CHAPTER 2

Synthesis and assignment of configuration of \textit{amp}-monomers and its incorporation into \textit{aeg}-PNA oligomers.
2.1 Introduction

Among all the DNA mimics, PNA introduced by Nielsen et. al\textsuperscript{1} in 1991 has been found to mimic many properties of DNA. The structure of PNA is remarkably simple, the repeating unit of PNA consisting of N-(1-aminoethyl)-glycine units linked by amide bonds and the nucleobases (A, G, C and T) attached to the backbone through methylene carbonyl linkages. Thus PNA lacks sugar and phosphate groups, making it acyclic, neutral, achiral and homomorphous. PNA is resistant to cellular enzymes and has strong affinity towards complementary DNA/RNA. Homopyrimidine PNA oligomers bind strongly to complementary DNA by Watson-Crick and Hoogsteen bonding to form [(PNA)\textsubscript{2}:DNA] triple helices that are much more stable than the corresponding DNA-DNA hybrids. The unique character of PNA is binding to duplex DNA by strand invasion forming both parallel (N-terminus of PNA to 5' end of the DNA) and antiparallel C-terminus to 5' end respectively) complexes.

![Chemical structure of DNA and PNA](image)

Figure 1 Chemical structure of DNA and RNA
In contrast to homopyrimidine sequences, mix sequences of PNA bind to complementary DNA/RNA with 1:1 stoichiometry to form duplexes. Here binding can be in either parallel or antiparallel mode, the latter mode having higher stability than parallel hybrids with high sequence specificity and affinity.\textsuperscript{2} These favorable hybridization properties\textsuperscript{2,3} with high chemical and bio-stability\textsuperscript{4} make PNA a promising lead for development as efficient antisense agents and medicinal drugs.\textsuperscript{5} However, despite of these advantages, PNA suffers from poor membrane permeability, inadequate solubility problems, ambiguity in binding orientation and lack of selectivity towards DNA/RNA. These characters limit PNAs potential as an antisense agent.

To address the limitations of PNA for biological applications several modifications have been introduced into the classical PNA monomer\textsuperscript{6} and many of these modifications have resulted in only marginal effects in terms of hybridization properties. However, these studies have pointed out the importance of rigidity and preorganization of PNA for effective complexation with complementary DNA. From this laboratory there have been several reports on the introduction of novel five membered pyrrolidine rings into PNA backbone to impart chirality as well as conformational constrain in the PNA backbone\textsuperscript{7} resulting in both neutral and positively charged PNA oligomers (Figure 2).
Figure 2 Conformationally constrained PNA analogues

2.2 Rationale behind the design:

The work presented in this thesis is directed towards the introduction of chirality and conformational constrain in the classical PNA backbone to control the orientation selectivity and specificity in binding by preorganization. This can be achieved in several
ways as shown in Figure 2 to obtain neutral and positively charged monomers. Bridging the β-carbon of ethylene diamine unit and α"-C of glycine unit with a methylene afforded the neutral ap-PNA analogues. Bridging the α-α" carbons of the classical PNA results the another class of neutral monomers (amp-PNA). Bridging the α-β', β-α' and α"-β' gave the positively charged preorganized PNA monomers. These kind of modifications involve the introduction of rigidity to the flexible aminoethyl glycyl backbone and or nucleobase side chain, simultaneously introducing chirality in the molecule with the generation of one or two asymmetric centers.

Figure 3 Rational behind the design

The present work is directed towards the synthesis of aminomethyl prolyl amp-PNA which were designed by bridging the α-α" carbons of the classical aeg-PNA with ethylene bond (Figure 3). This novel approach creates two chiral centers and less rigidity in aeg backbone compared to other cyclic modifications. This modification restricts movement in both the aminoethyl and the glycyl segments of the aegPNA and restrain the fluctuation region of γ and δ torsion angles.

A similar kind of modification but containing a sulphar atom in the ring was reported by Chassaing et. al in 2001(Figure 3a).6a
2.3 The Objectives of the present chapter are:

1. Functionalization of C5 of the proline ring to introduce a methoxy function that can be subsequently used for the conversion to the amino-methyl group, which is a part of the aminoethyl segment of the aeg PNA backbone.

2. Synthesis of N-(tert-butoxycarbonyl)-fluorenylmethoxycarbonyl-aminomethyl- L/D proline isomers (Figure 4) for the synthesis of the PNA oligomers starting from L/D-proline.
3. Synthesis and characterization of amp-PNA oligomers (Figure 5) employing both orthogonal and submonomer strategy.


![Figure 5 Chemical structure of PNA and amp-PNA](image)

2.4 Synthesis of amp-PNA monomers

**Synthesis of (2S,5S) and (2S, 5R) -N1-(tert-Butoxycarbonyl)-5-[(NFluorenlymethoxycarbonyl) aminomethyl]-Proline (9 and 10)**

The synthesis of target amp PNA monomers 9 and 10 (Figure 4 and Scheme 1) was achieved starting from L-proline 1, which on treatment with thionyl chloride in methanol afforded the 2-carboxymethyl ester 2 as its hydrochloride salt. The ring nitrogen (N1) of the ester 2 was protected as tert-butoxycarbonyl by treatment with tert-butyl carbazide and triethyl amine in dioxane/water to provide the N-(tert-
butoxycarbonyl) proline carboxymethyl ester 3. This was then subjected to electrochemical oxidation employing Ross-Eberson-Nyberg reaction.\textsuperscript{17} Earlier reports\textsuperscript{17} on use of this reaction on substituted pyrrolidine have revealed that the methoxylation occurs at the least substituted site adjacent to the nitrogen atom.

$$\text{Scheme 1: Synthesis of (2S,5R) and (2S,5S) nitriles}$$

The anodic oxidation involved the treatment of ester 3 with methanol as a solvent-reagent and tetrabutylammonium-tetrafluoroborate as a supporting electrolyte and passing a constant current of 0.06F/cm\textsuperscript{2} (260 mA) using graphite electrodes. The reaction mixture was cooled to ice temperature before passing the current and the temperature of the reaction was maintained between 0-10 °C for about 12 h. The efficiency of the reaction depends on controlling the temperature and current optimally to avoid the formation of C-2 and C-5 dimethoxylated products. Compound 4 formed in 95% yield, as
a non-separable diastereomeric mixture. The formation of C5-methoxylated compound was confirmed by the observance of multiple peaks for C5-methoxy (OCH$_3$) at δ 3.30-3.45 in $^1$H NMR and appearance of peaks for (OCH$_3$) at δ 51.64 in $^{13}$C NMR. The mechanism of methoxylation (Scheme 2) involves electrochemical removal of one electron from the lone pair on nitrogen in the initial step when inert supporting electrolytes (an electrolyte added to the solution for the sole purpose to increase the solution conductivity, while the electrolyte does not take part in any reactions) are used. The reaction was explored in the synthesis of C-5 methoxypoline, which is the key intermediate in the synthesis of amp submonomers.

![Scheme 2: Mechanism of electrochemical oxidation](image)

The diastereomeric mixture of compound 4 was treated with TMSCN in DCM employing catalytic amount of TMSTF at -35 °C for about 30 min, the reaction was quenched at -35 °C using dry methanol resulting the formation of C5-nitriles$^{19}$ 5 and 6 in 50% yield. The product obtained as diastereomeric mixture was separable by chromatography on neutral alumina. The formation of compounds 5 and 6 confirmed by
appearance of new peaks in the IR spectrum at 2260 cm$^{-1}$, and appearance of a signal at $\delta$
118 (C5-nitrile group) in the $^{13}$C NMR.

5 (minor) + 6 (major) $\rightarrow$

MeOH, Raney Ni, Net$_3$, 65 psi, 3h.

1) MeOH, 2N NaOH,
2) Dioxane/H$_2$O, 10% Na$_2$CO$_3$
30%.

(2S,5S) minor

(2S,5R) major

**Scheme 3**: Synthesis of (2S,5S) and (2S,5R) amp-PNA monomers

The diastereomers 5 and 6 showed different sign and magnitude of optical rotation of 5 ([α]$_D$ = −93.7) and 6 ([α]$_D$ = +41.8). These were individually subjected to hydrogenation at 65 psi, in MeOH employing Raney Ni as catalyst to afford the 5-methylaminoproline methyl esters 7 and 8. The identity of compounds 7 and 8 was confirmed by the appearance of peaks at 3100 and 3200 cm$^{-1}$ in the IR spectrum due to the 5-methyl amino group. These amino compounds upon hydrolysis in 2N NaOH in MeOH yielded the sodium salt of the acid, which on treatment with fluorenyl chloroformate afforded the required amp monomers 9 and 10 in 30% yield, along with
the by-product 10a (Scheme 3). Compound 10a was characterized by NMR and Mass spectral data. When the reduction reaction was carried out in the presence of Pd(OH)$_2$ formation of compound 10a was observed in high percentage compared to that of the required monomers 9 and 10 (Scheme 3). The formation of compounds 9 and 10 was supported by observance of characteristic signals for Fmoc aromatic protons at δ 7.26-7.78 in $^1$H NMR and appearance of peaks for (Fmoc-CO) at δ 141.2 in $^{13}$C NMR. The observed mass for compound 9 is 491.50 (M+Na) and for compound 10 is 491.15 (M+Na) was in agreement with calculated mass 466.

**Electro-oxidation**

Electroorganic chemistry can be classified into two categories, that is, direct and indirect reactions. In both categories, the reaction is initiated by transfer of electron between electrode and a substrate A, and as shown in eqn (1), the substrate A is transformed to an anion radical or a cation radical depending on the transfer of electron. When the starting substrate A is radical or ionic species, the pattern of transformation of A is such as shown in eqn 2, where -e means the removal of one electron, and -2e means removal of two electrons; +[e] means addition of electrons in appropriate numbers.

$$
\begin{align*}
A^2- &\xrightarrow{-e} A^- &\xrightarrow{+e} A &\xrightarrow{-e} A^+ &\xrightarrow{+e} A^{2+} &1 \\
A^- &\xrightarrow{-e} A &\xrightarrow{+e} A^+ &2
\end{align*}
$$

*Electro-organic chemistry is to investigate the chemical behavior of activated species of A in solution.* The generation of the similar activated species may be possible by using common organic reactions. The chemical behavior of the same activated species
is, however, often different between electro-organic chemistry and common organic chemistry. One of the major causes of this difference is that in an electro-organic reaction, the activated species is not formed uniformly in homogeneous solution, but is generated only on the surface of electrode, whereas in common organic reactions, the active species is uniformly distributed in the solution. This difference in location of generation of activated species and in distribution of activated species brings about great difference in the reaction of the activated species.

![Electro Chemical Cell](image)

Viewed from the standpoint of organic synthesis, electroorganic chemistry has remarkable characteristics. In common organic reactions the reaction generally takes place between nucleophilic reagents (Nu) and electrophilic reagents (E), while reaction between reagents of the same polarity is not possible. Therefore, the inversion of polarity of one of the reagents is essential for carrying out the reaction between Nu and Nu or E and E. The inversion of polarity of reagents (Umpolung) is, however, not facile in common organic reactions, whereas in electroorganic reaction, the process of formation
of the activated species is the Umpolung itself as shown in eqns (1) and (2). Thus, one of
the major characteristics of electro organic chemistry is the facility of Umpolung, which
makes a variety of organic syntheses possible.

2.5 Assignment of absolute stereochemistry at C₅ position of amp PNA monomers (9
and 10).

An important aspect remaining in the characterization of (2S,5S) and (2S,5R) amp
PNA is the assignment of the configuration at C5 position where a new chiral center was
generated upon anodic oxidation. This was achieved as described in Scheme-4.

![Scheme 4](image)

**Scheme 4** Configuration assignment of compound 9 and 10 amp monomers

Compounds 9 and 10 were individually treated with 50% DCM/DEA at rt for
about 8 h to furnish the amino acids 11 and 12. Compound 12 when treated with
HOBT/HBTU, in DMF, amino acid 12 furnished the compound 13. The formation of
compound 13 was confirmed by appearance of peaks in IR spectrum at 1690 cm⁻¹ and
observance of carbonyl (C=NH) peaks at δ 172 in ¹³C spectrum. The observed mass for
compound 13 is 227.11 (M+1) was in agreement with the calculated mass 226. In case of
amino acid 11, no cyclisation product was observed. In compound 12 and hence in 10 the
C5 and C2 substituents must be are cis to each other to obtain the cyclized product. Since
the configuration at C2 is known in acid 10, which is same as in starting material L-proline, the absolute configuration at C5 in the compound 10 that is cis to C2, would be R (2S,5R). The absolute configuration of acid 9 is therefore (2S,5S). In another approach the configuration at C5 was assigned by preparing (2S,5S) pyrrolidine dicarboxylic acid a marine natural product from the nitrile ester 5, which is discussed in chapter 4. \(^{19a}\)

![Chemical structure](image)

(2S,5S) PyrrolidinediCarboxylic acid

**Scheme 4a**

2.6 Synthesis of (2R,5S) and (2R,5R)-N1-(tert-Butoxycarbonyl)-5-[(N-Fluorenyl methoxycarbonyl) aminomethyl]-proline (22 and 23).

The synthesis of (2R)-5-[(N-fluorenymethoxycarbonyl) aminomethyl-N1-(tert-butoxycarbonyl) proline 22 and 23 were similarly achieved starting from D-proline 14 (Scheme 6). The steps involved esterification of D-proline, protection of the ring nitrogen, C5-functionalization by anodic oxidation and conversion of the 5-methoxy-N1-(tert-butoxycarbonyl) proline methyl ester 17 to the separable C5-nitrile diastereomers 18 and 19.
Scheme 5: Synthesis of (2R,5R) and (2R,5S) nitriles

The two nitrile compounds were subjected to reduction to get the amino-methyl ester 20 and 21, which were hydrolysed using 2N NaOH, followed by protection of the amino group employing Fmoc-Cl to furnish the required amp PNA monomers 22 and 23. The integrity of these monomers were confirmed by the appearance of peaks for (Fmoc protons) at δ 7.74-7.28 in 1H NMR and observance of peaks for (Fmoc aromatic carbons) at δ127.57-125.21 in 13C NMR. The observed mass for compound 22 is 467.16 (M+1) and for compound 23 is 467.48 (M+Na) both in agreement with the calculated mass 466.
Scheme 6: Synthesis of (2R,5R) and (2R,5S)-amp monomers.

2.7 Assignment of absolute stereochemistry at C5 position of compound 18 and 19

(amp monomers).

The absolute stereochemistry at C5 of the compounds 18 and 19 was assigned as described in Scheme-7. The two 5-cyano proline esters 18 and 19 were individually subjected to hydrolysis in 6 N HCl for about 14 h to afford the hydrochloride salt of the pyrrolidine 2,5-dicarboxylic acids 24 and 25. These diacids were subjected to esterification employing SOCl₂ in MeOH followed by neutralization to furnish the diester.
26 and 27 in 30% yield. The magnitude of the optical rotation of compound 26 is \([\alpha]_D = 0\) since the configuration at C2 in ester 18 is known as R, the stereochemistry at C5 of diester 26 is cis to C2 (meso) with R configuration, and hence the configuration of ester 18 is (2R,5S). The stereochemistry of compounds amp 22 and 23 are therefore (2R,5S) and (2R,5R) respectively.
2.8 4-Hydroxy *amp* monomer:

Rational behind the design

The *amp* monomers (9, 10) and (22, 23) enable the study of the constrain and steric effects on the hybridization properties of incorporated PNA oligomers with complementary DNA. It will not provide any information about conformational effect of the proline ring. Proline is a cyclic imino acid and the bridging of the α-carbon atom to the main chain amide nitrogen atom by 3 methylene bridge imposes further constrain on the main chain torsion angles $\phi$ and $\psi$ (Figure-5A).

![Diagram](image)

**Figure-5:** Main chain torsion angles of amino acid residues in polypeptides **A**; Endocyclic torsion angles in amino acid proline **B**.

Proline and substituted-proline rings exhibit two types of ring-pucker, and in analogy to ribo and deoxyribo sugars, they are named as *N* (γ-exo) and *S* (γ-endo) puckers. These ring-puckers are also expressed in terms of *endocyclic* torsion angles (Figure 5B). In proline the two puckers are almost equally preferred and the energy barrier to inter-conversion is very low.\(^{20}\) However, in 4-substituted prolines, depending on the steric and electronic effects exerted by the 4-substituent, pyrrolidine ring may prefer any one of the ring-pucker, as found in both its crystal structures and in solution.\(^{20}\)
This pucker preference has been attributed to the phenomenon of hydroxyl-amide gauche effect.\textsuperscript{21,22}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{gauche_effect_diagram.png}
\caption{Ring-puckers of the pyrrolidine ring in proline and substituted proline}
\end{figure}

**Gauche effect on the ring-pucker preferences**

Gauche effect may be described as “the preference of two electronegative atoms X and Y in vicinally substituted ethanes to remain gauche with respect each other rather than anti”.\textsuperscript{12} This effect surprisingly arises from a combination of dipole repulsion and steric effects among the electronegative atoms on the vicinal substituents that are expected to remain anti to each other. Many molecules containing N, O, P, S, F or Cl show a preference for gauche conformation. The origin of the gauche effect is not very clear and \textit{ab initio} quantum chemical calculations underestimate the gauche effect. σ-Hyper-conjugation\textsuperscript{22} and bent-bonds\textsuperscript{23} have been proposed to explain the phenomenon of the gauche effect. In 4-substituted prolines, the steric repulsion between the amide-ring nitrogen and the 4-substituent should result in a pseudo-equatorial positioning of the 4-substituent i.e., anti with respect to the ring amide-nitrogen. For example, 4\textit{R}-hydroxyproline and 4\textit{R}-fluoroproline may be expected to exhibit \textit{γ-endo} ring-pucker (Figure-7). However analysis of X-ray crystal structure, $^1$H-$^1$H and $^{19}$F-$^1$H coupling constant analyses confirm the pseudo-axial positioning of fluorine and the resulting \textit{γ-exo} ring-pucker\textsuperscript{24}. This suggests that the gauche effect may be a dominating factor in determining the ring-pucker preference for proline with 4-electronegative substituent.
Similarly, in 4-\textit{S}-fluoroproline with an opposite stereochemistry at C-4 the gauche effect leads to \textit{γ-endo} ring-pucker (Figure 7B).\textsuperscript{25}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Newman and saw-horse projections depicting the gauche effect and the gauche effect and the resulting pucker preferences in the prolines with electronegative A 4\textit{R}-substituent; B 4\textit{S}-substituent}
\end{figure}
In view of such 4-substituent effects on pyrrolidine ring conformation and hence consequently on the backbone, 4-substituted aminomethyl prolyl PNA monomers were synthesized.

2.9 Synthesis of 4-hydroxy aminomethyl prolyl (amp) monomer (35)

The synthesis of 4-hydroxy ampPNA monomer was achieved (Scheme 8a) starting from N-Boc hydroxyproline 28, which on treatment with DMS/K$_2$CO$_3$ in acetone afforded the methyl ester of hydroxyproline 29. This under Mitsunobu conditions using acetic acid yielded the acetate 30, which on electrochemical anodic oxidation afforded the methoxy compound 31 as a nonseparable distereomeric mixture. Treatment of 31 with TMSCN in presence of BF$_3$Et$_2$O in DCM, furnished the cyano derivatives 32 (minor) and 33 (major).

Scheme 8a Synthesis of 4-hydroxy-amp monomer 35

Reagents and conditions: (i) Acetone, K$_2$CO$_3$ (3 equiv.), DMS (2 equiv.), Reflux 5 h; (ii) THF, DEAD (1.2 equiv.), (Ph$_3$)P (1.1 equiv.), CH$_2$CO$_2$H (1.1 equiv.), rt, 8 h; (iii) MeOH, TBATFB, 260 mA, 0-5 °C, 6 h; (iv) DCM, BF$_3$Et$_2$O (2.2 equiv.), TMSiCN (3.5 equiv.), 0 °C-rt, 3 h; (v) DCM, (Boc)$_2$O, DMAP (Catalytic), rt, 12 h; (vi) (a) MeOH, RaneyNi, NEt$_3$ (3 equiv.), 60 Psi, rt, 4 h, (b) MeOH, 2 N NaOH, (c) Dioxane/H$_2$O, Na$_2$CO$_3$ (2 equiv.), Fmoc-Cl (1.2 equiv.).
The ring nitrogen in cyano derivative 33 was protected as Boc by treating with Boc anhydride in DCM employing catalytic amount of DMAP to provide nitriile compound 34. The formation of compound 34 was confirmed by the observance of peaks for (C5-CN) in IR at 2260 cm\(^{-1}\) and the appearance of the peak for (C5-CN) at \(\delta\) 118 in \(^{13}\)C NMR. The observed mass for the compound 34 is 313 (M+1) was in agreement with calculated mass 312. The configuration at C-5 was assigned from the small coupling constant of H-4 and H-5 as reported in literature\(^{26}\) (Scheme 8b). This was subjected to hydrogenation at 60 psi in the presence of Raney/Ni, followed by hydrolysis using 2N NaOH in MeOH to afford the sodium salt of 4-hydroxy aminomethyl proline (not isolated). This on treatment with Fmoc-Cl in presence of 10% Na\(_2\)CO\(_3\) in dioxane furnished the 4-hydroxy amp monomer 35.

![Scheme 8b Assigning the configuration at C5 of compound 34\(^{26a}\)](image)

The integrity of 4-hydroxy amp monomer 35 was confirmed by the observance of characteristic peaks for (Fmoc protons) at \(\delta\) 7.71-7.20 in \(^1\)H NMR and appearance of peaks for (Fmoc aromatic carbons) at \(\delta\)127.57-125.21 in \(^{13}\)C NMR. The observed mass for compound 35 is 505.13 (M + Na) which was in agreement with the calculated mass 482. Employing BF\(_3\).Et\(_2\)O during the cyanation reaction afforded the ester compound 33 as the major isomer, with the diastereomeric ratio being 80:20.

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2.10 Synthesis of Aminoethylglycyl (aeg) PNA monomers.

To study the influence of modified monomers on hybridization properties of PNA oligomers, *amp* PNA monomers were site specifically incorporated in *aeg* PNA oligomers along with the unmodified *aeg* PNA monomers, which were synthesized as described in Scheme 9.

The synthesis was carried out as reported in literature\textsuperscript{27-28} starting from the readily available 1,2-diaminoethane (Scheme 9). The monoprotected derivative of 36 was prepared by treating a large excess of 1,2-diaminoethane with di-\textit{t}-butyloxyanhydride in THF under high dilution conditions to minimize the formation of di-Boc derivative. The di-Boc compound being insoluble was removed by filtration. The N1-\textit{t}-Boc-1,2-diaminoethane was then subjected to N-alkylation using ethylbromoacetate and NE\textsubscript{T}\textsubscript{3} as base in acetonitrile to give compound 38. The aminoethyl glycine ester 38 was not stable.
for longer time at room temperature and was treated with chloroacetyl chloride in aqueous dioxane with Na₂CO₃ as a base to yield the chloro compound 39. The ethyl N-(t-Boc-aminoethyl)-N-(chloroacetyl)-glycinate 39 was used as a common intermediate for the preparation of all the four PNA monomers.

Alkylation of the ethyl N-(t-Boc-aminoethyl)-N-(chloroacetyl)-glycinate is regiospecific to N1 of thymine. Thymine was reacted with ethyl N-(t-Boc-aminoethyl)-N-(chloroacetyl)-glycinate using K₂CO₃ as a base to obtain N-(t-Boc-aminoethylglycyl)-thymine ethyl ester 40 in high yield. In case of cytosine, the exocyclic-amine N4 was protected as Cbz (Scheme 10b) to obtain 52,²⁸ which was used for alkylation employing K₂CO₃ as the base to provide the N1-substituted product 41. Adenine was treated with K₂CO₃ in DMF to give potassium adenylate, which was then reacted with ethyl N-(t-Boc-aminoethyl)-N-(chloroacetyl)-glycinate to obtain N-(t-Bocaminoethylglycyl)-adenine ethyl ester 42 in moderate yield. The alkylation of 2-amino-6-chloro purine with ethyl N-(t-Boc-aminoethyl)-N-(chloroacetyl)-glycinate was facile with K₂CO₃ as the base and yielded the corresponding N-(t-Boc-aminoethylglycyl)-(2-amino-6-chloro purine) ethyl ester 43 in excellent yield. All the compounds exhibited ¹H and ¹³C NMR spectra were consistent with the reported data.²⁷-²⁹ The ethyl esters except that of cytosine monomer were hydrolyzed in the presence of 2N NaOH to give the corresponding acids 44, 45, 47, which were used for solid phase synthesis.

In case of 2-amino-6-chloropurine monomer ester hydrolysis, the chloro group is oxidized to keto group to give guanine monomer 47. Cytosine monomer is more susceptible to Cbz deprotection in strong basic conditions and so in this case mild base LiOH was used for hydrolysis to afford the monomer acid 46.
The need for the exocyclic amino group protection for adenine and guanine was eliminated, as they were found to be unreactive under the conditions used for peptide coupling.

2.11 Alkylation of Nucleobases:

*amp* PNA oligomers were synthesized by employing the submonomer\textsuperscript{30a} strategy, in which the nucleobases were coupled to the ring nitrogen of *amp* monomer on solid support by converting to their N1-acetic acids, coupling was performed using HOBT/HBTU to obtain the *amp* PNA oligomers.

2.11.1 Synthesis of N\textsuperscript{1}-Thyminyl Acetic Acid (49)

Thymine 48 was alkylated\textsuperscript{20} as described in (Scheme 10a) using chloroacetic acid in aq KOH solution under reflux conditions for about 2h, maintaining the pH of the reaction by controlled addition of chloroacetic acid to the reaction mixture which is a key factor to obtain the acid 49 in good yields.

\begin{center}
\begin{equation}
\text{Scheme 10a: Synthesis of N\textsuperscript{1}-Thyminyl Aceticacid}
\end{equation}
\end{center}

2.11.1 Synthesis of N\textsuperscript{4}-Benzoxycarbonyl N\textsuperscript{1}-cytosinyl Acetic Acid (53)

For the synthesis of ester 52, protection of the exocyclic amino group of cytosine 50 is required in order to prevent the chain extension from this position during the peptide synthesis. The protection was done via its benzylxoycarbonyl derivative,\textsuperscript{29} using benzyl chloroformate in anhydrous pyridine which afforded the protected derivative N4-benzylxoycarbonyl cytosine C\textsuperscript{Cbs} 51 (Scheme 10b).
**Scheme 10b:** Synthesis of N\textsuperscript{1}-Benzyloxy carbonyl N\textsuperscript{1}-cytosinyl Acetic Acid

The N1-alkylation of C\textsuperscript{Cbz} 51 was carried out using ethylbromoacetate and potassium carbonate in dry DMF to furnish the ester 52. This on hydrolysis using 2N LiOH provided the required N4-(benzyloxy carbonyl) cytosin-1-yl acetic acid 53.

**2.11.2 Synthesis of (N\textsuperscript{6}-Benzyloxy carbonyl) N\textsuperscript{9}- Adenyl Acetic Acid (56)**

The exocyclic amine of adenine 54 was protected as benzyloxy carbonyl using benzyl chloroformate as in Scheme 10c in anhydrous DMF employing NaH as base to obtain the N\textsuperscript{6}-benzoyloxy derivative 55. This was subjected to alkylation with ethylbromoacetate in DMF using K\textsubscript{2}CO\textsubscript{3} as base and the resultant ester was hydrolyzed in 2N NaOH to afford the acid 56.

**Scheme 10c: Synthesis of (N\textsuperscript{6}-Benzyloxy carbonyl) N\textsuperscript{9} Adenyl Acetic Acid**
2.12 Solid Phase peptide Synthesis:

The solid phase peptide synthesis was devised by R. B. Merrifield in 1959. In this method the peptide is bound to an insoluble support in contrast to the solution phase method, and offers great advantages. The C-terminal amino acid is linked to an insoluble matrix such as polystyrene beads having reactive functional groups, which also acts as a permanent protection for the carboxylic acid (Figure 8). The Nα-protected amino acid is coupled to the resin bound amino acid either by using an active pentafluorophenyl (pfp) or 3-hydroxy-2, 3-dihydro-4-oxo-benzotriazole (Dhbt) ester or by in situ activation with carbodiimide reagents. The excess amino acid is washed out and the deprotection and coupling reactions are repeated until the desired peptide is achieved.

Figure 8 Schematic representation of solid phase peptide synthesis
The need to purify intermediates at every step is obviated. Finally, the resin bound peptide and the side chain protecting groups are cleaved in one step. The advantage of the solid phase synthesis are (i) all the reactions are performed in a single vessel minimizing the loss due to transfer, (ii) large excess of monomer carboxylic acid component can be used resulting in high coupling efficiency, (iii) excess reagents can be removed by simple filtration and washing steps and (iv) the method is amenable to automation and semi micro manipulation. The aeg-PNA oligomers and the modified amp PNA oligomers were synthesized by standard solid phase peptide synthesis protocols using both Boc and Fmoc strategies.

**Figure 9:** Some examples of resins used in SPPS
The readily available MBHA resin was chosen as the polymer matrix on which the \textit{aeg} PNA oligomers as well as the \textit{amp} PNA oligomers were assembled. This resin yields the peptides with C-terminal amides upon cleavage at the end of the synthesis. The first amino acid is attached via amide linkage that can be cleaved easily at the end of the synthesis either by acidolysis to get the free peptide acid or by aminolysis to get the carboxyamide.

In the present work the orthogonal strategy was employed to construct the \textit{amp} PNA oligomers followed by subsequent attachment of the nucleobases (sub monomer) strategy (Scheme 9). The \textit{aeg} PNA monomers used have the amino function protected as the tert-butoxycarbonyl group and the modified bifunctional \textit{amp} monomers (9, 10, 22, 23 and 35) have the primary amino function protected as the Fmoc group and the ring nitrogen as tert-butoxycarbonyl group.

In both the cases, HOBT/HBTU activation coupling strategy was employed.\textsuperscript{31} The use of Fmoc protection has drawback in PNA synthesis, as small amount of acyl migration is observed under basic conditions from the tertiary amide to the free amine liberated during the piperidine deprotection step.\textsuperscript{32} However, Fmoc protection employed in case of the \textit{amp} PNA monomers was found to be convenient for extending the oligomers. In the present study all the oligomers were built on MBHA resin using \textbeta- alanine as the C-terminal spacer- amino acid linker. Being achiral, its interference in spectral properties and hydrophobicity of the resulting PNA oligomers were negligible. The amine content on the resin was determined by the picrate assay and found to be 2 mmol/g and loading value was suitably lowered to approximately 0.250 mmol/g by
partial acetylation of total amine content using calculated amount of acetic anhydride. Free -NH$_2$ on the resin available for coupling was again estimated before starting synthesis. The PNA oligomers were synthesized using repetitive cycles (Scheme 11), each comprising of the following steps:

a) Deprotection of the N1-tert-butoxycarbonyl function using 50% TFA in DCM

b) Neutralization of the TFA salt to get the free amine using 5% DIPEA in DCM

c) Deprotection of Fmoc group using 20% piperidine in DMF.

d) Coupling of the amine on resin with 3 to 4 equivalents of free carboxylic function of the incoming amino acid using HOBT(1-hydroxybenzotriazole)/HBTU in DMF as solvent.

e) Capping of the unreacted amino groups using Ac$_2$O / Pyridine in CH$_2$Cl$_2$ in case coupling does not go to completion. Scheme 11 represents a typical Solid phase peptide synthesis cycle. The deprotection of the N-t-Boc protecting group and the coupling reactions were monitored by Kaiser’s test. Alternatively, chloranil$^{35}$ and De Clercq$^{36}$ tests are useful to detect the secondary amine. The t-Boc-deprotection step leads to a positive Kaiser’s test, where in the resin beads as well as the solution are blue in color (Rheumann’s purple). On the other hand, upon completion of the coupling reaction, the Kaiser’s test is negative, the resin beads remain colorless.
2.13 Synthesis of amp PNA oligomers

Homopyrimidine Oligomers

The various PNA oligomers synthesized in the present study are shown in Table 1. The unmodified PNA oligomers $T_8$ and $T_{10}$ were synthesized using the Boc protected $aeg$ PNA monomers (57 and 58). These were used as the control sequences for comparing the properties of the amp PNA oligomers. The synthesis of the oligomers (59 to 87) was achieved by incorporating the chiral, conformationally constrained modified amp PNA monomers at specific positions in the $aeg$-PNA oligomeric sequences. The
synthesis was done on solid support in a similar way, but using Fmoc chemistry for the *amp* monomer coupling and Boc chemistry for the *aeg* PNA monomer coupling to extend the oligomers. Since the *amp* monomers do not carry the nucleobases, these were introduced at desired positions by sub monomer coupling. After this, the *amp* amino acid that has acid stable Fmoc group at amino methyl terminus was treated with TFA to deprotect the ring nitrogen. The thyminyl acetic acid was then reacted with the resin using HOBT/HBTU as coupling agent to attach the nucleobases to the ring nitrogen. This was followed by treatment with 20% piperidine to remove base labile Fmoc group from aminomethyl terminus for initiating the next cycle (Scheme 11). This method involving orthogonal coupling proceeded with good efficiency leading to high purity products. It also circumvented the problem of pre-synthesis of *amp* monomers for each of the bases. *amp* PNA homo oligomers 65, 71, 77 and 83 were synthesized using sub monomer strategy,\(^{30a}\) each oligomer requiring 16 steps for completion of the sequence.

### 2.13.1 Purine-Pyrimidine mix sequence

The polypyrimidine sequences discussed in the previous section are known to form triplexes with the complementary DNA in 2:1 (PNA₂:DNA) stoichiometry. With the aim of studying the duplex forming potential of the present modification, *amp* monomers were also incorporated into a mixed decamer sequences 64, 70, 76, 82 and 87 containing both the purines and pyrimidines using the Boc and Fmoc chemistry. The corresponding unmodified control *aeg* PNA sequence is 58.
2.14 Cleavage of the PNA Oligomers from the Solid Support:

The oligomers were cleaved from the solid support, using trifluoromethanesulfonyl acid (TFMSA) in the presence of trifluoroacetic acid (TFA) ("Low, High TFMSA-TFA method")\(^{37}\) which yields peptide oligomers blocked as amides at their C-terminus. The synthesized PNA oligomers were cleaved from the resin (oligomer attached to β-alanine derivatized MBHA resin) using this procedure to obtain sequences bearing β-alanine-amide at their C-termini. A cleavage time of 60-90 min at room temperature was found to be optimum. The side chain protecting groups were also cleaved during this cleavage process. After cleavage reaction, the oligomer was precipitated from methanol with dry diethyl ether.

2.15 Purification and characterization of PNA oligomers

All the cleaved oligomers were subjected to initial gel filtration to remove small molecule impurities. These were subsequently purified by reverse phase HPLC (high pressure liquid chromatography) on a semi-preparative C8 RP column by gradient elution using an acetonitrile in water or by isocratic elution in 10% acetonitrile-water 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 confirmed by MALDI-TOF mass spectroscopic analysis.\(^{38}\) α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix and dry droplet method was employed in MALDI-TOF mass spectroscopic analysis. Some representative HPLC profiles and mass spectra are shown in appendix of this chapter. The purified PNA 57-87 sequences obtained are listed in Table 1
Table 1 PNA Oligomers synthesized for the present study

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence code</th>
<th>PNA Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>aegPNA 57</td>
<td>H-T-T-T-T-T-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<tr>
<td>3</td>
<td>ampPNA 59</td>
<td>H-(t₅R)-T-T-T-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<td>4</td>
<td>ampPNA 60</td>
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<td>5</td>
<td>ampPNA 61</td>
<td>H-T-T-T-T-T-T-(t₅R)-(CH₂)₂-CO₂NH₂</td>
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<tr>
<td>6</td>
<td>ampPNA 62</td>
<td>H-T-T-T-(t₅R)-T-T-T-(t₅R)-(CH₂)₂-CO₂NH₂</td>
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<tr>
<td>7</td>
<td>ampPNA 63</td>
<td>H-T-T-T-(t₅R)-T-T-T-(t₅S)-(CH₂)₂-CO₂NH₂</td>
</tr>
<tr>
<td>8</td>
<td>ampPNA 64</td>
<td>H-G-(t₅S)-A-G-A-(t₅R)-C-A-C-(t₅R)-(CH₂)₂-CO₂NH₂</td>
</tr>
<tr>
<td>9</td>
<td>ampPNA 65</td>
<td>H-(t₅R)ₖ-(CH₂)₂-CO₂NH₂</td>
</tr>
<tr>
<td>10</td>
<td>ampPNA 66</td>
<td>H-(t₅S)-T-T-T-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<td>11</td>
<td>ampPNA 67</td>
<td>H-T-T-T-(t₅S)-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<td>12</td>
<td>ampPNA 68</td>
<td>H-T-T-T-T-T-T-(t₅S)-(CH₂)₂-CO₂NH₂</td>
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<td>ampPNA 70</td>
<td>H-G-(t₅S)-A-G-A-(t₅S)-C-A-C-(t₅S)-(CH₂)₂-CO₂NH₂</td>
</tr>
<tr>
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<td>H-(t₅S)ₖ-(CH₂)₂-CO₂NH₂</td>
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<td>H-(t₅S)-T-T-T-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<td>ampPNA 77</td>
<td>H-(t₅S)ₖ-(CH₂)₂-CO₂NH₂</td>
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<td>22</td>
<td>ampPNA 78</td>
<td>H-(t₅R)-T-T-T-T-T-T-(CH₂)₂-CO₂NH₂</td>
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<td>ampPNA 79</td>
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<td>ampPNA 80</td>
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<td>25</td>
<td>ampPNA 81</td>
<td>H-T-T-T-(t₅R)-T-T-T-(t₅R)-(CH₂)₂-CO₂NH₂</td>
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<td>26</td>
<td>ampPNA 82</td>
<td>H-G-(t₅R)-A-G-A-(t₅R)-C-A-C-(t₅R)-(CH₂)₂-CO₂NH₂</td>
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<tr>
<td>27</td>
<td>ampPNA 83</td>
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<tr>
<td>28</td>
<td>ampPNA 84</td>
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<tr>
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<tr>
<td>31</td>
<td>ampPNA 87</td>
<td>H-G-T-A-G-A-(t₅S)-C-A-C-T-(CH₂)₂-CO₂NH₂</td>
</tr>
</tbody>
</table>

A/G/C/T = aeg PNA Adenine/Guanine/Cytosine/Thymine monomers, t₅R = (2S,5R)-amp-PNA Thymine monomer, t₅S = (2S,5S)-amp PNA Thymine monomer, t₅R = (2R,5S)-amp-PNA Thymine monomer, t₅S = (2R,4S,5R)-amp PNA Thymine monomer
2.16 Synthesis of Complementary Oligonucleotides

The oligodeoxynucleotides 88-91 (Table 2) were synthesized on a Pharmacia Gene Assembler Plus 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 as ascertained by RP HPLC on a C18 column was found to be more than 98% and were used without further purification in the biophysical studies of PNA. The RNA oligonucleotides 92-94 (Table 2) which are complementary to PNA oligomers 57-87 were obtained commercially from Genomechanix, Gainesville, FL, along with the HPLC purity and mass spectral data.

<table>
<thead>
<tr>
<th>Table 2 DNA/RNA Oligonucleotides</th>
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2.17 CONCLUSION

This chapter describes the synthesis of amp and aeg PNA monomers and sitespecific incorporation of amp monomers into aeg PNA oligomers. The monomers were synthesized starting from L/D proline, with additional methoxy functionality at C5 being introduced by electrochemical anodic oxidation. These were then transformed to the aminomethyl group to form part of the chiral PNA backbone. The modified PNA
monomers were introduced at desired places into the PNA oligomers by solid phase synthesis using submonomer strategy. This has the advantage for introduction of any of the bases during the solid phase synthesis, without making all pre-formed monomers. The oligomers was cleaved from the resin, purified by HPLC and characterised by MALDI-TOF.

Next chapter describes the hybridization studies of above synthesized *amplaeg*-PNA oligomers with DNA and RNA.

2.18 Experimental

All reagents were obtained from commercial sources and used without further purification. NaH was obtained from Aldrich as 60% suspension in paraffin oil and the paraffin coating was washed off with pet-ether before use to remove the oil. The supporting electrolyte tetrabutyl ammonium tetrafluoroborate was obtained from Aldrich and used as such without further purification. All the solvents were dried according to literature procedures. IR spectra were recorded on a Perkin Elmer 599B instrument. $^1$H NMR (200 MHz), $^{13}$C NMR (50 MHz) spectra were recorded on Bruker ACF200 spectrometer fitted with an Aspect 3000 computer. All chemical shifts are with reference to TMS as an internal standard and are expressed in d scale (ppm). The values given are directly from the computer printout and rounded to the decimal place. TLCs were carried out on (E.Merck 5554) precoated silica gel 60 F254 plates. TLCs were visualized with UV light and/or ninhydrin spray, followed by heating after exposing the HCl for the deprotection of the tert-butoxycarbonyl group. Optical rotations were measured on JASCODIP-181 polarimeter. All TLCs were run in pet-ether containing appropriate amount of ethyl acetate or dichloromethane containing appropriate amount of methanol.
to get the rf value 0.5. All the compounds were purified by column chromatography using 100-200 silica gel obtained from Sisco Research Laboratory. In NMR spectra that show splitting of peaks due to the presence of rotameric mixtures, arising from the tertiary amide linkage, the major rotamer is designated as maj. and the minor rotamer as min. The ratio of major: minor is 80:20 unless otherwise mentioned. In cases, where minor isomer is <10% only the peaks of major rotamer are reported. Melting points of the compounds reported are uncorrected.

**Methyl (2S) N1-(tert-Butyloxycarbonyl) Proline carboxylate 3**

L-Proline 1 (10 g, 87 mmol) was suspended in methanol (100 mL) and cooled to 0 °C with stirring. To this was added thionyl chloride (7 mL, 95.7 mmol, 1.1 eq.) dropwise over a period of 10 min. Stirring was continued at 0 °C for 4 h. and then at ambient temperature until the completion of the reaction (12 h). Removal of methanol under vacuum gave an oily ester hydrochloride salt 2 (14.1 g, 97.9%). This hydrochloride salt was used for the next step without further purification.

Ester hydrochloride 2 (10 g, 60.4 mmol) was dissolved in dioxane/water (1:1 v/v, 100 mL). To this were added triethylamine (23 mL, 166 mmol, 2.75 eq.) and BocN₃ (11.2 g, 78.5 mmol, 1.3 eq.) and the mixture was stirred at 50 °C for 24 h, under argon atmosphere. After the completion of the reaction, the mixture was concentrated to a paste by rotary evaporation, the mixture was diluted with H₂O (75 mL), and extracted with ether (3 x 50 mL). Combined ether layer was washed with H₂O (2 x 25 mL), followed by brine and dried over sodium sulphate. Evaporation of the solvent and purification by column chromatography using 60-120 silica gel and 10% ethyl acetate-
petroleum ether as eluent afforded 12.1 g of 3 as a yellow oily liquid. Yield 12.1 g, 82.9%.

**Methyl (2S)-N1-(tert-Butoxycarbonyl)-5-methoxy proline carboxylate 4**

N-(tert-Butoxycarbonyl)-L-proline methyl ester 3 (8 g, 34.9 mmol) was dissolved in a 0.5 M solution of tetrabutylammonium tetrafluoroborate in methanol (100 mL). The reaction flask was cooled to 5 °C in an ice bath. The stirred solution was oxidized at a carbon anode and cathode using a constant current (270 mA). After the completion of reaction (12h.) solvent was evaporated under reduced pressure and the residue was treated with ether (3 x 75 mL) leaving the supporting electrolyte as a crystalline solid. The combined ether layers were concentrated under vacuum to get the crude product as an oil which was purified by flash chromatography on 60-120 silica gel by isocratic elution using 10% ethyl acetate/petroleum ether as eluant to get the methoxylated product 4 (7.8 g, 86%).

![Structure 4]

\(^1\text{HNMR (CDCl}_3\text{200 MHz)}\) δ 5.35-5.10 (m, 1H, H2), 4.45-4.15 (m, 1H, H5), 3.80-3.70 (m, 3H, -OCH\textsubscript{3} of ester), 3.45-3.30 (m, 3H, -OCH\textsubscript{3}), 2.50-1.65 (m, 4H, H3 & H4), 1.55-1.35 (d, 9H, Boc); 13\textsuperscript{C NMR (CDCl}_3\text{200 MHz)}\) δ 173.0, 172.8 (-C=O ester); 89.7, 88.4 (C5); 80.3 (tertiary carbon); 59.1, 58.7 (C2); 55.9, 55.6, 54.9; 51.6 (-OCH\textsubscript{3}); 32.3, 30.9, 30.0, (C3); 28.0 (Boc methyl); 26.9 (C4).

**Methyl (2S)-N1-(tert-butoxycarbonyl)-5-cyano proline carboxylate 5 and 6**

To a solution of 4 (2.46.0 g, 9.5 mmol) in anhydrous dichloromethane (25 mL) was added TMSTf (0.25 mL) at −35 °C, followed by slow addition of TMSCN (1.46 mL, 10.9 mmol, 1.15 eq.). After the completion of the reaction (30 minutes) methanol was added to the reaction mixture and the solvents were evaporated under reduced pressure.
The residue was purified by column chromatography using neutral alumina (Al₂O₃) with 10% ethylacetate/petether as eluant to get the diastereomeric nitriles 5 (minor isomer, upper spot on tlc Rₜ 0.30 in 10% ethylactate-petroleum ether) 0.69 g & 6 (major isomer, top spot on tlc Rₜ 0.3 in 10% ethylactate-petroleum ether) 1.43 g. The diastereomeric ratio is (35:70).

**Minor isomer 5**

\[ \text{NC} \quad \text{CO}_2\text{CH}_3 \]

\[ \text{O} \quad \text{O} \]

\[ \text{1H NMR (CDCl}_3 \text{ 200 MHz)} \delta 4.77-4.64 (dd, J = 2.5 Hz, 1H, H2), 4.46-4.33 (dd, J = 3.75, 1H, H5), 3.74 (s, 3H, OCH}_3), 2.55-2.2 (m, 4H, H3 & H4), 1.51-1.42 (d, 9H, 3 x CH}_3); \text{13C NMR (CDCl}_3 \text{ 400 MHz): } \delta 171.90 & 171.71 \]  
(ester.CO), 152.4 (Boc.CO), 18.2 (cayano), 82.3, 81.8 (C-(CH}_3)\text{3}, 59.4, 59.0 (C2), 52.3 (C5), 47.5, 47.2 (COOCH}_3), 30.3, 29.8 (C3), 28.1 (C4), 28.0 (CH}_3); Ms (m/z): (M+1) = 255 (5 %), 188.04 (85 %), 155.06 (100 %). [α]_D^{25} = -93.7 (C = 0.365, CHCl}_3).

**Major isomer**

\[ \text{NC} \quad \text{CO}_2\text{CH}_3 \]

\[ \text{O} \quad \text{O} \]

\[ \text{1H NMR (CDCl}_3 \text{ 200 MHz): } \delta 4.70-4.55 (m, 1H, H2), 4.52-4.26 (m, 1H, H5), 3.75 (s, 3H, ester -OCH}_3), 2.45-2.19 (m, 4H, H3 & H4), 1.52-1.43 (d, 9H, (CH}_3)\text{3); } \text{13C NMR} \]
(\text{CDCl}_3, 400 MHz): \δ 172.4, 172.2 (ester.CO), 152.7, 152.6 (Boc.CO), 118.8, 118.7 (cayano), 82.4, 81.9 (C-(CH}_3)\text{3}, 58.9, 58.5 (C2), 51.9 (C5), 47.7, 47.6 (COOCH}_3), 30.0, 29.5(C3), 29.2(C4), 28.2 (CH}_3). Ms (m/z): (M+1) = 254.41 (5%), 277.41 (100 %), 221 (10 %), 144.60 (13 %), 95 (100 %); IR cm⁻¹ (neat): 2246, 1755, 1747, 1713. [α]_D^{25} = +41.8 (C = 0.665, CHCl}_3).

(2S, 5R)-N1-(tert-butoxycarbonyl)-[5-(flioureylethoxycarbonyl)aminomethyl]

**Proline 9**

To a solution of 5 (0.4 g, 1.55 mmol) in methanol (2 mL) was added NEt₃ (0.5 mL) followed by raney Ni (400 mg). The mixture was subjected to hydrogenation at 65 psi , after completion of the reaction (4h) , reaction mixture was filtered through celite pad and the solvent was evaporated under reduce pressure to get amino ester 7 (not
isolated) as a oily liquid. This was subjected to hydrolysis using 2N aq. NaOH (2mL) and methanol (2 mL). After 30 minutes excess of sodium hydroxide was neutralized using potassium bisulfate and the pH was adjusted to 7.0. Methanol was removed by rotary evaporation and the residue was redissolved in 10% Na₂CO₃ (2 mL) the reaction mixture was cooled to 0 °C in an ice-bath. To this was added of dioxane (2 mL) (peroxide free) followed by the slow addition of Fmoc-Cl (0.44 g, 1.7 mmol, 1.1 eq.) in dioxane at 0 °C. Stirring was continued at 0 °C for 4 h. followed by room temperature stirring for 18 h. The reaction was monitored by TLC, after the completion of the reaction contents were poured in ice-water and extracted with ether (2 x 20 mL) to remove the unreacted chloroformate. The aqueous phase was chilled in ice and acidified by the addition of saturated KHSO₄ solution. The pH of the solution was brought to 2.0 at which the compound started getting separated as foam. This was then extracted with ethyl acetate (3 x 10 mL) and dried over MgSO₄ and the solvent was removed under vacuum to get the crude product as a solid. This was purified by flash column chromatography on 60-120 silica gel using ethyleacetate/petether (0.3 Rf) as eluant to get 0.27 g, 37 % of the desired product 9.

![Chemical Structure](image)

**¹H NMR** (CDCl₃, 400 MHz) δ 7.78-7.345 (m, 8H), 5.95-5.90(s,1H), 4.50-4.14(m,5H), 3.36-3.30(m,2H), 2.26-1.75(m,4H), 1.44(s,9H). **¹³C NMR** (CDCl₃, 400 MHz): δ 177.4, 175.5 (acid CO), 156.8 (Boc.CO), 154.9 (Fmoc.CO), 143.7, 143.6, 127.4, 126.8, 124.9, 124.6, 119.7 (Fmoc.carbons), 80.9 (C-(CH₃)₃), 65.6, 66.2, 59.8, 57.5, 52.5, 46.9, 45.0, 44.0, 28.3, 28.0 (Boc.(CH₃)₃), 26.6. Ms (m/z) : 491.50 (M+Na), (10%) , 475 (100%). [α]²⁵D = + 40.5 (C = 0.2, CHCl₃)
(2S,5S) -N1-(tert-butoxycarbonyl))-5-(flourenylethoxycarbonyl)aminomethyl

Proline 10

To a solution of 6 (0.4 g, 1.55 mmol) in methanol (2 mL) was added NEt₃ (0.5 mL) followed by raney Ni (400mg). The mixture was subjected to hydrogenation at 65 psi, after completion of the reaction (4h), reaction mixture was filtered through celite pad and the solvent was evaporated under reduce pressure to get amino ester 7 (not isolated) as a oily liquid. This was subjected to hydrolysis using 2N aq. NaOH (2mL) and methanol (2 mL). After 30 minutes excess of sodium hydroxide was neutralized using potassium bisulfate and the pH was adjusted to 7.0. Methanol was removed by rotary evaporation and the residue was redissolved in 10% Na₂CO₃ (2 mL). The reaction mixture was cooled to 0 °C in an ice-bath. To this was added of dioxane (2 mL) (peroxide free) followed by the slow addition of Fmoc-Cl (0.44 g, 1.7 mmol, 1.1 eq.) in dioxane at 0 °C. Stirring was continued at 0 °C for 4 h. followed by room temperature stirring for 18 h. The reaction as monitored by TLC, after the completion of the reaction contents were poured in ice-water and extracted with ether (2 x 20 mL) to remove the unreacted chlороformate. The aqueous phase was chilled in ice and acidified by the addition of saturated KHSO₄ solution. The pH of the solution was brought to 2.0 at which the compound started getting separated as foam. This was then extracted with ethyl acetate (3 x 10 mL) and dried over MgSO₄ and the solvent was removed under vacuum to get the crude product as a solid. This was purified by flash column chromatography on 60-120 silica gel using ethyleacetate/petether (0.3 Rf) as eluant to get 0.27 g, 37 % of the desired product 10 along with undesirable 10a as the major product 0.48 g, 45 %.
\[ ^1\text{HNMR (CDCl}_3, 400 \text{ MHz)} \delta 7.74-7.72 (d, J = 2.5, 2H), 7.60-7.58 (d, J = 2.5, 2H), 7.38-7.27 (m, 4H) 6.21-6.15 (bs, 1H), 4.44-4.40 (m, 5H), 3.52-3.43 (m, 2H, H2), 2.24-1.83 (m, 4H), 1.43 (s, 9H); \]
\[ ^{13}\text{C NMR (CDCl}_3, 400 \text{ MHz)}: \delta 177.6, 177.1 \text{ (acid.CO)}, 157.0 \text{ (Boc.CO)}, 154.8, 154.4, 144.2, 143.8, 144.2, 143.8, (Fmoc.CO), 127.6, 126.7, 125.2, 119.8, (Fmoc.aromatic carbons), 81.2 \text{ (C-(CH}_3)_3), 67.0, 66.9, 58.3, 57.5, 47.0, 44.3, 43.5, 28.6, 28.2, 28.1 \text{ (Boc.(CH}_3)_3); \]
\[ \text{Ms (m/z): 491.15 (20\%) (M+1+Na), 265.04 (100\%), } [\alpha]^{25}\text{D} = -35.5 \text{ (C = 0.4, CHCl}_3) \]

**Synthesis of compound 13**

To a solution of 12 (50 mg, 0.204 mmol) in DCM (2 mL) was added diethyl amine (1 mL) at room temperature the mixture was stirred for 4h, after completion of the reaction solvent was evaporated under reduced pressure to get the 12 (not isolated). To a solution of 12 in DMF, HOBT(43.48mg, 0.321 mmol) and HBTU (121.75 mg, 0.321 mmol) were added at room temperature and stirred at for about 8h, DMF was evaporated and the mixture was dilute with H2O (5 mL) and extracted with ethyleacetate (2 x 10 mL). Drying of the combined organic phases (Na2SO4), evaporation of the solvent, and purification by column chromatography afforded 10 mg of 13 (46.1%) as a white solid.

\[ ^{1}\text{HNMR (CDCl}_3, 200 \text{ MHz)} \delta 6.5-6.25 (t, 1H, amide), 4.43-4.36 (m, 2H, H2 & H5), 3.71-3.64 (dd, J = 10, 4 Hz, 1H), 3.03-2.96 (dd, J = 7, 14 Hz, 1H), 2.20–2.05 (m, 3H), 1.80-1.74 (m, 1H). 1.43 (s, 9H, 3 x CH3); \]
\[ ^{13}\text{C NMR (CDCl}_3, 400 \text{ MHz)}: \delta 172.4, 153.6, 80.7, 59, 50.6, 47.5, 30.1, 28.2, 27.9; \]
\[ \text{IR } \gamma 3240 \text{ (br), 1690, cm}^{-1}; \text{Ms (m/z): } (\text{M+1}) = 227.11 \text{ (25\%)}, \text{ (2M}^+\text{) 453 (85\%)}, \text{ (2M+Na) 475 (100\%)}. \]
Methyl (2R)-N1-(tert-Butoxycarbonyl) proline carboxylate 17

To a solution of 14 (6 g, 52.2 mmol) in methanol (50 mL) was added thionyl chloride (4.2 mL, 57.4 mmol, 1.1) as discussed in the case of compound 1. Removal of methanol gave the ester 15 as a hydrochloride salt (8.30 g, 96%). This was used as such for the next step without further purification. The ester 15 (5 g, 30.2 mmol) was dissolved in 1:1 dioxane/water, treated with triethylamine (10.5 mL, 75.5 mmol,) and BocN₃ (5.2 mL, 36.2 mmol) under argon atmosphere at 50 °C. Usual work up and purification as mentioned for the L-isomer gave the tertbutoxycarbonyl derivative 16. Yield: 6.1 g, 88%.

![Chemical Structure of 16](image1)

$^1$H NMR (CDCl₃, 200 MHz): δ 4.35-4.10 (m, 1H, H2), 3.72 (s, 3H, CH 3), 3.60-3.25 (m, 2H, H5A & H5B), 2.30-1.70 (m, 4H, H3 & H4), 1.38 (d, 9H, 3 x (CH₃)₃); $^{13}$C NMR (CDCl₃, 200 MHz): δ 173.3 (ester CO), 153.4 (Boc.CO), 79.2 (C(CH₃)₃), 58.8 & 58.5 (C2), 51.5 (-OCH₃), 46.1 (C5), 30.6 & 29.6 (C3), 27.9 ((CH₃)₃), 23.4 (C4); [α]$^D$_{25} = + 61.30 (C = 1.135, CHCl₃). IR cm⁻¹ (neat): 2977, 1747, 1699;

Methyl (2R)-N1-(tert-Butoxycarbonyl)-5-methoxy proline carboxylate 17

The ester 16 (4.5 g, 19.7 mmol) was oxidized electrochemically to get the 5-methoxy product 17 as a diastereomeric mixture after the work up and purification as mentioned for the L-isomer 3. Yield 4.4 g, 88%.

![Chemical Structure of 17](image2)

$^1$H NMR (CDCl₃, 200 MHz): δ 5.35-5.05 (m, 1H, H2), 4.40-4.15 (m, 1H, H5), 3.80-3.60 (m, 3H, ester methyl), 3.50-3.25 (m, 3H, methoxy), 2.50-1.70 (m, 4H, H3 & H4), 1.60-1.30 (d, 9H, 3 x CH₃); $^{13}$C NMR (CDCl₃, 200 MHz): δ 159.5 (Boc.CO), 153.5 (ester.CO), 88.7, 88.0, 87.9, 80.1, 79.9, 59.1, 58.7, & 58.3, 55.5, 55.3 & 54.7, 51.7 & 51.4, 47.7, 32.4, 31.7, 30.6, 29.6, 27.6, 26.5.
Methyl (2R)-N1-(tert-Butoxycarbonyl)-5-cyano proline carboxylate 18 & 19

To a solution of 17 (2.46.0 g, 9.5 mmol) in anhydrous DCM (25 mL) were added TMSCN and TMSTf (1 %) at -35 °C to get the product 18 and 19 as separable diastereomeric mixture after the work up and purification as mentioned for the L-isomer. Major isomer 18 and minor isomer 19 in 68:32 ratio.

**Major isomer 18**

1H NMR (CDCl3, 400 MHz): δ 4.78-4.65 (dd, 1H), 4.46-4.38 (dd, 1H), 3.74 (s, 3H), 2.60-2.13 (m, 4H), 1.52-1.43 (d, 9H). 13C NMR (CDCl3, 400 MHz): δ 172.0, 171.8 (ester CO), 152.5 (Boc.CO), 118.0 (cyano), 82.4, 82.0 (C-(CH3)3), 59.5, 59.1 (C2), 52.4 (C5), 47.6 (COOCH3), 30.4 (C3), 29.9, 29.6 (C4), 28.6, 28.1 (Boc.(CH3)3); LC-MS (EI) m/z (M+1) = 254, (M + Na) = 277.54 (100%), 158.68 (20%) [α]D25 = -39.8° (C = 0.4, CHCl3); IR cm⁻¹ (neat): 2934, 2243, 1747, 1715;

**Minor isomer 19**

1H NMR (CDCl3, 400 MHz): δ 4.78-4.67 (dd, J = 2 Hz, 1H), 4.46-4.38 (dd, J = 2 Hz, 1H), 3.74 (s, 3H), 2.60-2.13 (m, 4H), 1.60-1.25 (d, 9H); 13C NMR (CDCl3, 400 MHz): δ 172.2, 172.0, (ester.CO), 152.5 (Boc.CO), 118.6, 118.8 (cyano), 82.2, 81.7 (C-(CH3)3), 58.7, 58.4 (COOCH3), 52.3, 52.2 (C2), 48.1 (C5), 47.6, 29.7 (C3), 28.4 (C4), 28.0 (Boc.(CH3)3); LC-MS (EI) m/z (M⁺) = 254, (M+Na) = 277 (5%), 128.66 (100%); [α]D25 = +101.5° (C = 0.476, CHCl3); IR cm⁻¹ (neat): 2243, 1747, 1716.
(2R,5S)-N1-(tert-butoxycarbonyl)-[5-(flourelynmethoxycarbonyl)aminomethyl]

Proline 22.

\[
\begin{align*}
\text{1H NMR (CDCl}_3, 400 \text{ MHz): } & \delta 7.74-7.22 \text{ (d, } J = 2 \text{ Hz, } 2\text{H,)}, \ 7.60-7.59 \text{ (d, } J = 1 \text{ Hz, } 2\text{H,)}, \ 7.45-7.15 \text{ (m, } 4\text{H, aromatic,))}, \ 6.20-5.95 \text{ (s, } 1\text{H, -NH,)}, \ 4.43-4.04 \text{ (m, } 5\text{H, -CH, -CH}_2\text{CH}_2\text{,}}, \ 3.60-3.25 \text{ (m, } 2\text{H,)}, \ 2.45-1.65 \text{ (m, } 4\text{H, H}_3\text{ & H}_4\text{)}, \ 1.43 \text{ (s, } 9\text{H, 3 x CH}_3\text{);} \ \text{13C NMR (CDCl}_3, 
\end{align*}
\]

(2R,5R)-N1-(tert-butoxycarbonyl)-[5-(flourelynmethoxycarbonyl)aminomethyl]

Proline 23.

\[
\begin{align*}
\text{1H NMR (CDCl}_3, 400 \text{ MHz): } & \delta 7.74-7.72 \text{ (d, } J = 2 \text{ Hz, } 2\text{H, aromatic),} \ 7.58-7.65 \text{ (m, } 2\text{H, aromatic),} \ 7.37-7.28 \text{ (m, } 4\text{H, aromatic),} \ 6.00 \text{ (bs, } 1\text{H, -NH,)}, \ 4.45-4.00 \text{ (m, } 5\text{H, -CH, -CH}_2\text{,}}, \ 3.77-3.69 \text{ (m, } 1\text{H,)}, \ 3.31-3.05 \text{ (m, } 2\text{H,)}, \ 2.28-1.73 \text{ (m, } 4\text{H, H}_3\text{ & H}_4\text{)}, \ 1.42 \text{ (s, } 9\text{H, 3 x CH}_3\text{);} \ \text{13C NMR (CDCl}_3, 400 \text{ MHz): } & \delta 177.4 \text{ (acid CO,)}, \ 158.9 \text{, (Boc.CO,)}, \ 155.2 \text{, 154.8, (Fmoc.CO,)}, \ 143.9 \text{, 141.4, 141.3, 127.6, 127.0,} \ 125.1 \text{, 124.8, 119.9, 119.9, 91.2, 80.8, 67.0 (C-(CH)_3),} \ 66.8 \text{, 57.7, 47.2, 45.5, 47.3, 45.5, 28.6, 28.4, 28.2 (Boc.CH}_3), \ 27.0; \ \text{Le-Ms (EI) m/z (M+1) = 467.48 (5%) 245.15 (100%). [}\alpha^p_{250} = +38^\circ \text{ (C = 0.027, CHCl}_3)\].
\end{align*}
\]

(2R,5S)-Pyrrolidine-2,5-dimethyldicarboxylate 26

To a solution of 18 (0.4 g, 1.57 mmol) 6 N HCl (10 mL) was added refluxed for 24 h, and reaction mixture was concentrated under vacuo to obtain the hydrochloride salt of dicarboxylic acid 24, yield 0.28 g (92%). The resultant salt was dissolved in methanol and cooled to 0 °C and SOCl₂ (0.25 mL) was added in a drop wise fashion. The reaction mixture was further stirred at room temperature for 12 h and concentrated under vacuo.

The residue was treated with saturated aqueous NaHCO₃ solution and the aqueous layer

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was extracted with ethyl acetate (3 x 10 mL), dried over Na₂SO₄, concentrated in vacuo to afforded 200 mg of 26, yield (75%). R₇ = 0.25 (Ethylacetate/Petether = 1:0).

\[ \text{H}_2\text{CO}_2\text{C} \quad \text{N} \quad \text{H} \quad \text{NH} \quad \text{CO}_2\text{CH}_3 \]

\[ 26 \]

\[ \text{^1H NMR} \quad \text{(CDCl}_3, \quad 500 \text{ MHz)}: \delta \quad 3.81\text{-}3.83 \quad (t, \quad J = 10 \text{ Hz}, \\
2\text{H}, \text{H}_2\text{H}_5), \quad 3.73 \quad (s, \quad 6\text{H}, \text{CO}_2\text{CH}_3), \quad 2.95 \quad (bs, \quad 1\text{H}, \quad \text{-NH}), \\
2.14 \quad (m, \quad 2\text{H}), \quad 1.80 \quad (m, \quad 2\text{H}); \quad \text{^13C NMR} \quad \text{(CDCl}_3, \quad 125 \text{ MHz)} \quad 174.1 \quad (\text{COCH}_3), \quad 59.9 \quad (\text{C}_2 \& \text{C}_5), \quad 51.8 \quad (\text{OCH}_3), \\
29.4 \quad (\text{C}_3 \& \text{C}_4). \quad \text{[α]}^D_{25} = 0 \quad (0.01, \quad \text{CHCl}_3) \]

\[ \text{(2R,5R)-Pyrroldine-2,5-dimethylidicarboxylate} \quad 27 \]

\[ \text{H}_2\text{CO}_2\text{C} \quad \text{N} \quad \text{H} \quad \text{CO}_2\text{CH}_3 \]

\[ 27 \]

\[ \text{^1H NMR} \quad \text{(CDCl}_3, \quad 500 \text{ MHz)}: \delta \quad 4.0\text{-}3.98 \quad (t, \quad J = 10 \text{ Hz}, \\
2\text{H}, \text{H}_2\text{H}_5), \quad 3.73 \quad (s, \quad 6\text{H}, \text{CO}_2\text{CH}_3), \quad 2.18 \quad (m, \quad 2\text{H}), \quad 1.96 \quad (m, \quad 2\text{H}); \quad \text{^13C NMR} \quad \text{(CDCl}_3, \quad 125 \text{ MHz)} \quad 174.8 \quad (\text{COCH}_3), \\
59.4 \quad (\text{C}_2 \& \text{C}_5), \quad 51.8 \quad (\text{OCH}_3), \quad 29.4 \quad (\text{C}_3 \& \text{C}_4); \quad \text{[α]}^D_{25} = +32^{\circ} \quad (0.01, \quad \text{CHCl}_3) \]

**Compound 29**

To a solution of 28 (10 gm, 43.29 mmol.) in acetone (500 mL) K₂CO₃ (23.37 gm, 129 mmol.) was added followed by slow addition of dimethyl sulphate (5.76 gm, 4.42 mL) and refluxed for 5h, after completion of the reaction concentrated the reaction mixture and dilute with H₂O (35 mL), followed by extraction with ethyl acetate (3 x 50 mL). Drying of the combined organic phases (Na₂SO₄), evaporation of the solvent and purification by column chromatography afforded 9.5 gm of 29 (89.6%) as white solid.

\[ \text{HO} \quad \text{N} \quad \text{Boc} \quad \text{CO}_2\text{CH}_3 \]

\[ 29 \]

\[ \text{^1H NMR} \quad \text{(CDCl}_3, \quad 200 \text{ MHz)}: \delta \quad 4.44\text{-}4.3 \quad (m, \quad 2\text{H}, \text{H}_3, \text{H}_2), \\
3.70 \quad (s, \quad 3\text{H}, \text{-CO2CH}_3), \quad 3.57\text{-}3.26 \quad (m, \quad 2\text{H}, \text{H}_5), \quad 2.35\text{-}2.15 \quad (m, \quad 1\text{H}, \text{H}_3), \quad 2.10\text{-}1.90 \quad (1\text{H}, \text{H}_3), \quad 1.42\text{-}1.37 \quad (d, \quad 9\text{H}, \quad J = 10 \text{ Hz}, \text{-}3 \text{H}, \text{CH}_3); \quad \text{^13C NMR} \quad \text{(CDCl}_3, \quad 200 \text{ MHz)}: \delta \quad 173.4, \\
173.2 \quad (\text{ester CO}), \quad 154.3, \quad 153.7 \quad (\text{Boc CO}), \quad 80.0, \quad 60.2, \quad 68.5, \quad (\text{C}_4), \quad 57.6, \quad 57.2 \quad (\text{C}_2), \quad 54.3 \quad (\text{C}_5), \quad 51.7 \quad (\text{OCH}_3), \quad 38.6, \quad 37.9 \quad (\text{C}_3), \quad 27.8 \quad (\text{Boc CO}); \quad \text{IR} \gamma 3018, \quad 1745, \quad 1720 \text{ cm}^{-1}. \]
Compound 30

To a solution of 29 (4 gm, 16.32 mmol) in THF (60 mL), was added triphenylphosphine (4.7 gm, 17.95 mmol) as THF solution (15 mL) and reaction mixture was cooled to ice temperature, to this acetic acid (1.18 gm, 17.95 mmol) followed by DEAD (3.41 gm, 16.32 mmol) were added drop wise fashion and the reaction mixture was stirred at ambient temperature for 10h, after completion of the reaction solvent was removed and diluted with H₂O (25 mL) followed by extraction with ethyl acetate (3 x 40 mL). Drying of the combined organic phases (Na₂SO₄), evaporation of the solvent and purification by column chromatography by 100-200 silica-gel afforded 4.3 gm (93.4%) of 30 as a oily liquid.

![Compound 30](image)

\[ ^1H \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta \text{ 5.26-5.22 (m, 1H, H3), 4.52-} \]
\[ \text{4.34 (dd, J = 2 Hz, 1H, H1), 3.75 (s, 3H, -CO}_2\text{CH}_3), 2.37-3.49} \]
\[ \text{(m, 2H, H4), 2.60-2.20 (m, 2H, H2), 2.01 (s, 3H, -OCOCH}_3), \]
\[ \text{1.48-1.43 (d, J = 10 Hz, 9H, -CO}_2\text{C(CH}_3)_3)}; \]
\[ ^13C \text{NMR (CDCl}_3, 200 \text{ MHz): } \delta \text{ 172.1, 171.8 (-COCH}_3), 169.8} \]
\[ \text{ (OCOCH}_3), 153.7, 153.2 (OCOC(CH}_3)_3), 179.9 (tBu-C), 72.9, 71.8 (C4), 57.9, 57.5} \]
\[ \text{(C2), 52.4, 52.4 (CO-OCH}_3), 52.2, 51.9 (C5), 36.4, 35.5 (C3),} \]
\[ 28.5, 28.4, (Boc-C(CH}_3)_3), 21.0 (OCO-CH}_3); \text{ IR } \gamma \text{ 3020,} \]
\[ 1737,1693 \text{ cm}^{-1}; \text{ Ms: m/z = 288 [M+1] (6%), 277 (10%), 228} \]
\[ (93%), 188 (100%), 128 (96%); [\alpha]_{D}^{25} = -25.33 \text{ (C = 0. 073, DCM).} \]

Compound 31

To a solution of 30 (6 gm, 20.90 mmol) in methanol was added tetrabutylammonium tetrafluoroborate (8 gm). The reaction flask was cooled to 5 °C in an ice bath. The stirred solution was oxidized at a carbon anode and cathode using a constant current (270 mA). After the completion of reaction 8h, solvent was evaporated under reduced pressure and the residue was treated with ether (3 x 75 mL) leaving the supporting electrolyte as a crystalline solid. The combined ether layers were concentrated under
vacuum to get the crude product as an oil which was purified by column chromatography
on 100-200 silica gel by isocratic elution using 10% ethyl acetate/petroleum ether as
eluant to get the methoxylated product 31 (3.5 gm, 66%).

\[ \text{1H NMR (CDCl}_3\text{, 200 MHz)} \delta 5.36-5.26 (m, 1H, H4),
4.95-4.73 (m, 1H, H2), 4.50-4.17 (m, 1H, -H5), 3.72-3.70
(3H, -OCH}_3\), 3.47-3.43 (3H, OCH}_3\), 2.64-2.50 (m, 1H,
H3), 2.18-1.99 (4H, H3, COOCH}_3\), 1.48-1.41 (m, 9H, Boc
methyl); \text{13C NMR (CDCl}_3\text{, 200 MHz)} \delta 172.6, 171.5
(COOCH}_3\), 170.2, 189.9 (OCOC(CH}_3\)), 153.7
(COOC(CH}_3\)), 80.9, 84.7, 81.4, 80.8, (OCH}_3\), 75.9, 74.7,
71.4, 70.8, (C4), 58.4, 57.8 (C2), 55.6, 54.7 (CO}_2\text{CH}_3\),
33.4, 32.4, 31.0, 30.2, (C3), 28.2, 28.0, (COOC(CH}_3\)),
20.7, (OCOC(CH}_3\)); \text{Ms: m/z [M+1]} = 260 (33%), 277
(55%), 128 (100%); \text{IR} \gamma 3020, 2401, 1737.74, 1706.88
cm\(^{-1}\)

\[
\text{Compound 33}
\]

To a solution of 31 (1 gm, 3.15 mmol) in DCM was added TMSICN (0.812mL,
6.3 mmol) and reaction mixture was cooled to ice temperature, to this BF\(_3\)-O(Et\(_2\))
(0.997 mL, 7.875 mL) was added drop wise fashion and stirred at ambient temperature
for 14h, after completion of the reaction Na\(_2\text{CO}_3\) (160 mg, 0.5 mmol) was added and
stirred for 2h to quench the excess Lewis acid present in the reaction. Evaporation of the
solvent, and purification by column chromatography using neutral alumina afforded 150
mg of 33 (23%) as an oily liquid.

\[ \text{1H NMR (CDCl}_3\text{, 200 MHz)} \delta 5.31-5.28 (d, J = 6 Hz, 1H,
H4), 4.23 (s, 1H, H2), 4.06-3.99 (dd, J = 4 Hz, H5), 3.77
(s, 3H), (2.72-2.57 (m, 1H, H2), 2.33-2.26 (m, 1H, H2),
2.03 (s, 3H, COOCH}_3\); \text{13C NMR (CDCl}_3\text{, 200 MHz)} \delta
173.4 (CO}_2\text{CH}_3\), 169.5 (OCOC(CH}_3\)), 118.0 (CN), 76.0
(CO}-OCH}_3\), 58.0 (C5), 53.7 (C4), 52.3 (C2), 34.5 (C3),
20.4 (OCO-CH}_3\); \text{IR} \gamma 3022, 2954, 1741

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Compound 34

To a solution of 33 (150 mg, 0.678) in DCM was added di teritylbutyloxy carbonate (591.8 mg, 2.712 mmol) followed by catalytic amount of DMAP (16.56 mg, 0.135 mmol) at ice temperature and stirred at ambient for 8h. evaporation of the solvent and purification by flash chromatography afforded 220 mg of 34 (97%) as a oily liquid.

\[
\begin{align*}
\text{H NMR} \ (\text{CDCl}_3 \ 200 \text{ MHz}) \ &\delta \ 1.54-1.45 \ (d, \ J = 18 \text{ Hz}, \ COOC(CH_3)_3) ; \ 2.05-2.04 \ (d, \ J = 2 \text{ Hz}, -OCO-CH_3), \ 2.47-2.40 \ (d, \ J = 16 \text{ Hz}, H3), \ 2.81-2.64 \ (m, 1H, H3), \ 3.76 \ (s, \ 3H), \ 4.61-4.47 \ (m, 1H) \ 4.68-4.64 \ (d, \ J = 8 \text{ Hz}, m, 1H), \ 5.38-5.36 \ (d, \ J = 4 \text{ Hz}, 1H, H4), \ 13C \ NMR \ (\text{CDCl}_3 \ 200 \text{ MHz}) \ &\delta \ 171.0, \ 170.6 \ (\text{CO}_2\text{CH}_3), \ 169.4, \ 169.0 \ (\text{OCO-CH}_3), \ 152.5, \ 152.21 \ (\text{COOC(CH}_3)_3), \ 115.7, \ 115.6 \ (\text{CN}), \ 82.8, \ 82.1, \ (\text{COOC(CH}_3)_3), \ 76 \ (\text{OCH}_3), \ 58.0, \ 57.7 \ (C2), \ 53.6, \ 53.5 \ (C2), \ 53.6, \ 53.5 \ (C5), \ 52.5, \ 52.3 \ (C4), \ 35.2, \ 34.1 \ (C3), \ 20.5 \ (\text{OCO-CH}_3); \ \text{Ms: } m/z = 333 [M+Na] (3%), \ 235.21(20%), \ 102.11 \ (100%) \).
\end{align*}

Compound 35

To a solution of 34 (220 mg, 0.709 mmol) in methanol (2 mL) was added NEt\textsubscript{3} (0.29 mL, 2.127 mmol) followed by raney Ni (250 mg). The mixture was subjected to hydrogenation at 65 psi, after completion of the reaction (4h), reaction mixture was filtered through celite pad and the solvent was evaporated under reduce pressure to get amino ester 7 (not isolated) as a oily liquid. This was subjected to hydrolysis using 2N aq. NaOH (1mL) and methanol (2 mL). After 30 minutes excess of sodium hydroxide was neutralized using potassium bisulfate and the pH was adjusted to 7.0. Methanol was removed by rotary evaporation and the residue was redissolved in 10% Na\textsubscript{2}CO\textsubscript{3} (1 mL) the reaction mixture was cooled to 0 °C in an ice-bath. To this was added of dioxane (2 mL) (peroxide free) followed by the slow addition of Fmoc-Cl (201 mg, 0.719 mmol, 1.1 eq.) in dioxane at 0 °C. Stirring was continued at 0 °C for 4 h. followed by room
temperature stirring for 18 h. The reaction as monitored by TLC, after the completion of the reaction contents were poured in ice-water and extracted with ether (2 x 15 mL) to remove the unreacted chloroformate. The aqueous phase was chilled in ice and acidified by the addition of saturated KHSO₄ solution. The pH of the solution was brought to 2.0 at which the compound started getting separated as foam. This was then extracted with ethyl acetate (3 x 10 mL) and dried over MgSO₄ and the solvent was removed under vacuum to get the crude product as a solid. This was purified by flash column chromatography on 60-120 silica gel using ethylacetate/petether (0.3 Rf) as eluant to get 135 mg, (39 %) of the desired product 35 as a cream color solid.

\[ \text{\textbf{1H NMR (CDCl₃ 200 MHz)}} \delta \begin{align*} & 7.69-7.65 \text{ (d, } J = 8 \text{ Hz, 2H)} , 7.53-7.49 \text{ (d, } J = 8 \text{ Hz, 2H)} , 7.31-7.17 \text{ (m, 4H)} , 5.94 \text{ (bs, } 1H, -NH) , 4.40-3.91 \text{ (m, 6H)} , 3.40-3.26 \text{ (m, 1H)} , 3.21-3.07 \text{ (m, 1H)} , 2.29-1.94 \text{ (m, 2H, H3)} ; \text{\textbf{13C NMR (CDCl₃ 400 MHz)}} \delta \begin{align*} & 176.0, 159.0 \begin{align*} & 154.4, 143.8, 143.26, 127.7, 127.0, 125.2, 124.9, 119.9, 81.3, 73.2, 67.3, 52.6, 47.23, 43.4, 36.4, 28.2; \text{ Ms: } m/z = 505.13 \text{ [M+Na] (3%), 277 (20%), 199 (75%), 155 (100%)}
\end{align*} \end{align*} \end{align*} \]

\textbf{N1-(t-Boc)-1,2-diaminoethane (37)}

1,2-diaminoethane (20 g, 0.33 mol) was taken in THF (500 ml) and cooled in an ice-bath. Boc-anhydride (5 g, 35 mmol) in THF (150 ml) was slowly added with stirring. The mixture was stirred for at ambient temperature for 16 h and the resulting solution was concentrated to 100 ml. The \textit{N1, N2-di-Boc} derivative not being soluble in water, precipitated, and it was removed by filtration. The corresponding \textit{N1-mono-Boc} derivative was obtained by repeated extraction from the filtrate in ethyl acetate. Removal
of solvents yielded the mono-Boc-diaminoethane 37 (3.45 g, 63%, Rf = 0.25, DCM: MeOH; 9:1).

\[ ^1H\text{ NMR (CDCl}_3, 200\text{ MHz)} \delta \ 5.21 (\text{br s, 1H, NH}), 3.32 (\text{t, 2H, J=8 Hz}), 2.54 (\text{t, 2H, J=8 Hz}), 1.42 (\text{s, 9H}). \]

**Ethyl N-(2-Boc-aminoethyl)-glycinate (38)**

The N1-(Boc)-1,2-diaminoethane 37 (3.2 g, 20 mmol) was treated with ethyl bromoacetate (2.25 ml, 20 mmol) in acetonitrile (100 ml) in the presence of N(Et)_3 (6.05 gm, 60 mmol) at 0 °C, the mixture was stirred at ambient temperature for 12 h. The reaction mixture was concentrated to paste and diluted with H_2O (20 ml), and extracted with ethyl acetate (5 x 20 mL). Drying of the combined organic phases (Na_2SO_4) and evaporation of the solvent afforded 4.3 gm (83%) of 38 as oily liquid.

\[ ^1H\text{ NMR (CDCl}_3, 200\text{ MHz)} \delta \ 5.02 (\text{br s, 1H, NH}), 4.22 (\text{q, 2H, J = 8Hz}), 3.35 (\text{s, 2H}), 3.20 (\text{t, 2H, J = 6Hz}), 2.76 (\text{t, 2H, J = 6Hz}), 1.46 (\text{s, 9H}), 1.28 (\text{t, 3H, J = 8Hz}). \]

**Ethyl N-(Boc-aminoethyl)-N-(chloroacetyl)-glycinate (39)**

The ethyl N-(2-Bocaminoethyl)-glycinate 14 (4.0 g, 14 mmol) was taken in 10% aqueous Na_2CO_3 (75 ml) and dioxane (60 ml). Chloroacetyl chloride (6.5 ml, 0.75 mmol) was added in two portions with vigorous stirring. The reaction was completed 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-(Bocamoethyl)-N-(chloroacetyl)-glycinate 39 as a colorless oil in good yield (4.2 g, Yield, 80%; \( R_f = 0.3 \), ethyelacetate:petroleum ether; 2:8).

\[ \text{H NMR (CDC3, 200 MHz)} \delta \ 5.45 \text{ (br s, 1H)}, \ 4.1 - 4.9 \text{ (S, 2H)}, \ 4.00 \text{ (s, 2H)}, \ 3.53 \text{ (t, 2H)}, \ 3.28 \text{ (q, 2H)}, \ 1.46 \text{ (s, 9H)}, \ 1.23 \text{ (t, 3H, } J=8Hz)\].

\[ \text{N-(Boc-aminoethylglycyl)-thymine ethyl ester (40)} \]

Ethyl N-(Boc-aminoethyl)-N-(chloroacetyl)-glycinate 39 (1.0 g, 3.1 mmol) was stirred with anhydrous K\(_2\)CO\(_3\) (0.47 g, 3.4 mmol) in DMF with thymine (0.41 g, 3.25 mmol) to obtain the desired compound 40 in good yield. DMF was removed under reduced pressure and the oil obtained was purified by column chromatography to afford 40. (1 g, Yield 83%; \( R_f = 0.2 \), MeOH:DCM; 5:95).

\[ \text{H NMR (CDCl3, 200 MHz): } \delta \ 9.00 \text{ (br s, 1H, T-NH), 7.05 (min) & 6.98 (maj) (s, 1H, T-H6), 5.65 (maj) & 5.05 (min) (br s, 1H, NH), 4.58 (maj) & 4.44 (min) (s, 1H, T-CH2), 4.25 (m, 2H, OCH2), 3.55 (m, 2H), 3.36 (m, 2H), 1.95 (s, 3H, T-CH3), 1.48 (s, 9H), 1.28 (m, 3H); } \text{C NMR (CDCl3) } \delta \text{: 170.8, 169.3, 167.4, 164.3, 156.2, 151.2, 141.1, 110.2, 79.3, 61.8, 61.2, 48.5, 48.1, 47.7, 38.4, 28.1, 13.8, 12.2} \]

\[ \text{N-(Boc-aminoethylglycyl)-(N4-benzyloxy carbonyl cytosine)ethyl ester (41)} \]

A mixture of NaH (0.25 g, 6.2 mmol) and N4-benzyloxy carbonyl cytosine 17 (1.24 g, 6.2 mmol) was taken in DMF and stirred at 75 °C till the effervescence ceased. The mixture was cooled and ethyl N-(Boc-aminoethyl)-N-(chloroacetyl)-glycinate 39 (2.0 g, 6.2 mmol) was added. Stirring was then continued at 75 °C to obtain the cytosine
monomer, \( N\)-(Boc-aminoethylglycyl)-(N4-benzylloxycarbonyl cytosine)ethyl ester 41, in moderate yield (1.75 g, 69\%; ).

\[ ^1H \text{ NMR (CDCl}_3, \text{ 200 MHz)}: \delta \ 7.65 \text{ (d, 1H, C-H6, } J = 8\text{Hz)}, \ 7.35 \text{ (s, 5H, Ar), 7.25 (d, 1H, C-H5, } J = 8\text{Hz)}, \ 5.70 \text{ (br s, 1H, NH), 5.20 (s, 2H, Ar-CH2), 4.71 (maj) & 4.22 (min) (br s, 2H), 4.15 (q, 2H), 4.05 (s, 2H), 3.56 (m, 2H), 3.32 (m, 2H), 1.48 (s, 9H), 1.25 (t, 3H).} \]

\( N\)-(Boc-aminoethylglycyl)-adenine ethyl ester (42)

NaH (0.25 g, 6.1 mmol) was taken in DMF (15 ml) and adenine (0.8 g, 6.1 mmol) was added. The mixture was stirred at 75 °C till the effervescence ceased and the mixture was cooled before adding ethyl \( N\)-Boc-aminoethyl\()-\( N\)-(chloroacetyl)-glycinate 39 (2.0 g, 6.1 mmol). The reaction mixture was heated once again to 75 °C for 1 h, 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 \( N\)- (Bocaminoethylglycyl)-adenine ethyl ester 42. (Yield 75\%; \( R_t = 0.2, \text{ MeOH:DCM; 5:95).} \)
$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 8.32 (s, 1H), 7.95 (m) & 7.90 (m) (s, 1H), 5.93 (m & 5.80 (m) (br, 2H), 5.13 (m & 4.95 (m), 4.22 (m) & 4.05 (m) (s, 2H), 4.20 (m, 2H), 3.65 (m) & 3.55 (m) (m, 2H), 3.40 (m) & 3.50 (m) (m, 2H), 1.42 (s, 9H), 1.25 (m, 3H).

$N$-(Boc-aminoethylglycyl)-2-amino-6-chloropurine ethyl ester (43)

A mixture of 2-amino-6-chloropurine (1.14 g, 6.8 mmol), K$_2$CO$_3$ (0.93 g, 7.0 mmol) and ethyl $N$-(Boc-aminoethyl)-$N$-(chloroacetyl)-glycinate 39 (2.4 g, 7.0 mmol) were taken in dry DMF (20 ml) and stirred at room temperature for 4 h. K$_2$CO$_3$ was removed by filtration, and the DMF, by evaporation under reduced pressure. The resulting residue was purified by column chromatography to obtain the $N$-(Boc-aminoethylglycyl)-2-amino-6-chloropurine ethyl ester (43) in excellent yield (2.55 g, 90%; $R_f = 0.25$, MeOH:DCM; 5:95).

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 7.89 (m) & 7.85 (m) (s, 1H), 7.30 (s, 1H), 5.80 (br s, 1H, NH), 5.18 (br, 2H), 5.02 (m & 4.85 (m) (s, 2H), 4.18 (m) & 4.05 (m) (s, 2H), 3.65 (m) & 3.16 (m) (m, 2H), 3.42 (m) & 3.28 (m) (m, 2H), 1.50 (s, 9H), 1.26 (m, 3H).

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 acids (21-24) in excellent yield (>85%). In case of cytosine monomer ethyl ester, mild base 0.5 M LiOH was used to avoid deprotection of the exocyclic amine-protecting group by strong bases.

**Thymine N1-acetic acid 49**

To thymine 48 (5 g, 39.7 mmol) and potassium hydroxide (3.8 g, 79.4 mmol) in \( \text{H}_2\text{O} \) (30 mL) was added chloroacetic acid slowly (3.1 g, 39.7 mmol) in water (12 mL). The pH of the solution was adjusted and kept at 10 by drop wise addition of aq. KOH solution. After refluxing for 2h, the solution was cooled and acidified to pH 2 with conc. HCl. The resulting precipitate was filtered, washed with cold water and dried to obtain the crude product, recrystallized from water to get pure 49 (4.9 g, 66%) as a white solid. mp 258 °C (260-261 °C).

**N4-Benzylxycarbonyl cytosine 4**

Cytosine 50 (1 g, 9 mmol) was suspended in dry pyridine (15 mL) and cooled to 0°C. To this was added benzylxycarbonyl chloroformate (3.0 mL, 18 mmol.) drop wise over a period of 15 min. under nitrogen atmosphere. The reaction mixture was stirred overnight. The pyridine suspension was evaporated to dryness in vacuo. To this were added water (10 mL) and 4N hydrochloric acid to bring the pH to 1. The resulting white precipitate was filtered off, washed with water and partially dried. The wet precipitate was boiled in absolute ethanol (25 mL) for 10 min. cooled to 0°C, filtered, washed thoroughly with ether, and dried, in vacuo to get 52 as a white solid.

\(^1\text{H NMR (DMSO D}_6\):} \ 7.85-7.70 (d, 1H, H6), 7.45-7.20 (m, 5H, aromatic), 7.00-6.80 (d, 1H, H5), 5.15 (s, 2H, benzyl CH2). Mass (m/e) 245 (M+).
Ethyl (N4-Benzylxocarbonyl-N1cytosinyl) acetate 52

A suspension of N4-Benzylxocarbonyl cytosine 51 (0.5 g, 2 mmol) and K₂CO₃ (0.28 g, 2 mmol) in dry DMF was cooled to 0°C and ethyl bromoacetate (0.133 mL, 1.2 mmol, 0.6 eq.) was added drop wise and the mixture was stirred vigorously overnight, filtered and evaporated to dryness. Water (7 mL) and 4 N HCl (0.2 mL) were added and the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2 x 10 mL). The isolated precipitate was purified by column chromatography on silica gel using ethylacetate/petether as an eluent to get the pure ester 52 as a white solid. Yield 0.48g, 71%.

\[ \text{H NMR (CDCl}_3, \text{ 200 MHz): } \delta \text{ 7.75 (bs. NH), 7.60-7.45} \]
\[ \text{ (d, 1H, H6), 7.45-7.30 (m, 5H, aromatic), 7.30-7.20 (d,} \]
\[ \text{ 1H, H5), 5.20 (s, 2H, benzyl CH2), 4.60 (s, 2H, N-CH2),} \]
\[ \text{4.35-4.15 (q, 2H, -CH2), 1.35-1.20 (t, 3H, CH3). Mass} \]
\[ \text{(m/e) 331 (M+1).} \]

N4-Benzylxocarbonyl cytosine-N1-acetic acid 53

Ester 52 (0.25 g, 0.74 mmol) in water (1.5 mL) was treated with 2N NaOH (1.5 mL) for 15 min. filtered, cooled to 0°C and neutralized with 4N HCl (0.5 mL). The product acid was isolated by filtration and the precipitate was washed thoroughly with water to get the free acid 53 as a white solid. Yield 0.195 g, 92 %.
2.19 Functionalization of MBHA resin

The commercially available MBHA resin has loading value of (2meq/g) which is not suitable for oligomer synthesis. Hence it is required to minimize the loading value to 0.17-0.27 meq/g to avoid the syntheses problems due to aggregation of developing oligomer. Dry resin (2 gm) was taken in solid phase funnel and swelled in DCM (20 mL) for 1 h. The solvent was drained off and the resin was treated with calculated amount of acetic anhydride in 5% DIPEA /DCM solution for about 15 min then the solvent was drained off, resin was washed with DCM and DMF thoroughly to remove the traces of acetic anhydride and dried in vacuum descicator. The dried resin was taken in to solid phase funnel and swelled in DCM for about 1h and functionalized with Boc protected β-allanine.

2.19.1 Picric acid assay for the estimation of the amino acid loading

The functionalized dry resin (5 mg) was taken in a sintered funnel and swelled in CH₂Cl₂ for 1 h. The solvent was drained off and the resin was treated with 50% TFA/DCM for 15 min (1 mL x 2) each time. The resin was thoroughly washed with CH₂Cl₂ and the TFA salt was neutralized with 5% diisopropyl ethylamine for 2 min (1 mL x 3). The free amine was treated with picric acid 0.1 M in DCM (2 mL x 3) 3 min for
each time. The resin was thoroughly washed with CH₂Cl₂ to remove the unbound picric acid. The picrate bound to amino groups was eluted with 5% diisopropyl ethylamine in CH₂Cl₂, followed by washing with CH₂Cl₂. The eluant was collected into a 10 ml volumetric flask and made up to 10 ml using CH₂Cl₂. An aliquot (0.2 ml) of picrate eluant was diluted to 2 ml with ethanol and the optical density was measured at 358 nm, and the loading value of the resin (0.27 meq/g) was calculated using the molar extinction coefficient of picric acid as $\varepsilon_{358}=14,500 \text{ cm}^{-1} \text{ M}^{-1}$ at 358 nm.
2.20 Reference:


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2.21 Appendix

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<td>Compound 18; $^1$H NMR and Mass spectra</td>
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<td>Compound 27; $^{13}$C and DEPT spectra</td>
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<td>Compound 34; $^1$H NMR and Mass spectra</td>
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<td>Compound 34; $^{13}$C NMR and DEPT spectra</td>
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<td>Compound 49 and 53; $^1$H NMR and spectra</td>
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<td>MALDI-TOF spectra of <em>ampplaeg</em> PNA oligomers</td>
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</tr>
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</table>
$^1$H NMR, CDCl$_3$

![Chemical structure image]

Compound 5

$M_{\text{calculated}} = 254$

$M_{\text{observed}} = 255.10$

Figure 10 $^1$H NMR and Mass spectra of compound 5
Compound 5, $^{13}$C NMR, CDCl$_3$

Compound 5, DEPT, CDCl$_3$

Figure 11 $^{13}$C and DEPT spectra of compound 5
Figure 12 $^1$H NMR and Mass spectra of compound 6

$^1$H NMR, CDCl$_3$

Compound 6

$[M + Na]$  

$M_{\text{calculated}} = 254$  

$M_{\text{obs}} = 254.41$
Compound 6, $^{13}$C NMR, CDCl$_3$

Figure 13 $^{13}$C and DEPT spectra of compound 6
Figure 14 $^1$H NMR and Mass spectra of compound 9

Compound 9
$M_{\text{calculated}} = 466$
$M_{\text{observed}} = 491.50 \,(M + Na)$
Compound 9, $^{13}$C NMR, CDCl$_3$

Figure 15 $^{13}$C and DEPT spectra of compound 9
Figure 16 $^1$H NMR and Mass spectra of compound 10
Compound 10, $^{13}\text{C}$ NMR, CDCl$_3$

Figure 17 $^{13}\text{C}$ and DEPT spectra of compound 10
Figure 18 $^1$H NMR and Mass spectra of compound 13
Compound 13, $^{13}$C NMR, CDCl$_3$

Figure 19 $^{13}$C and DEPT spectra of compound 13
**Figure 20** $^1$H NMR and Mass spectra of compound 18
Compound 18, $^{13}$C NMR, CDCl$_3$

Compound 18, DEPT, CDCl$_3$

Figure 21 $^{13}$C and DEPT spectra of compound 18
Figure 22 $^1$H NMR and Mass spectra of compound 19
Compound 19, $^{13}$C NMR, CDCl$_3$

Figure 23 $^{13}$C and DEPT spectra of compound 19
Compound 22

$M_{\text{calculated}} = 466$

$M_{\text{observed}} = 467.16$

$M+1 = 467.16$

Figure 24 $^1$H NMR and Mass spectra of compound 22
Figure 25 $^{13}$C and DEPT spectra of compound 22
Compound 23
$M_{\text{calculated}} = 466$
$M_{\text{observed}} = 467.48$
Compound 23, $^{13}$C NMR, CDCl$_3$

Figure 27 $^{13}$C and DEPT spectra of compound 23
Figure 28 $^1$H NMR and Mass spectra of compound 26
Figure 29 $^{13}$C and DEPT spectra of compound 26
Figure 30 $^1$H NMR and Mass spectra of compound 27

Calculated = 187
[M + 1] = 188.05

Compound 27
Figure 31 $^{13}$C and DEPT spectra of compound 27
Compound 34
M + Na

$M \text{ calculated } = 312$
$M \text{ observed } = 313$ (M + 1)

Figure 32 $^1$H NMR and Mass spectra of compound 34
Figure 33 $^{13}$C NMR and DEPT spectra of compound 34
Compound 35

$M_{calculated} = 482$

$M_{observed} = 505.13 \ [M+Na]$
Figure 35 $^{13}$C and DEPT spectra of compound 35
Figure 36 $^1$H NMR and spectra of compound 49 and 53
Table 3 HPLC and MALDI-TOF mass spectral analysis of modified *amp-*PNAs

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<td>27</td>
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<td>10.286</td>
<td>2423</td>
<td>2425.09[M + 2H]</td>
</tr>
<tr>
<td>28</td>
<td>SRR-amp PNA 84</td>
<td>8.617</td>
<td>2257</td>
<td>2261.60 [M +9]</td>
</tr>
<tr>
<td>29</td>
<td>SRR-amp PNA 85</td>
<td>8.891</td>
<td>2257</td>
<td>2259.70 [M +3H]</td>
</tr>
<tr>
<td>30</td>
<td>SRR-amp PNA 86</td>
<td>8.379</td>
<td>2257</td>
<td>2252.83 [M - 4H]</td>
</tr>
<tr>
<td>31</td>
<td>SRR-amp PNA 87</td>
<td>9.247</td>
<td>2835</td>
<td>2836.47 [M + 1H]</td>
</tr>
</tbody>
</table>
Figure 37 Reverse phase HPLC- Profiles of amplaeg PNAs

Figure 37a HPLC- Profiles of amplaeg PNAs 57-63
Figure 37b HPLC-Profiles of amp-PNAs 64-69
Figure 37c HPLC-profiles of amp-PNAs 70-75
Figure 37d HPLC-Profiles of *amp*-PNAs 76-81
Figure 37e HPLC-Profiles of amp-PNAs 82-87
Figure 38 MALDI-TOF spectra of *amplaeg* PNA oligomers

- **PNA 57**
  - $M_{calc} = 2218$
  - $M_{obs} = 2218.58$

- **PNA 58**
  - $M_{calc} = 2793$
  - $M_{obs} = 2832.28$

- **PNA 59**
  - $M_{calc} = 2241$
  - $M_{obs} = 2240.28$

- **PNA 60**
  - $M_{calc} = 2241$
  - $M_{obs} = 2240.83$

- **PNA 61**
  - $M_{calc} = 2241$
  - $M_{obs} = 2241.37$

- **PNA 62**
  - $M_{calc} = 2267$
  - $M_{obs} = 2265.86$

Figure 38a MALDI-TOF spectra of *amp*-PNAs 57-62
Figure 38b MALDI-TOF spectra of amp-PNAs 63-69
Figure 38c MALDI-TOF spectra of amp-PNAs 70-75
Figure 38d MALDI-TOF spectra of amp-PNAs 76-81
Figure 38e MALDI-TOF spectra of *amp*-PNAs 82-87