DNA sequences. The binding of modified oligomers with DNA was also seen in diagnostic

gel mobility shift experiment where even a single modification effected significant retardation.

These preliminary results are encouraging and invoke the possibility of development of designed antisense oligo mimics with minimum structural modifications. The (3*S*,5*S*) diastereomer might prefer axial nucleobase orientation and equatorial backbone orientation as in the case of hexitol nucleic acid.

4.10. EXPERIMENTAL

All chemicals used were of reagent or analytical grade. All the solvents used were purified according to the literature procedures[24] and the reactions were monitored for completion by TLC. Column chromatography was performed for purification of compounds on LOBA chemie silica gel (100- 200 mesh). TLCs were carried out on pre-coated silica gel GF_{254} aluminium sheets (Merck 5554). TLCs were run in either dichloromethane with an appropriate quantity of methanol or in petroleum ether with an appropriate quantity of added ethyl acetate for most compounds. Free acid was chromatographed on TLC using a solvent system of iso-propanol: acetic acid: water in the proportion 9:1:1. The compounds were visualized with UV light and /or by spraying with ninhydrin reagent subsequent to Boc-deprotection (exposing to HCl vapors) and heating.^{1}H (200 MHz/ 300MHz) and ^{13}C (50MHz) NMR spectra were recorded on a Bruker ACF 200 spectrometer fitted with an Aspect 3000 computer and all the chemical are referred to internal TMS for ^{1}H and chloroform-d for ^{13}C. The chemical shifts are quoted in δ (ppm) scale. In compounds that bear a tertiary amide group, splitting of

NMR signals was observed due to the presence of rotamers. Optical rotations were measured on a JASCO DIP-181 polarimeter. Mass spectra were recorded on a Finnigan-Matt mass spectrometer, while MALDI- TOF spectra were obtained from a KRATOS PCKompact instrument.

(2*S*,4*R*)-*N*-1-(benzyl)-4-hydroxyproline-methylester[21] 3

Trans-4-hydroxy-L-proline (10g, 76mmol) was dissolved in 120 ml of dry methanol. Under dry conditions thionyl chloride (9ml, 76 mmol) was added drop-wise in to the continuously stirred reaction mixture while keeping the flask in ice bath. After the addition was over, it was refluxed for 4 h and then evaporated the solvent completely. Then this residue was dissolved in dry DMF (100ml), and to it K_2CO_3 (20g, 147 mmol) was added while stirring. To this mixture benzyl chloride (14.79ml, 118mmmol) was added in one portion and the mixture was stirred at 60^0C for 4 h. After completion of reaction solvent was removed completely under vacuum, and residue was taken in water, extracted with ethyl acetate (3x 50ml). The organic layers were pooled together, washed with water, brine and then dried over sodium sulfate and concentrated to give the crude product, which was purified by silica gel column chromatography (17g, 95%). ^{1}H NMR 200mz, $CHCl_3$ d δ: 7.4 (m, 5H, Ar), 4.4 (m, 1H, H_4), 4-3.7 (dd, 2H, CH_2-Ar), 3.6 (s, 4H, COOMe, H5), 3.4 (m, 1H, H5'), 3.2 (bs, 2H, OH), 2.6 (m, 1H, H_2), 2.4-2 (m, 2H, H3),

(2*S*,4*R*) *N*-1-(benzyl)-4-[(t*ert.* butyldimethylsilyl)-oxy]-2-hydroxymethyl)-pyrrolidine[21] 5

Compound **3** (10.0g, 47mmol) was dissolved in dry DMF (150ml), and DMAP (1.9g, 15mmol), Et_3N (5ml, 56 mmol) was added to it. Reaction mixture was stirred in ice bath, tetrabutyl-dimethysilylchloride (7.8g, 51mmol) was added while maintaining the temperature. After completion of reaction, solvent was removed completely, residue was taken in water and extracted with ethyl acetate. The organic layers were pooled together, washed with water, brine and then dried over sodium sulphate and concentrated to get the crude product (13.0g, 90%). This ester **4** was then taken in dry THF (50ml), and reaction mixture was cooled to 0°c for 20 min. To a stirred mixture of this, lithium borohydride (1.0g, 46mmol) was added portion wise while maintaining the temperature. After stirring for 4 h under nitrogen atmosphere, the pH of the solution was adjusted to 4 with saturated solution of ammonium chloride. THF was evaporated under vacuum and water was added to the residue. The resulting solution was washed with ethyl acetate (3x 40ml) and the organic washings were pooled, washed with water followed by brine. The solution was dried over unhy. Na_2SO_4, and concentrated to afford **5** (10.4g, 80%).

$[\alpha]_D^{20}$ -44 (c = 0.5, MeOH)

^{1}H NMR (200mz, $CDCl_3$) δ: 7.34 (s, 5H, Ar), 4.41-4.24 (m, 1H, H4), 4.10-3.96 (m, 1H, CH_2-Ar), 3.77-3.3 (m, 3H, CH_2-OH, CH_2-Ar), 3.25-3 (m, 2H, H_5), 2.5- 2.2 (m, 3H, H_2, OH), 2.19-2 (m, 1H, H3), 1.99-1.81 (m, 1H, H3') 0.91 (s, 9H, TBDMS), 0.05 (s, 6H, TBDMS)

^{13}C NMR ($CDCl_3$) δ: 138.4, 127.9, 127.6, 126.3 (Ar), 70.1, 63.0 (C5), 61.1(C3) (N-CH_2-Ph), 61.0 (C2), 58.4 (C6), 37.2 (C4), 25.1, 17.2, 5.52.

(3*R*,5*R*)-*N*-Benzyl-5-[(*tert*.butyldimethylsilyl)oxy]-piperidin-3-ol[21] 6

Trifluroacetic unhydride (8.5ml, 40mmol), was added drop wise to a solution of pyrrolidine **5** (10 g, 31mmol) in THF (300 ml), cooled to -78^0c. After 3h, Et_3N (18ml, 125 mmol) was added drop wise. The reaction mixture was stirred for 15 min at -78^oC and then heated at reflux for 20 h. After addition of an aqueous 2.5M NaOH solution (100ml), the mixture was stirred for 3 h at room temperature and then extracted with ethyl acetate (3x 50 ml), dried with Na_2SO_4, and evaporated to dryness in vacuum. The residue was purified by column chromatography on silica gel (6g, 65%).

$[\alpha]_D^{20}$ -34 (c = 0.5, MeOH)

^{1}H NMR (200mz, $CDCl_3$) δ: 7.38 (s, 5H, Ar), 4.18-4.03 (m, 2H, H3', H5'), 3.74-3.55 (q, 2H, Ar-CH_2), 2.95-2.75 (m, 2H,H_2), 2.28-1.98 (m, 4H, H6, OH, H4), 1.51-1.38 (m, 1H, H4'), 0.94 (s, 9H, TBDMS), 0.12 (m, 6H, TBDMS)

^{13}C NMR ($CDCl_3$) δ: 174, 159.5, 137.4, 128.8, 127.9, 126.8(Ar), 69.9 (C5), 65.9 (C3), 62.0 (N-CH2-Ar), 59.6 (C2), 59.1(C6), 39.4 (C4), 25.5, 17.7, 5.1.

(3*R*,5*R*)-3-*O*-mesyl-*N*-benzyl-5-[(*tert*. butyl)dimethylsilyl)-oxy]-piperidine 7

To a stirred ice cooled solution of compound **6** (3g, 9.3 mmol) in dry pyridine (20ml), was added drop wise methanesulphonylchloride (1.6 ml, 13.96 mmol). After 3h, upon completion of reaction, the pyridine was removed under vacuum and the residue was taken in water and extracted with ethyl acetate (4x15ml). The organic layer was dried

over sodium sulphate and concentrated to get the crude product, which was purified by column chromatography on silica gel (1.60 g, 45%).

$[\alpha]^{D}_{20}$ -10.7 (c .013, MeOH)

^{1}H NMR (200mz, $CDCl_3$) δ: 7.4 (m, 5H, Ar), 4.6-4.1 (m, 2H, H3, H5), 4.1-3.6 (m, 3H, N-CH_2-Ar) , 3.2-3.0 (bs 1H, H6) , 2.9-2.6 (m, 2H, H2, H6') , 2.6-2.5 (s, 1H, H2') , 2.1-1.9 (m, 3H,OMs), 0.9 (s, 10H, TBDMS, H4) , 0.2 (s , 6H, TBDMS),

ESIMS calculated for C_{19} $H_{33}NO_4SSi$ 399 found 400,

(3*S*,5*R*) –*N*-Benzyl-5-[(*tert.* butyl)dimethylsilyl)-oxy]-3 azido-piperidine 8

Compound **7**, (3g, 7.4 mmol) was taken in dry DMF (30 ml) and to this was added NaN_3 (8g, 123 mmol) The mixture was stirred at 65°c for 12 h, when the reaction was found to be complete by TLC. The solvent was completely removed in *vacuo* and the residue was diluted with water. The product **5** was extracted in ethyl acetate (3x 20 ml), organic layers were pooled, washed with water (2x 10 ml), brine (1x 10 ml) and concentrated to give thick oil which was then purified by column chromatography on silica gel (1.47g, 56%).

$[\alpha]_{D}^{20}$ +47.27 (c .010, MeOH)

^{1}H NMR (200mz, $CDCl_3$) δ: 7.4 (s, 5H, Ar)**,** 4.3 (m, 1H, H3), 4 (m, 1H, H5), 3.5 (m 1H, N-CH_2-Ar), 3.48-3.3 (m, 1H, NCH_2-Ar), 3.2-3.0 (m, 2H, H6), 2.6 (m, 1H, H2), 2.47(m, 1H, H2'), 2.0-1.8 (m, 2H, H4), 1.6 (m, 1H, H4'), 0.9 (s, 9H, TBDMS), 0.2 (s, 6H, TBDMS)

ESIMS calculated for $C_{18}H_{30}N_4OSSi$ 350 found 351

(3*S*,5*R*)-*N*-Benzyl-5-[(*tert.*butyldimethylsilyl)-oxy]-3-(*tert.*butyloxycarbonylamino) piperidine 9

Compound **8**, (3g, 8.57mmol) was taken in ethyl acetate. To this Raney Nickel (2.1 ml, suspension in EtOH) and di-tert.butyldicarbonate (2ml, 9.17mmol) was added. This mixture was subjected to hydrogenation for 2 h at 40 psi in a Parr hydrogenation apparatus. Ra/Ni suspension was removed by filtration through celite. The filtrate was concentrated to give crude product, which was then purified by column chromatography (3.2 g, 85%).

^{1}H NMR (200mz, $CDCl_3$) δ: 7.3 (s, 5H, Ar), 5.2 (m, 1H), 4.1-3.6 (bs, 2H, H5), 3.65-3.4 (m, 2H, N-CH_2Ar), 2.9-2.8 (m, 1H, H3), 2.7-2.5 (m, 1H, H5), 2.3-1.6 (m, 4H, H2, H6), 1.4 (m, 9H, Boc), 0.8 (s, 9H, TBDMS) 0.0 (s, 6H, TBDMS)

^{13}C NMR ($CDCl_3$) δ: 155, 137.8, 128.7, 128.2, 127.0, 78.9, 65.4, 62.3, 61.1, 56.8, 45.9, 38.4, 28.3, 25.7, 18.0, 4.7.

ESIMS calculated for $C_{23}H_{40}N_2O_3Si$ 421 Found 421

(3*S*,5*R*)-*N*-Benzyl-5-[(*tert.*butyldimethylsilyl)-oxy]-3-(*tert.*butyloxycarbonylamino) piperidin *N*-1 ethyl acetate 10

Compound **9** (2.7 g, 6.42 mol) was taken in methanol and to this $Pd(OH)_2$, (0.27g) was added. The mixture was subjected to hydrogenation at 50 psi for 8 h. $Pd(OH)_2$ suspension was removed by filtration and filtrate was evaporated to dryness. This free

amine was immediately subjected to alkylation reaction. To the solution of amine in dry THF, DIPEA (1.02ml, 6.1mmol), and ethyl bromoacetate (1.08 ml, 7.5 mmol) was added drop-wise. Reaction mixture was stirred at room temperature for 2h.When no starting material was observed by TLC, the solvent was evaporated and the residue was purified by column chromatography (2.2g, 90%).

^{1}H NMR (200mz, $CDCl_3$) δ: 5.4-5.3 (m, 1H, NH-Boc), 4.3-4.1 (q, 2H, C$\underline{H}_2$CH$_3$), 4-3.8 (m, 2H,H3, H5), 3.2 (s, 2H, N-C$\underline{H}_2$Ar), 3-2.8 (m, 1H, H6), 2.7-2.6 (m, 1H, H2), 2.5-2.4 (m, 1H, H2'), 2.3-2 (m, 2H, H4) 1.4 (s, 9H, Boc), 1.3 (t, 3H, CH$_2$C$\underline{H}_3$), .9 (s, 10H, TBDMS), 0.2 (s, 6H, TBDMS)

(3*S*,5*R*)- 3-*N*-(*tert*.butyloxycarbonylamino)-*N*-1 ethyl acetate -5-(hydroxy)-piperidine 11

To the above product (2.5 g, 5.9 mmol) in dry THF (120ml), TBAF (2.35 ml, 9 mmol) was added and the reaction mixture was stirred for 5 h. When TLC indicated complete disappearance of starting material solvent was removed under vacuum and the residue was purified by column chromatography to get pure product **11**. (1.4g, 85%)

$[\alpha]_D^{20}$ +7.5 (c 0.004, MeOH).

^{1}H NMR (200mz, $CDCl_3$) δ: 4.88 (bs, 1H), 4.22-4.11 (q, 2H), 3.96 (bs, 2H), 3.29 (s, 2H), 2.81 (bs, 2H), 2.5 (bs, 2H), 1.71 (bs, 2H), 1.44 (s, 9H), 1.28 (t 3H).

^{13}C NMR ($CDCl_3$) δ: 170.2 ($COOCH_3$), 159, 128.6 (C3), 124.91 (C5), 60.1 ($COOCH_2CH_3$), 58.5 (NCH_2-CO), 58.26 (C2) 56.9 (C6), 36.5 (C4), 28.0 ($C(CH_3)_3$, 12.0 (OCH_2CH_3).

ESIMS calculated for $C_{14}H_{26}N_2O_5$ 302 found 302

(3*S*,5*S*)-3-*N*-(*tert.*butyloxycarbonylamino)-5-*N*3-benzoylthymin-1-yl-*N*-1 ethyl acetate 12

To a stirred solution of compound **11**, (0.17g, 0.56mmol), *N*3-benzoylthymine (0.25g, 1.09mmol) and PPh_3 (0.29g, 1.10mmol) in dry THF (10ml) at room temperature, diisopropylazodicarboxylate (0.19ml, 1.09mmol) was added drop-wise. After completion of the reaction as indicated by TLC (12h), the solvent was removed and residue was purified by column chromatography (90mg, 30%).

^{1}H NMR (200mz, $CDCl_3$) δ: 8.15 (s, 1H,Thy-H6), 7.90 (d, 1H), 7.61-7.41 (m, 5H, Ar), 4.97 (m, 1H,H3), 4.18 (q, 2H, OCH_2CH_3, H5), 3.6-3.32 (m, 4H, H6, NCH_2-CO), 2.82-2.4 (m, 2H, H2), 2 (s, 3H, Thy-CH_3), 1.93 (m, 1H, H4), 1.72 (m, 1H, H4'), 1.41(s, 9H, Boc), 1.25 (t, 3H,OCH_2CH_3).

^{13}C NMR ($CDCl_3$) δ: 170.6 (COOEt), 168.8, 162.1 (Thy-C4), 155.1 (Thy-C2), 149.4 (Thy-C6),134.0, 131.6, 131.08, 130.0, 128.8, 128.3 (Ar), 128.1($C(CH_3)_3$), 109.4, 79.0 ($C(CH_3)_3$, 60. (O-CH_2CH_3), 59.7 (C3), 58.6 (C5), 53.69 (N-CH_2-COO), 50.7 (C2), 49.3 (C6), 35.8 (C4), 28.0 ($C(CH_3)_3$, 13.9 (O-CH_2CH_3), 11.9 (Thy-CH3).

ESIMS calculated for $C_{26}H_{34}O_7N_4$ 514 Found 514

(3*S*,5*S*)- 3-*N*-(*tert.*butyloxycarbonylamino)-5-thymin-1-yl-*N*-1-acetic acid 13

To a solution of (3*S*,5*S*)-3(*tert.*butylooxycarbonylamino)–*N*3 benzoylthymin-1-yl-*N*-1 ethyl acetate **12** (0.11g) in methanol 5ml, was added 1ml aqueous NaOH (2N). The reaction was stirred at room temperature for 16 h. After the completion of reaction, the

solvent was evaporated and residue was washed with ethyl acetate. Aqueous layers were pooled together and then excess NaOH was neutralized by Dowex H^+ resin. The resin was removed by filtration washed with methanol and the filtrate was concentrated to get the product as white foam. (90mg, 89%)

$[\alpha]_D^{20}$ -16.0 (C,0.005, MeOH)

^{1}H NMR (200mz, D2O) δ: 7.7 (d, 1H, Thy-H6), 4.1-3.7 (m, 2H, H3, H5), 3.7-3.4 (m, 2H, H6) 3.4-3.2 (m, 1H, H2'), 2.6 (m, 1H, H2), 1.9 (m, 2H, H4) 1.7 (s, 3H, Thy-CH_3) 1.4 (s, 9H, $C(CH_3)_3$).

^{13}C NMR (D_2O) δ: 179.2, 174.5, 170.5 (COOH), 170.1, 166.4 (NHCO), 157.0, 152.8, 142.3, 138.8 (C2-Thy), 135.1 (C6-Thy), 111.7 (C5-Thy), 109.9 (C3), 104.6 (C5), 65.15 (N-CH_2COOH), 59.9 (C2), 47.8 (C6), 32.8 (C4), 27.46 ($C(CH_3)_3$, 21.9, 11.0 (Thy-CH3),

ESIMS calculated for $C_{17}H_{26}N_4O_6$ 382 Found 382

(3*S*,5*S*)- **3-(*N*-*tert.*butyloxycarbonylamino)-5-*O*-benzoyl-(piperidin)-*N*-1 ethyl acetate 14**

To a mixture of **11** (0.5 g, 0.165mmol), benzoic acid, (0.22g, 0.18mmol), PPh_3 (0.48g, 0.181mmol) and DIAD (0.4 ml, 0.19mmol) in freshly dried THF were added. Reaction was kept for stirring for 4 h when TLC indicated complete disappearance of starting material. Solvent was removed under vacuum and residue was purified by column chromatography to obtain pure product **14**.

^{1}H NMR (200mz, $CDCl_3$) δ: 8.15-8.03 (m, 4H), 7.66-7.37 (m, 6H, OBZ), 7.33-7.27 (m, 5H,Ar), 5.38-5.07 (m, 2H, H3,H5), 4.11 (bs, 1H, NHBoc), 3.7 (m, 2H, NCH_2Ph), 3.1-3(m, 1H, H6), 2.57 (s, 2H, H6',H2), 2.5-2.49 (m, 1H, H2'), 2.12-1.74 (m, 2H, H4), 1.45 (s, 9H, $C(CH_3)_3$)

^{13}C NMR (200mz, $CDCl_3$) δ: 170.4 (OCOAr), 165 (BocCO), 136.4, 132.8, 132.7, 130.0, 129.7, 129.4, 128.0 (Ar), 127.1 ($C(CH_3)_3$, 79.4 ($C(CH_3)_3$, 76.8 (C3), 76.2 (C5), 67.75 (N-CH_2), 56.9 (C6), 55.6 (C2), 34.5 (C4), 28.1 ($C(CH_3)_3$.

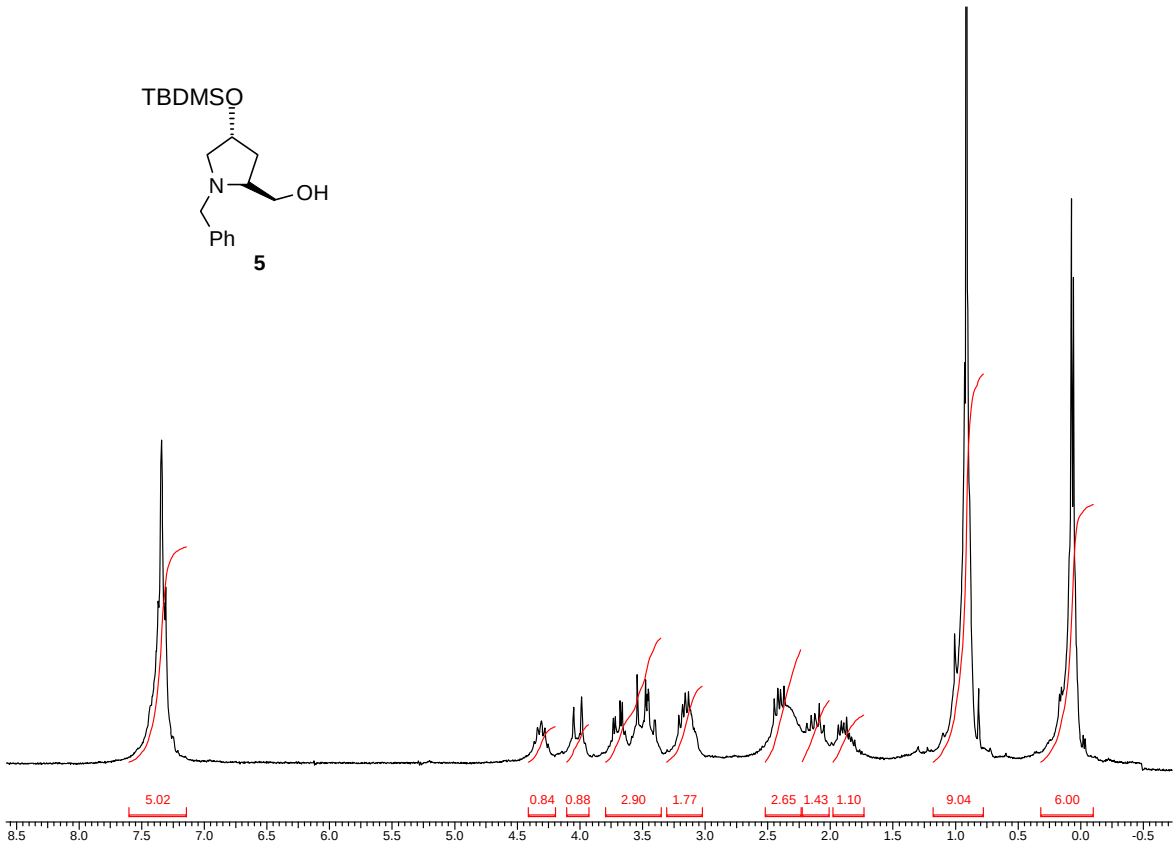
(3*S*,5*S*)- 3-(*N*-*tert.*butyloxycarbonylamino)-5-hydroxy-(piperidin)-*N*-1 ethyl acetate 15

Compound 14 (0. 2g, 0.26mmol) was taken in absolute methanol and sodium metal (50 mg) was added to it while maintaing the temperature. Reaction mixture was stirred for 1 h when TLC indicated complete disappearance of starting material. Aqueous $KHSO_4$ was added to neutralize the base and the mixture was concentrated in *vacuum*. The residue was taken in EtOAc and washed with water and brine. The organic layer was dried over anhydrous Na2SO4 and concentrated to give residue, which was further purified by column chromatography.

^{1}H NMR (200mz, $CDCl_3$) δ: 7.49 (s, 5h, Ar), 4.67 (s, 1H, NHBoc), 4.0 (m, 2H, N-CH_2Ar), 3.52 (m, 2H, H3, H5), 2.9-1.9 (m, 4H, H2, H6) 1.8-1.6 (m, 2H, H4), 1.5 (s, 9H, $C(CH_3)_3$).

4.11. Appendix

♦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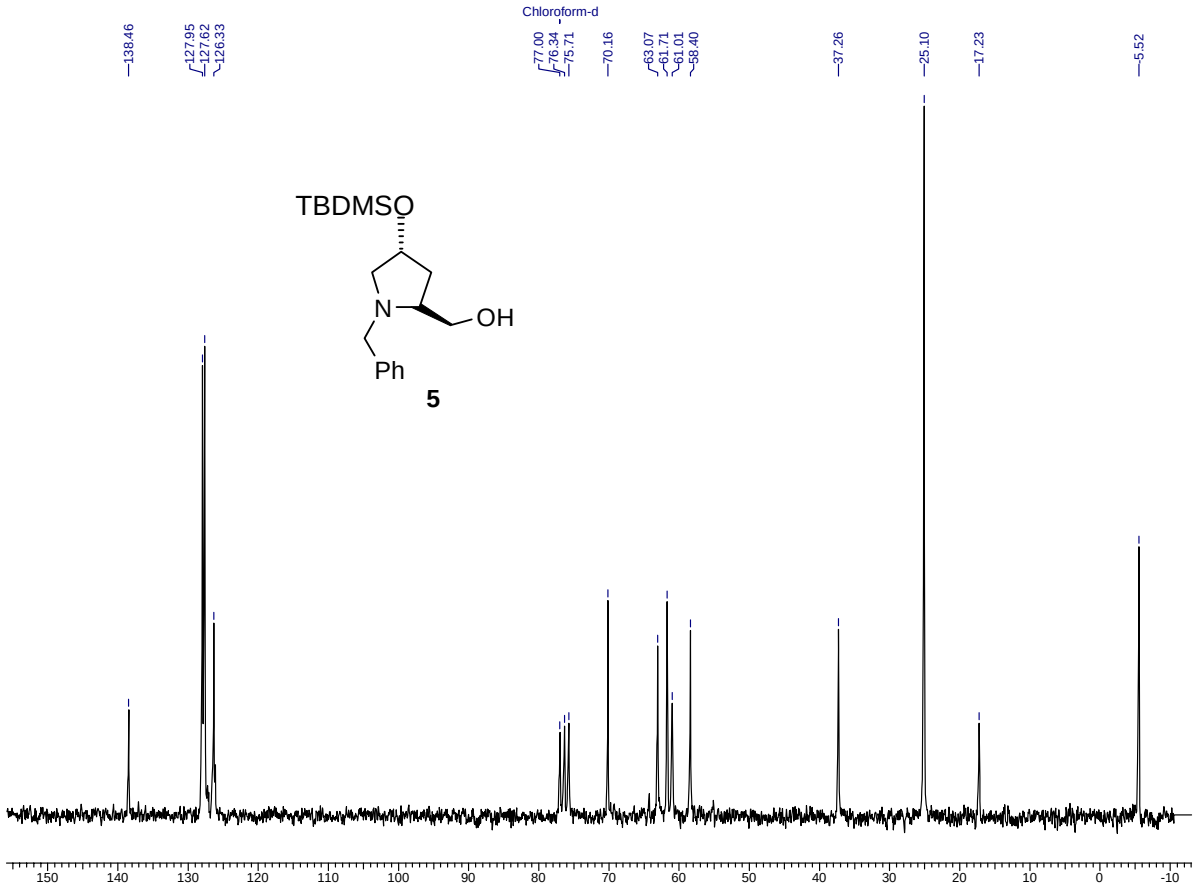
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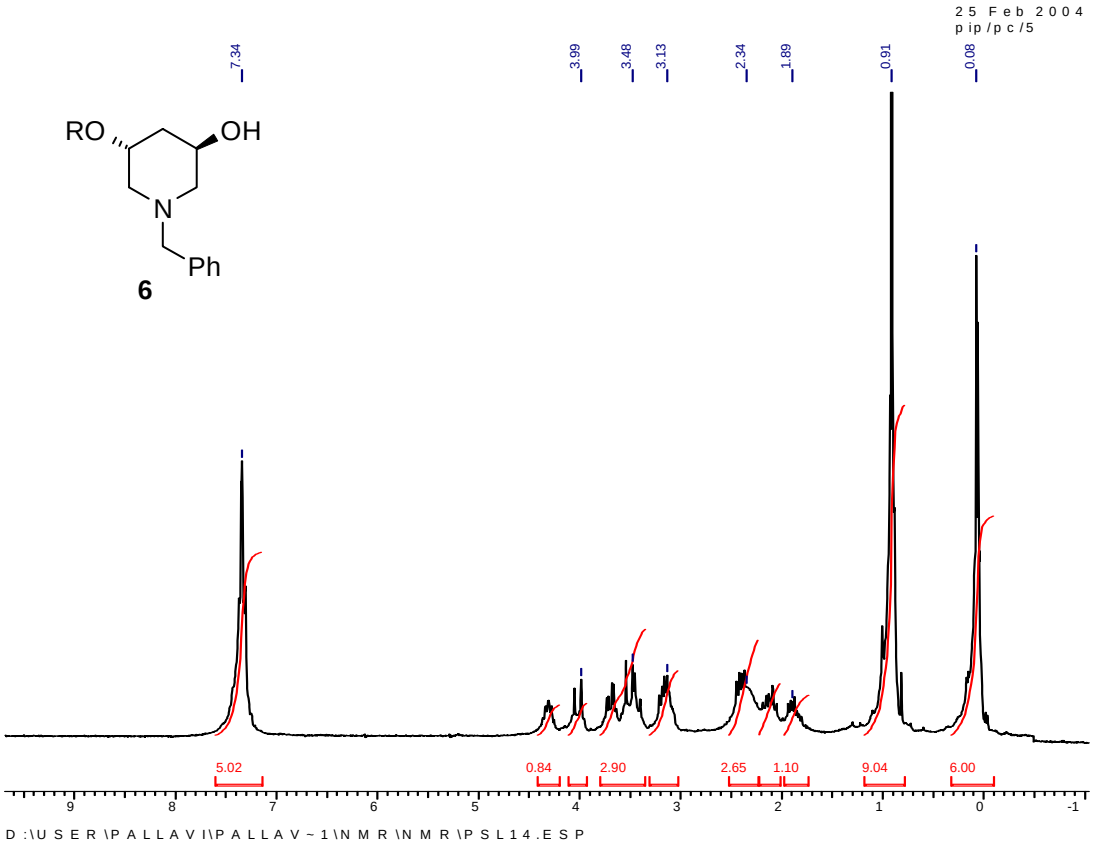
25 Feb 2004
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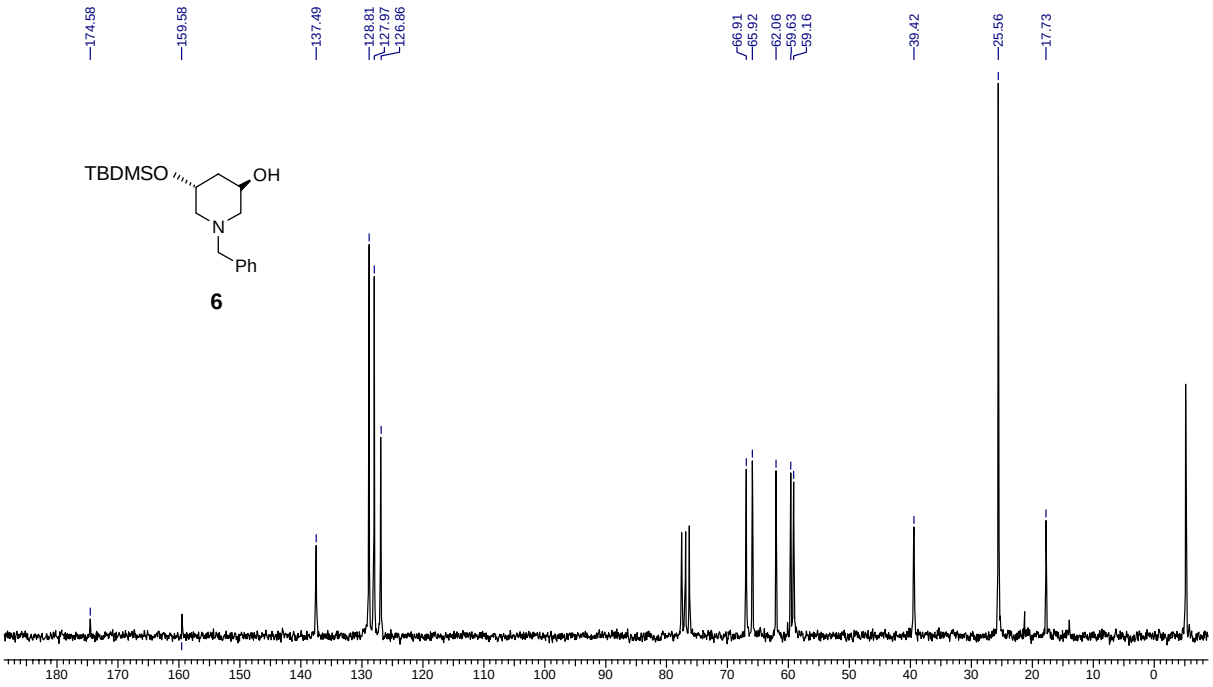
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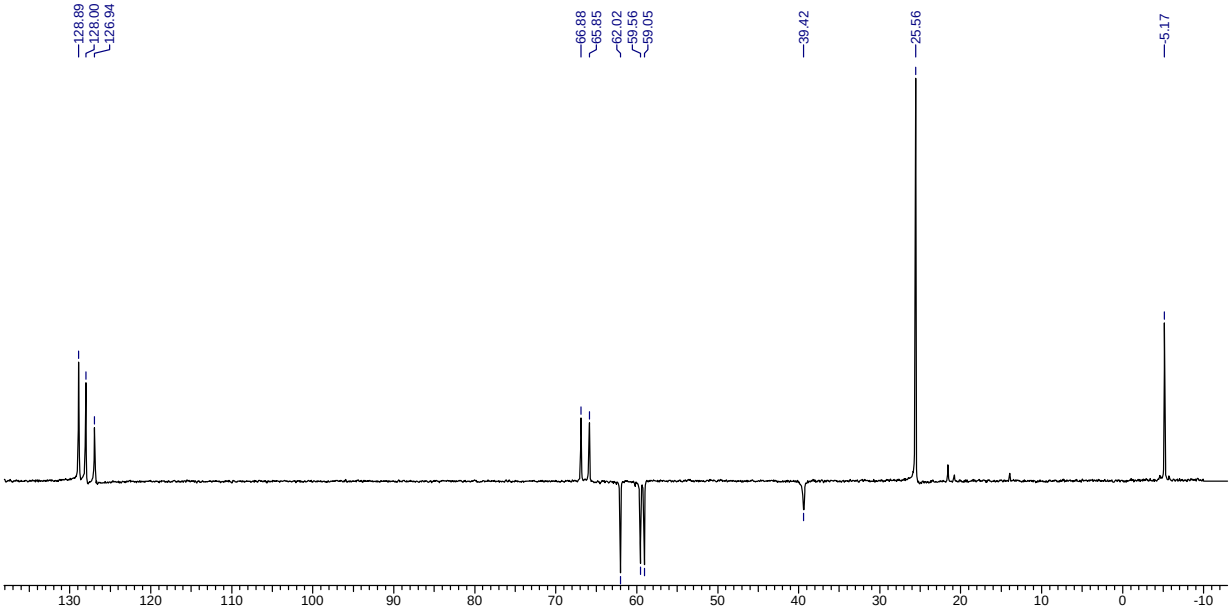
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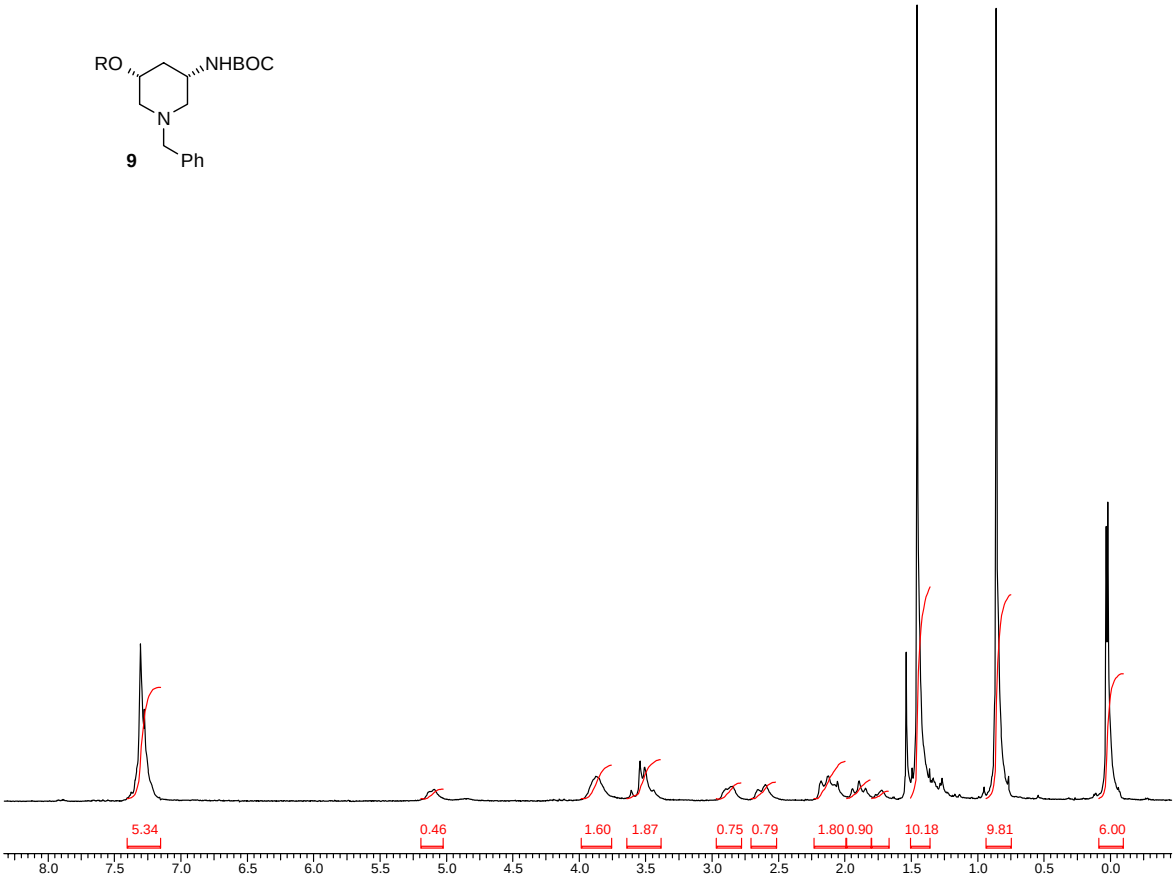
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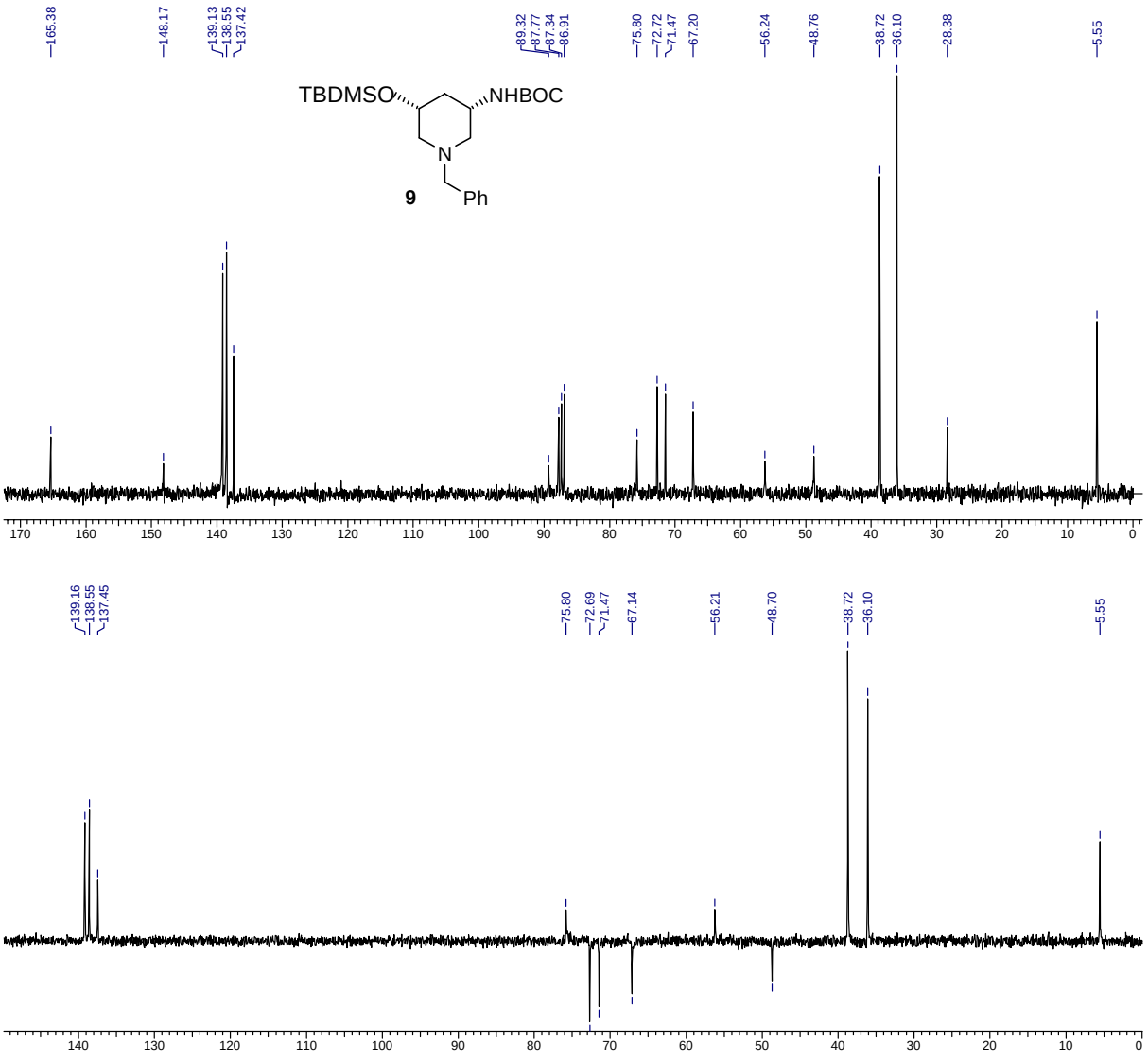
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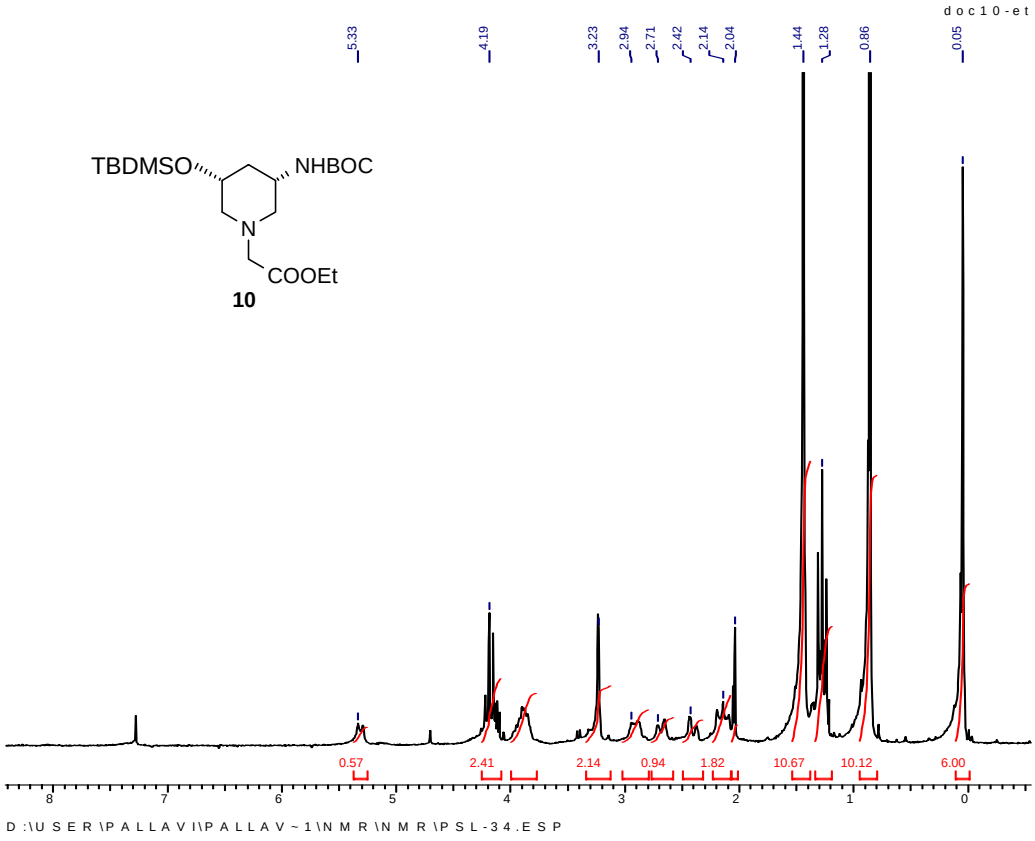
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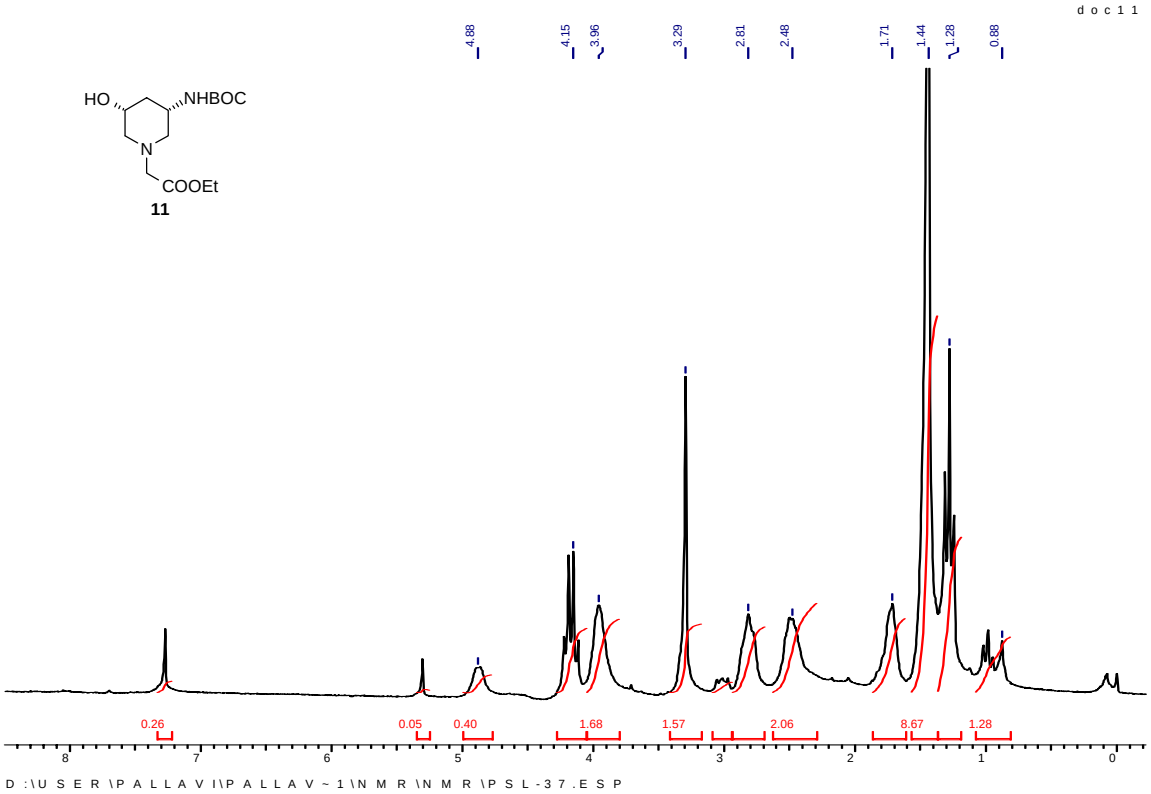
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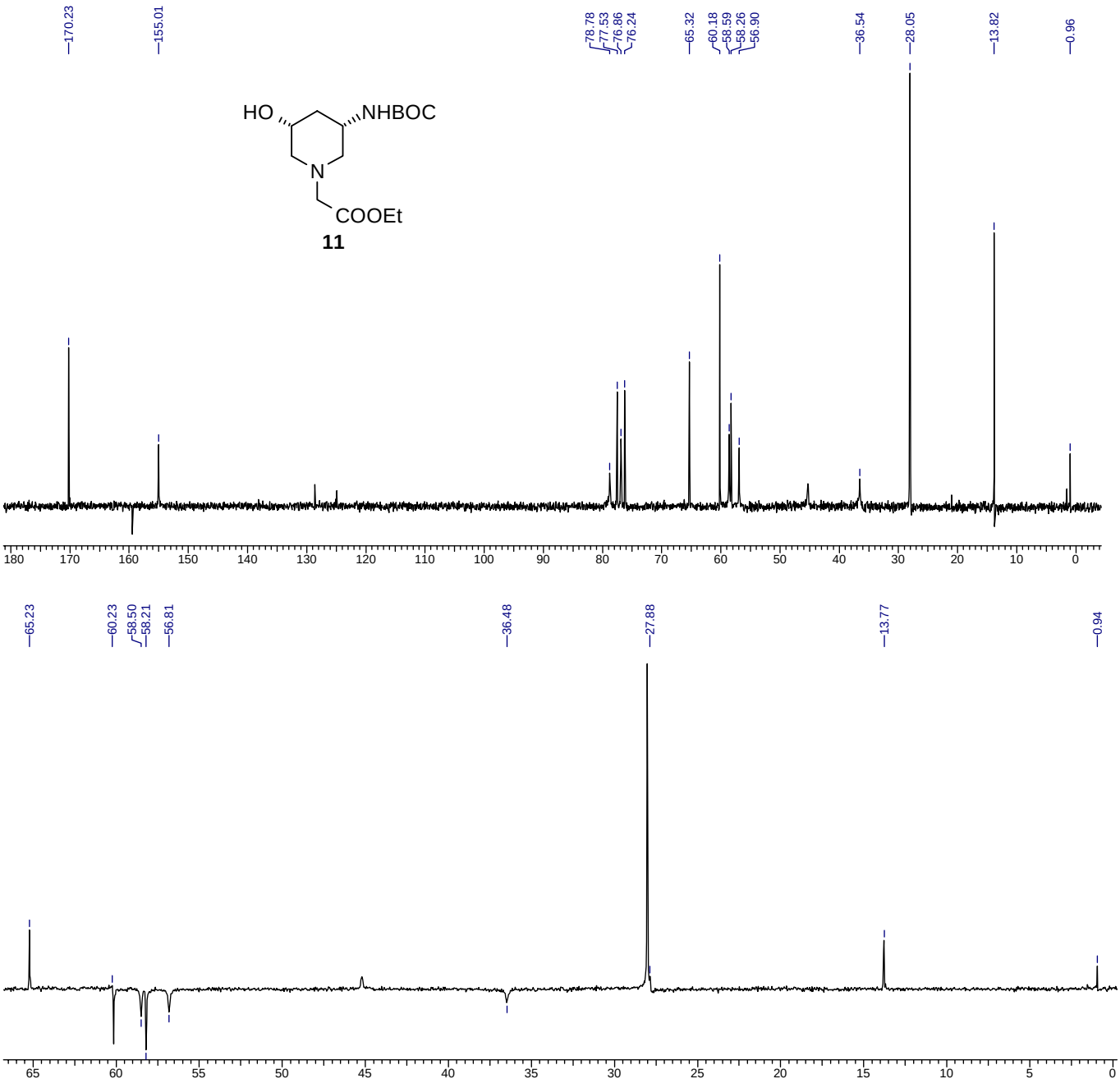
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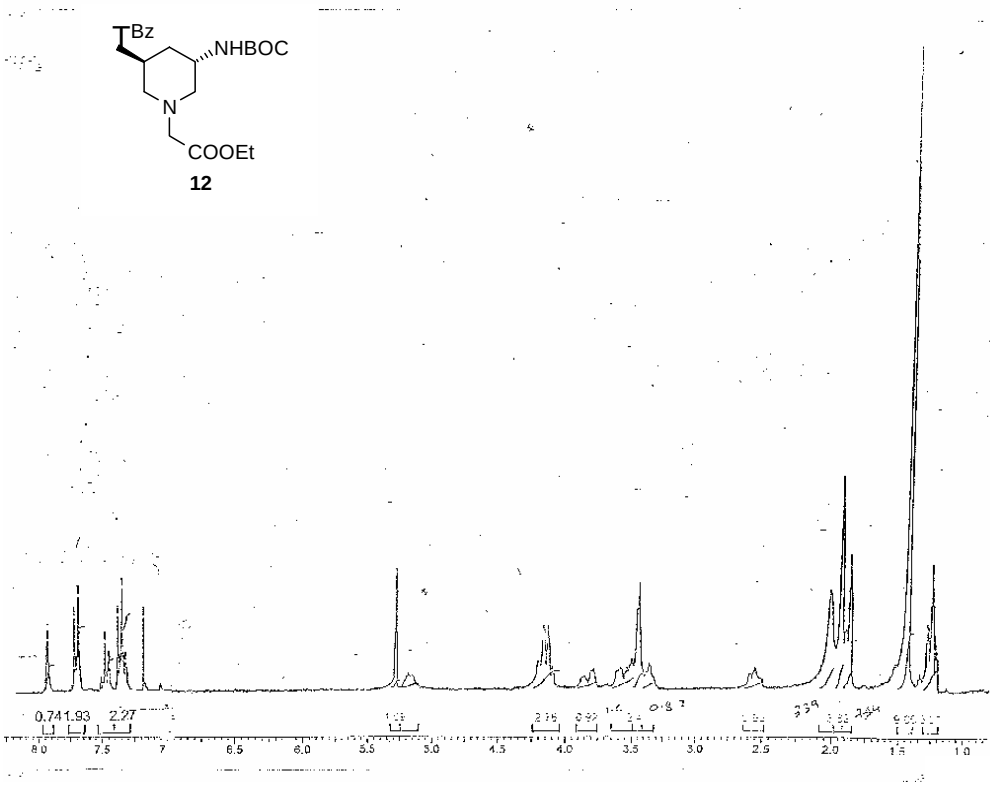
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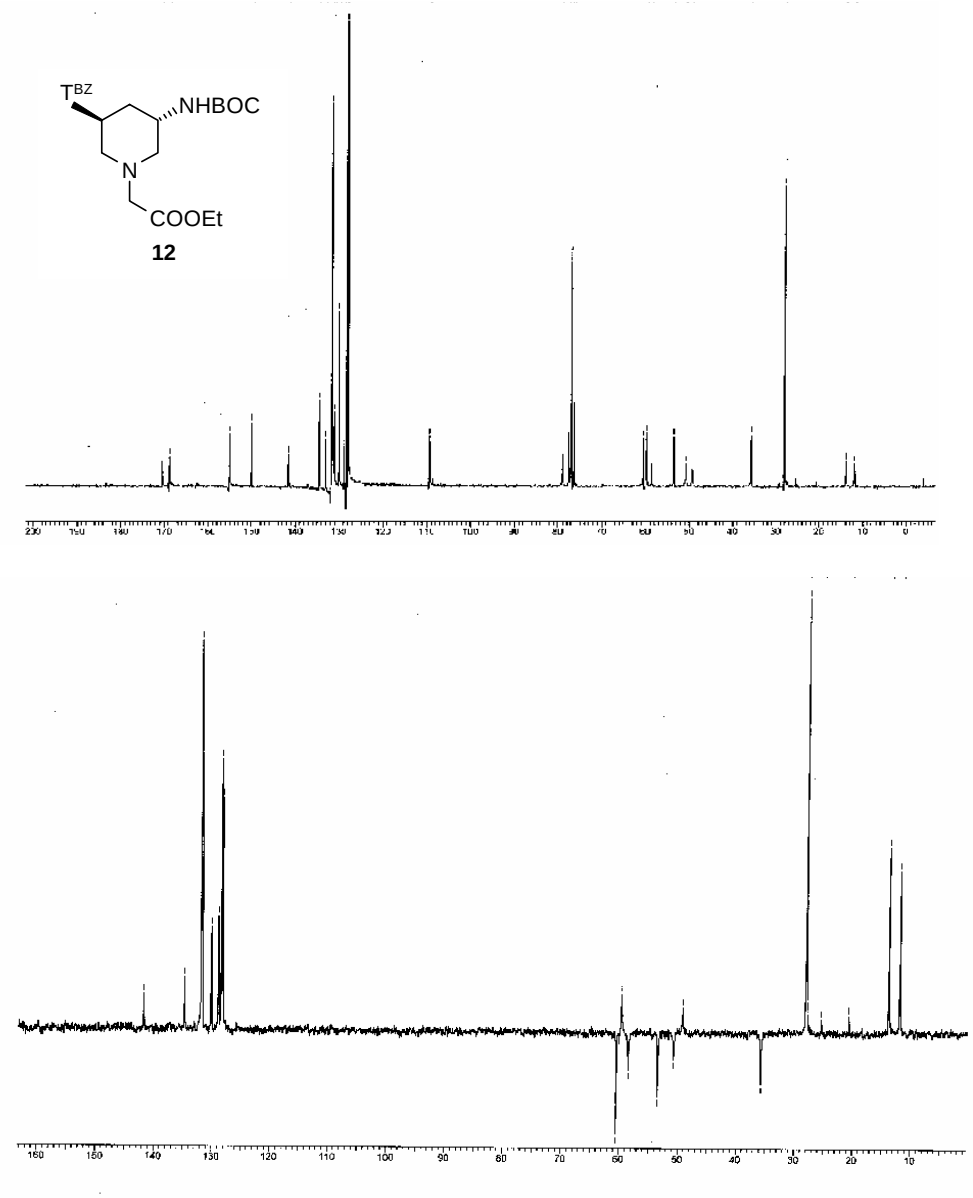
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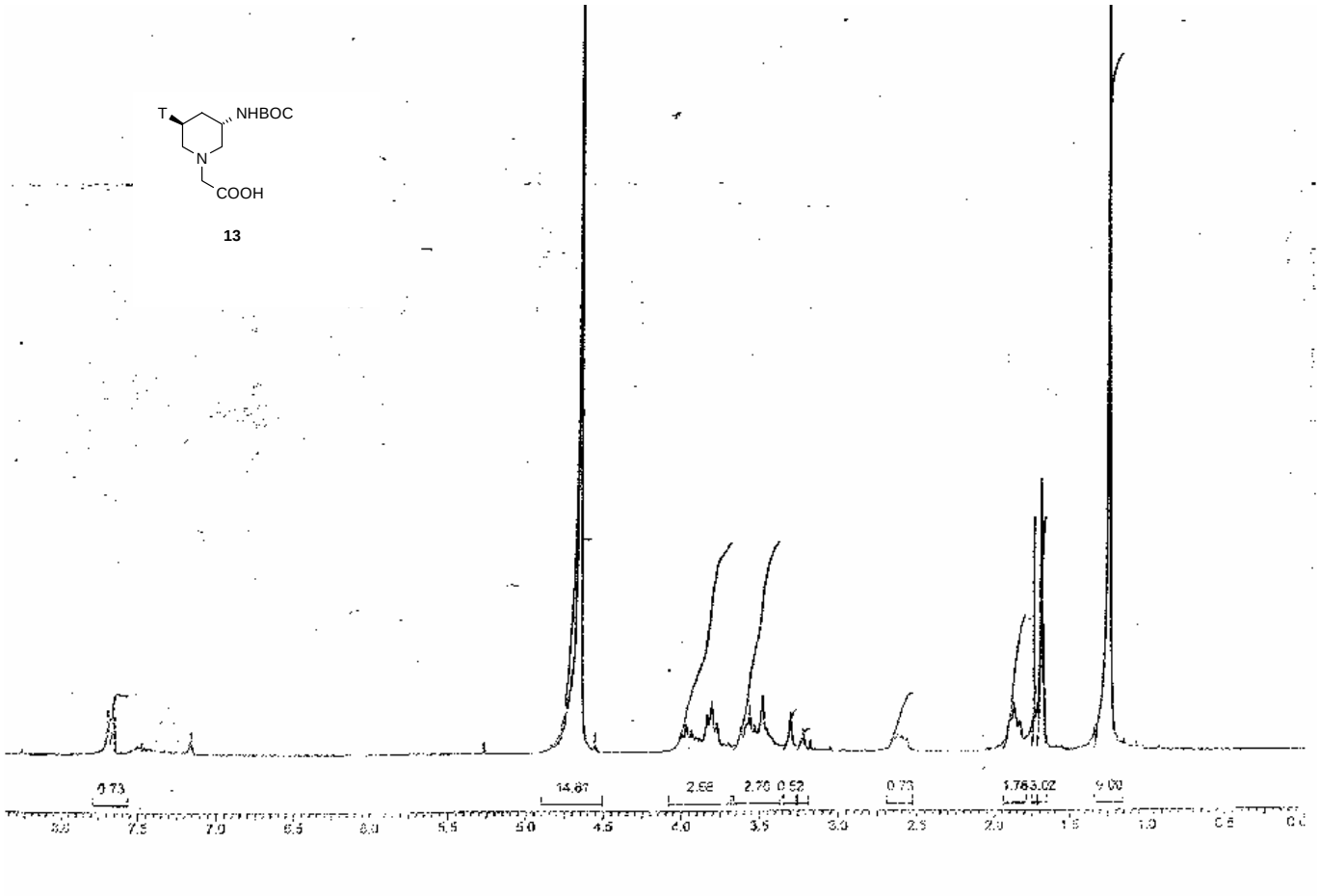
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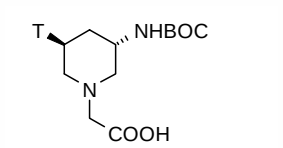
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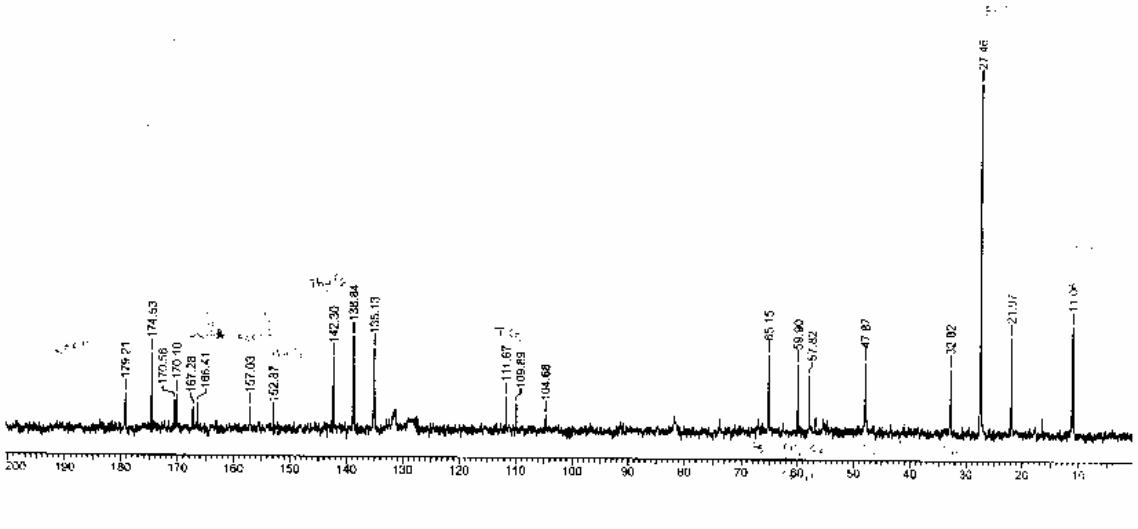
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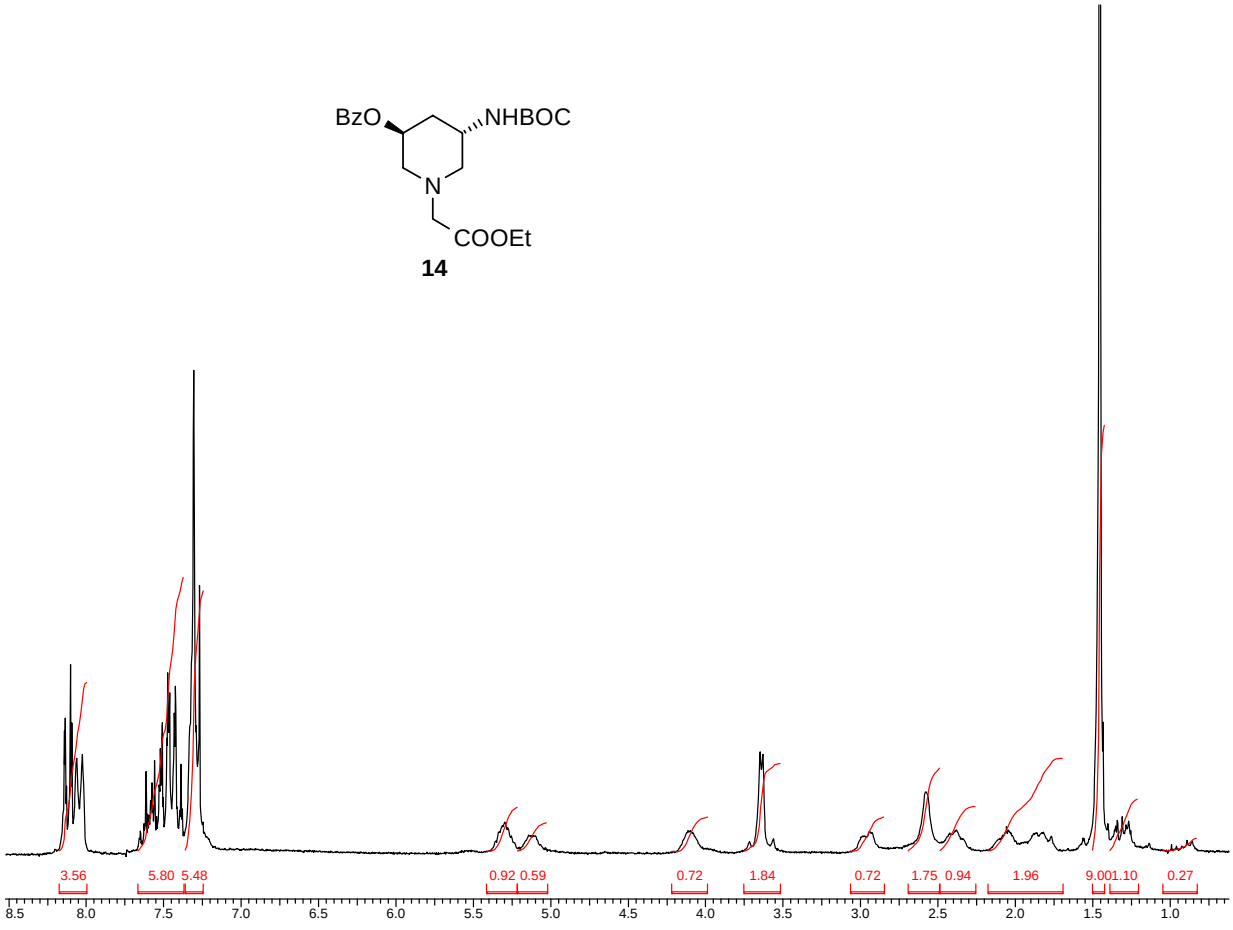
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4.13. References

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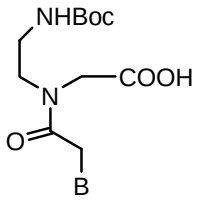
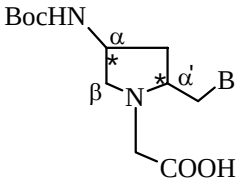
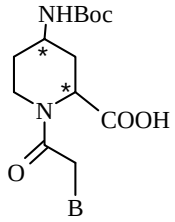
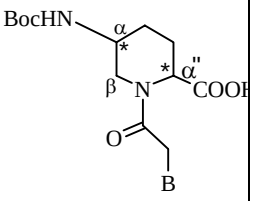
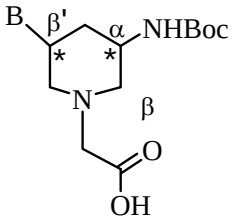
Summary and outcome

PNA	DNA/RNA binding	Solubility	*p/ap* orientation
***aeg*PNA** 	Binds to both DNA and RNA equally	Very low water-solubility	Bind to DNA/RNA both in parallel and antiparallel modes - ambiguity in binding
Pyrrolidine PNA 	(2*S*,4*R*) –T **stabilizes** triplex while (2*R*,4*S*) highly destabilizes triplex. Duplex-(2*S*,4*R*) & (2*R*,4*R*)-T **stable complexes**. **RNA-stable complexes** with all four diastereomers.	soluble in water	(2*S*,4*R*)- T larger *p-ap* orientational selectivity.
4-aminopipecolyl PNA 	Hybridizes with DNA but with low affinity. Duplex-Stabilizes complex with RNA rather than DNA	Less soluble in water	Larger *p-ap* orientational selectivity.
5-aminopipecolyl PNA 	Triplex-strong binding affinity for DNA. Duplex-destabilizes complex with Both DNA as well as RNA.	Less soluble in water	*p-ap* orientational selectivity.not observed-
Piperidinyl PNA 	Stable triplexes as well as duplexes with complememntary DNA.	soluble in water	Very good *p-ap* orientational selectivity.