Synthesis of Ethyl N-[(2-Boc-amino)-ethyl] glycinate and PNA Monomer Containing 5-Iodouracil

Wenjun Zhu

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Aston University

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List of Abbreviations

Bhoc----benzhydryloxycarbonyl

Cbz----benzyloxycarbonyl

DhbtOH----3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine

DCC-N, N----dicyclohexyl carbodiimide

DIPCDI ----N,N'-diisopropylcarbodiimide

DMF----dimethylformamide

Ds---- double stranded

FISH---- fluorescence in situ hybridization

TFA----trifluoroacetic acid

HBTU----hexafluorophosphate

TEMSA----trifluoromethanesulfonic acid

PNA----peptide nucleic acid

Boc----N-tert-butoxycarbonyl

Fmoc----9-fluorenylmethoxycarbonyl

^{1}H NMR---- proton nuclear magnetic resonance

DMSO---- dimethyl sulphoxide

DNA---- deoxyribonucleic acid

TLC----thin layer chromatography

UV---- ultraviolet spectroscopy

THF---- tetrahydrofuran

To my parents

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Summary

Peptide nucleic acids, or PNAs, are oligonucleotide analogs in which the sugar-phosphate backbone is replaced with a polyamide structure. The PNAs were first synthesized about more than 15 years ago, and have received great attention for their potential as a drug in antisense or antigene applications and as diagnostic and biological tools.

The PNAs have several favorable particular nature and unique properties including resistance to the digestion of nucleases and proteases, high stability in serum and cell extracts, and strong affinity and specificity to complementary sequences of DNA and RNA that form single and double-stranded DNA or RNA targets.

PNAs constitute an attractive new class of applications introduced in cytogenetics used as a promising procedure for *in situ* hybridization assays. The PNA technology is already used as an essential tool in a wide range of research and diagnostic molecular protocols. During recent years, the new applications of PNAs have appeared in genetics and cytogenetics such as genome mapping, antigene therapy, mutation detection or aneuploidy screening.

In this project, a convenient and economic synthesis of ethyl N-[(2-Boc-amino) ethyl] glycinate, together with its hydrochloride, was developed. These are key intermediates for the synthesis of peptide nucleic acid monomers and are usually the necessity to prepare N (2-aminoethyl) glycine derivatives. We have found a reliable and easily scalable route to ethyl N-[(2-Boc-amino) ethyl] glycinate.

At the same time, the PNA monomer (thymine-monomer) successfully has been obtained by the reaction based on the reported method. The PNA monomer can be used in future synthesis of PNA oligomers. And also, the preparation of other PNA monomers can be fulfilled referring to this development.

Finally, a modified uracil PNA monomer was also synthesized, which can be used for the synthesis of modified peptide nucleic acid by post-synthetic modification. The internal modification of PNAs could conceivably modulate their antisense/antigene efficacy and the potency of interruption of nucleic acid processing enzymes.

Chapter I Introduction

1.1 Background of Peptide nucleic acid (PNA)

The antisense and antigene strategy has attracted wide attention in the treatment of diseases at the level of gene expression in medicinal chemistry. The binding of an oligonucleotide (or an oligonucleotide analogue) to DNA or RNA can accomplish interference with gene expression by standard base pairing rules and result in the straightforward design of therapy agents.

Natural oligonucleotides have been shown to exhibit both antisense and antigene properties *in vitro* [1, 2, 3]. However, nucleases degrade both DNA and RNA rapidly *in vivo*. In order to overcome this serious problem, a large number of oligonucleotide analogues have been synthesized during the last 20 years. However, it is important to keep the DNA and RNA binding affinity and specificity of the analogues and to obtain desired pharmacokinetic behavior in applications.

In 1991, Danish researchers used a computer model to search for a molecule able to recognize nucleic acid sequences via triple helix formation [4, 5]. The outcome of this study led to a new type of DNA mimics in which the DNA backbone is replaced by repeating N-2- (2-aminoethyl) glycine units, while the nucleobases are attached via methylenecarbonyl linkages which make this DNA mimic with remarkable properties.

Although there were previous attempts to combine some fragments of nucleic acids and proteins within nucleopeptides or nucleoamino acids to acquire a new functionality, none of them was as prosperous as in the PNA case. The success of PNA has stimulated the synthesis of numerous PNA modifications and the search for other related nucleic acid analogs.

After the development of a solid-phase procedure for the construction of oligomers of peptide nucleic acid (PNA), it was shown that besides their ability to form triple

helixes PNAs can also interact with complementary single-stranded RNA with higher affinity than the corresponding DNA sequence. Moreover, PNAs also recognize the complementary nucleic acid strand more specifically, as PNA-DNA duplexes demonstrate to be less tolerant to mismatches with the corresponding DNA-DNA duplexes.

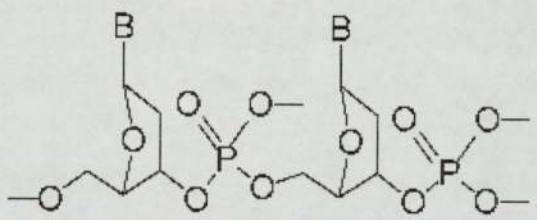
Additionally, the unique unnatural backbone of PNAs different from DNA, RNA and protein makes them particularly resistant to normal protease, peptidase and nuclease degradation and shows high chemical and enzymatic stability and low toxicity [6]. The lifetime of PNAs is extended both *in vivo* and *in vitro* because of this kind of property to the enzyme degradation.

These biophysical properties of PNAs make it one of the most valuable nucleic acid mimics, with a high potential for application either as a genetic diagnostic agent or as a therapeutic agent in the anti-sense and anti-gene strategy. However, the applicability of PNA is hampered by low water-solubility, a tendency to form aggregates and, in particular, poor cell membrane permeability [7]. To overcome these drawbacks, much attention has been focused on the new developments of PNA.

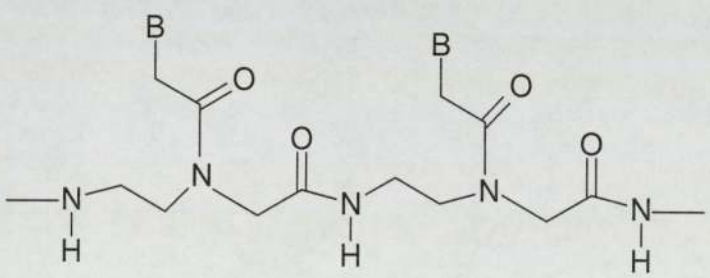
All these novel polyamide-based nucleic acid derivatives significantly extend the abilities of DNA/RNA mimics in terms of their future biomedical applications. This booming growth of the PNA-related family indicates that this clever concept has a strong creative potential in the field of development and gene therapeutic drugs, target validation in medicine and functional genomics studies in (molecular) biology.

1.2 The structure of PNA

The PNA is afforded by replacement of the natural deoxyribose phosphate backbone of DNA by repeating N-(2-aminoethyl)glycine units with the nucleobases attached through methylene carbonyl linkers. The purine and pyrimidine bases are attached to the backbone and extend out in a conformation that is remarkably similar, both in spacing and geometry, to standard oligonucleotides. The chemical structures of DNA and PNA are shown in Figure 1.



DNA



PNA

Figure 1. The chemical structures of PNA and DNA.
B=nucleobase

PNAs are depicted like peptides because the N-terminus is at the first (left) position and the C-terminus is at the right. Compared with DNA, the structural difference of PNA is obvious as the backbone of PNA is acyclic and neutral. Although, unlike DNA or DNA analogs, no sugar moieties or phosphate groups are contained in PNAs, it is surprising that PNAs mimic the behaviors of DNA in many respects, and in some applications they have demonstrated superior properties.

PNA can bind complementary polynucleotide targets in at least two modes. PNAs containing mixed purine/pyrimidine nucleobases form duplexes by recognizing complementary nucleic acid targets based on Watson-Crick hydrogen bonding. They are called duplex-forming PNAs [8, 9, 10]. However, PNAs only containing pyrimidines form PNA_2/nucleic acid triplexes via Watson-Crick and Hoogsteen hydrogen bonds and are termed as triplex-forming PNAs [11, 12] (Figure 2).

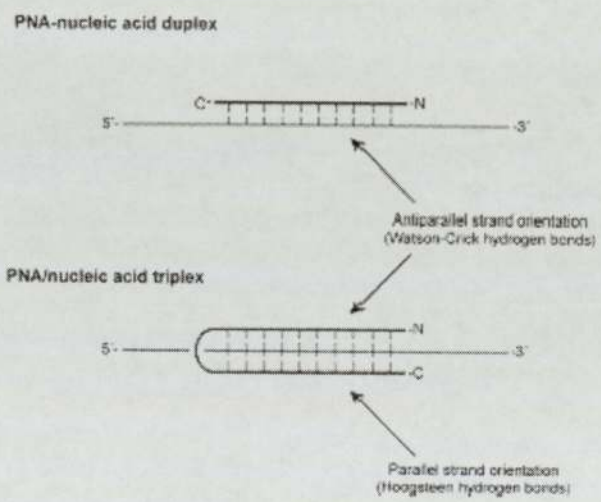


Figure 2. PNA-nucleic acid duplex and PNA/nucleic acid triplex [13]

In the structure of PNA-nucleic acid duplex and PNA/nucleic acid triplex, there are no charges in the PNA backbone and consequently, no electrostatic repulsion when PNA hybridizes to the target of a nucleic acid sequence. Therefore, it induces a higher stability of PNA-DNA or PNA-RNA duplexes than the natural homo- or hetero-duplexes of DNA or RNA [14]. This property has enabled PNA oligomers to show remarkably high affinity and stability when binding to complementary sequences in either RNA or DNA.

The achirality of PNA in structure offered it advantages to replace the glycine moiety of the backbone with chiral amino acids. The side chains of the amino acids are valuable for controlling binding affinity, specificity, hydrophobicity and attachment of ligands when a chiral PNA monomer developed (Figure 3). Moreover, chirality might be used to control the binding orientation of PNA to DNA [15]. But, the flexibility of PNA might be reduced for substituents in the glycine part of the PNA backbone.

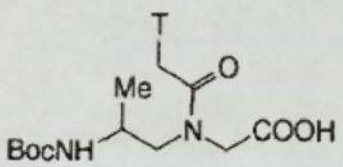


Figure 3. Chiral PNA monomer

1.3 Synthesis of PNAs

1.3.1 Synthesis of PNA monomers

In the synthesis of PNA monomers and oligomers, two protection schemes are used. One is the tert-butyloxycarbonyl (Boc) group, which is applied to protect the primary amino group of the backbone: N-(2-Boc-aminoethyl)glycine. The other one is the benzyloxycarbonyl (Cbz), which is used to protect exocyclic amino groups of the nucleobases [16-17]. These different protection schemes have provided product in high yields and purity.

It seemed that acid labile protective groups were more appropriate for use because alkaline conditions were found to induce a rearrangement of the N-terminal nucleobase to the primary amino group of the backbone (Figure 4). However, it was reported by some researchers that acyl migration is a slow process and was not observed during piperidine treatment of growing PNA chains [18].

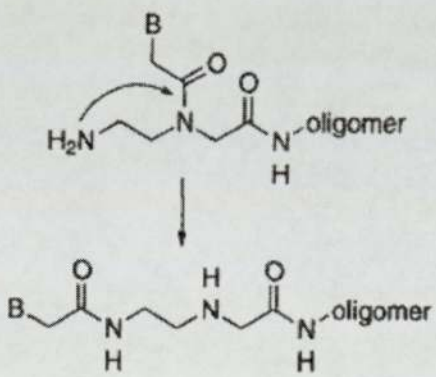


Figure 4. The N-acyl transfer reaction

B=nucleobase

Initially, the synthesis of PNA monomers involves alkylation of the nucleobases with bromoacetate. The thymine monomer was reported firstly because this nucleobase has no need to use protective groups and can be alkylated by methyl bromoacetate easily (Figure 5). After the resultant methyl ester of the desired thymin-1-ylacetic acid is hydrolysed, the thymine monomer is obtained [19].

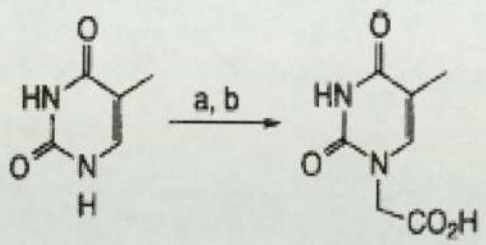
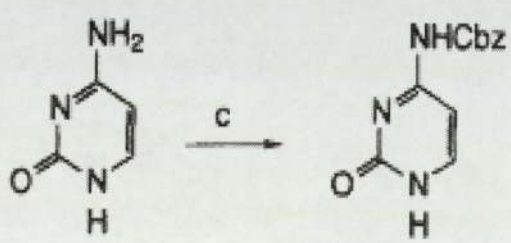


Figure 5. Synthesis of thymin-1-ylacetic acid

a= $BrCH_2CO_2CH_3/K_2CO_3$

b= NaOH (aq)

The synthesis of cytosine monomer requires protection in order to prevent chain extension from the exocyclic amino group or acetylation during the capping procedure of oligomerization of PNA [20]. It is more appropriate to introduce the Cbz group prior to alkylation, partly because Cbz group is a lipophilic group, which can considerably improve the solubility of cytosine in organic solvents (Figure 6).



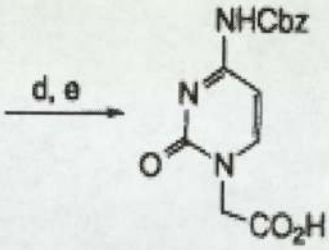


Figure 6. Synthesis of (N^4-(benzyloxycarbonyl) cytosine-1-yl) acetic acid

c= $PhCH_2OCOCl$/Pyridine

d= $BrCH_2CO_2CH_3/K_2CO_3$

e= NaOH (aq)

In the synthesis of adenine monomer, adenine was firstly alkylated by ethyl bromoacetate in DMF using either sodium hydride or potassium carbonate only at 9-amino position of adenine, which was verified by X-ray crystallography [21]. Subsequently the Cbz group was introduced on the 6-amino group using "Rapoport's reagent", as an acylating agent. The reverse order involving Cbz protection prior to the alkylation was also reported [22] (Figure 7). But it had lower yields in the result.

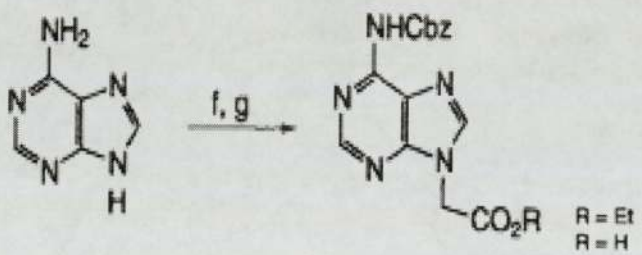


Figure 7. Cbz protection in synthesis of Ethyl (N6-(benzyloxycarbonyl)adenine-9-yl)acetate and (N6-(benzyloxycarbonyl)adenine-9-yl)acetic acid

f= $BrCH_2CO_2Et$/NaH

g= $PhCH_2OCOlM^+EtBF_4^-$

In the synthesis of guanine monomer, 2-amino-6-chloropurine was employed as the starting material to prepare the G-monomer, since guanine itself cannot be alkylated to exclusively obtain the 9-isomer. The reaction between 2-amino-6-chloropurine and bromoacetic acid can yield a mixture of 9-alkylated and 7-alkylated products [21]. However, the 9-isomer is the major compound and can be easily separated from the mixture of two compounds (Figure 8).

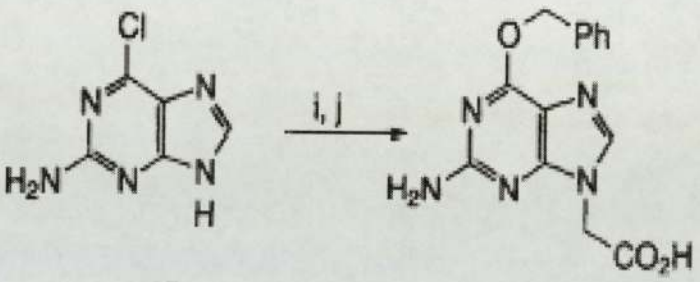


Figure 8. Synthesis of (2-Amino-6-(benzyloxy)purin-9-yl)acetic acid

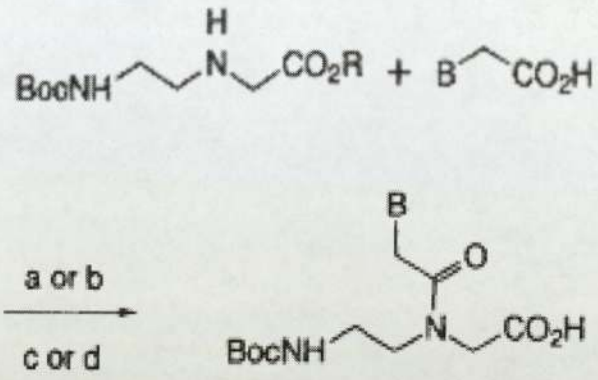
i= $BrCH_2CO_2CH_3/K_2CO_3$

j= $PhCH_2ONa$

Subsequently, the chloro group was replaced by a benzyloxy group to increase the solubility of alkylated product. The benzyloxy group will be removed during the treatment with TFA in oligomerization procedure. But it failed to protect the alkylated product with benzyloxy group; the treatment with acetic anhydride for capping in the oligomerization caused partial acetylation. To avoid this kind of unwanted acetylation, the capping is omitted after introducing the first guanine residues in the synthesis of oligomer by this G-monomer [17].

Following the alkylation of the nucleobases with or without carboxylmethyl protection, nucleobase-acetic acid was activated and coupled with the Boc-protected backbone N-(2-Boc-aminoethyl) glycine and then the monomer ethyl esters were hydrolyzed with LiOH in H_2O/THF to afford the T-, N4-Cbz C-, and N6-Cbz-A-

monomers (Figure 9). Finally, they are hydrolyzed to free acids in aqueous sodium hydroxide [23, 24].



a= DCC, DhbtOH b= PyBrop, DIEA

c= LiOH, THF, H_2O d= NaOH, EtOH, H_2O

Figure 9. Coupling of the nucleobases to the backbone

1.3.2. Synthesis of PNA oligomers

Most syntheses of PNA oligomers described in the literature [16, 17, 19] are based on the standard procedures for solid phase peptide synthesis by using Boc (*tert*-butyloxycarbonyl)/Z(benzyloxycarbonyl)-protected intermediates supported by a (4-methylbenzhydryl)amine polystyrene resin with repetitive TFA (trifluoroacetic acid) deprotection during chain elongation and final HF or trifluoromethane sulfonic acid cleavage to release the PNAs resin (Figure 10).

PNA synthesis based on Boc/Z protection relies on differential acid lability between the two groups. The Boc group is removed by TFA, and either HF or, more frequently, TEMSA is used to remove the Z-group. But the harsh HF or TEMSA treatment is required for cleavage from the resin and deprotection, which renders this strategy incompatible with the synthesis of many types of modified PNAs, such as the synthesis of PNA-DNA chimeras due to the sensitivity of DNA to strong acids [18].

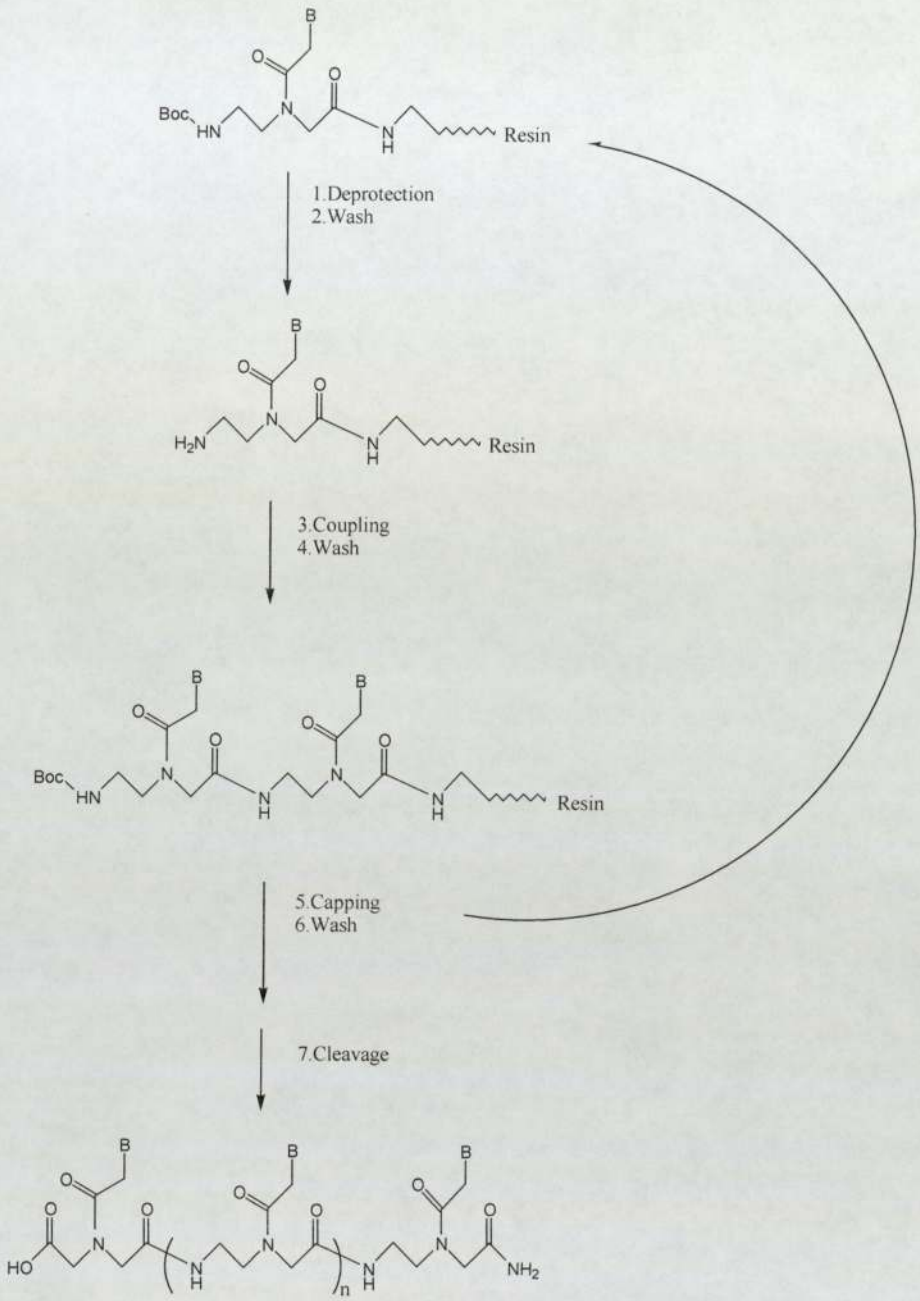


Figure 10. Schematic representation of the solid phase PNA synthesis

In 1995, the synthesis of Fmoc/benzyloxycarbonyl protected PNA monomers was reported and the method for their oligomersation was outlined [18]. The preparation of PNA-peptide conjugates may be allowed by this Fmoc strategy combined with Boc peptide synthesis. However, a strong acid (for example HF) deprotection is still needed for the Fmoc-protected monomers in the final cleavage step.

By well-established solid phase peptide synthesis protocols, however, oligomers composed of modified amino acid building blocks (PNA monomers) can be assembled and this has supplied oligomers in milligram to gram quantities. For example, homothymine oligomers were synthesized by activated pentafluorophenyl ester monomers in a good yield. However, this kind of activation was not efficient for the cytosine residues, whereas DCC coupling resulted in a quantitative work up of both T- and C-monomers. Although the adenine and guanine residues were also able to be incorporated utilizing this coupling scheme, N, N'-diisopropylcarbodiinide (DIPCDI) was reported to be a more potent coupling reagent for adenine and guanine monomers [16, 21].

In the recent study, all the coupling reagents showed very similar coupling results, but O-(benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) constantly gave the best yields. The exact coupling conditions, such as coupling reagents, capping reagents, solvents, concentration of reactants, are optimized to be able to ensure more than 95% coupling yields, which allow the synthesis of oligomers at least up to various residues by using all four natural nucleobases. Additionally, reporter groups, intercalators, metal binders, etc. can be attached through either the N-terminal amino group or the C-terminal carboxylic acid [17].

Of course, there are other PNA synthesis strategies being developed today. The focus of these strategies is to make PNA synthesis fully compatible with the synthesis of other biopolymers, e.g. DNA. If general PNA is synthesized under much milder conditions, it will allow the production of PNA-DNA chimeras and other acid sensitive PNA-conjugates.

1.4. Modifications of PNA

DNA analogues can be designed successfully under the guidance of structure-activity studies on already existing analogues. However, it is essential to understand the undesirable features of these analogues and so much effort has been directed towards this issue. One approach is preparing closely related compounds by modification and comparing their characteristics.

The backbone of PNAs can be subjected to further chemical modifications in that the glycine unit can easily be envisaged substituted by all naturally occurring amino acids. This can test the limits of what the PNA structure will allow without compromising the hybridization properties of PNA. A few modified nucleobases of PNAs have also been studied and may be used in some recognition applications [25, 26].

Retro-inverso PNA stands for a modified PNA where a methylene group transferred to glycine part from the aminoethyl part of backbone. This change resulted in an N-aminomethyl –alanine backbone [27, 28] (Figure 11). It is reported that the synthesis of modified retro-inverso PNA monomers such as Cbz-A- and T-monomers have been developed and incorporated into PNA oligomers by standard oligomerization chemistry. But the hybridization properties of these oligomers to complementary oligonucleotides have not yet been published.

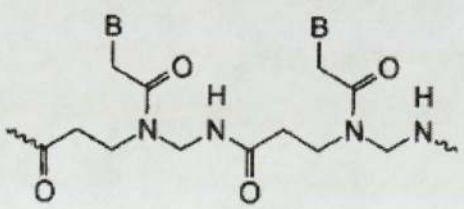


Figure 11. Retro-inverso PNA

Another method of PNA modification is to extend the units of PNA. There are the same number of bonds connecting the nucleobases in PNA and DNA. This induces a question whether it is a necessary condition for DNA analogues such as PNA to get the desired properties. In a recent study, a single methylene group has been linked to either the backbone or the nucleobases on PNA oligomer residues. And then, their hybridization properties have been examined [25]. It was found that the modified PNAs showed a preference for the antiparallel binding orientation when forming duplexes with complementary DNA or PNA. This behavior is like unmodified PNA [26].

There is another alternative interchange of groups called a heterodimer in the PNA backbone. The amide connects the two units which is positioned carbonyl group towards the C-terminal not the N-terminal [29] (Figure 12). When this oligomer binding with complementary DNA, there was no finding about influence to the thermal stability of hybrids. However, no data were reported to show whether the sequence selectivity of hybrids was kept.

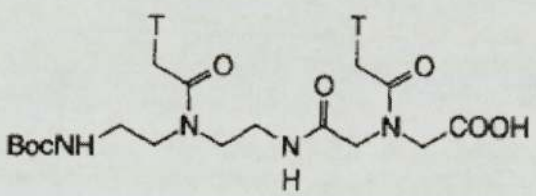


Figure 12. PNA heterodimer

However, it has been shown that all modified PNA-DNA hybrids were thought less stable than unmodified complexes according to a decrease in Tm [30]. The decreased stability is most likely caused by geometric constraints in the PNA and/or a larger loss in entropy upon complex formation. These results tell that it is important to maintain the correct distance between the nucleobases. But also, when all units of PNA are modified, it suggests that the backbone distances might be more important than the

distance between the backbone and the nucleobases.

As with base modified nucleosides, there will be a rich area of PNA chemistry to be accessed by some strategy. For example, internal derivatization of an oligomer can be used as a route to branched or dendrimeric systems [31], however, these have not been reported in PNA yet.

The use of internal thiol to form bis-PNA type structures may be another strategy. This structure could arise from homo- or hetero-oxidative crosslinking with a second thiol labeled PNA oligomer. There will maybe result in bent PNA or to bend PNA:NA hybrid duplexes by this functionalizing with a thiol group [32]; at the same time, the extension of this method may improve solubility of PNAs such as hydroxypropynyl PNAs and aminopropynyl PNAs [33]. The cationic aminopropynyl derivative especially may have interesting properties [34].

1.5 Properties of PNA

1.5.1 Hybridization

PNAs have an additional semantic and structural consequence of the polyamide backbone which is they do not have a 5' to 3' orientation as do phosphodiester backbones, but rather have an amino (NH_2) to carboxyl (CO_2H) orientation. Because the geometry and the spacing of the bases in PNA are nearly identical to that found in a native DNA or RNA strand, PNAs can hybridize to complementary sequences of DNA or RNA through classic Watson-Crick base pairing.

The unique chemical structure gives PNAs the strong hybridization capability to form PNA-nucleic acid duplexes. PNA is able to hybridize to complementary sequences of DNA and RNA with high affinity and specificity according to the

Watson-Crick hydrogen-binding scheme. In contrast to DNA, PNA can bind in either parallel or anti-parallel binding. Antiparallel binding is the amino terminal of a PNA facing the 3'-end of the oligonucleotide, while parallel binding is the amino terminal of PNA facing a 5'-end of the oligonucleotide. The PNA-DNA or PNA-RNA duplexes have a higher stability than the natural homo- or hetero-duplexes, resulting in higher thermal melting temperature (Tm) [35, 36].

The higher thermal stability of PNA-DNA duplexes is because the PNA backbone is not charged, inducing no electrostatic repulsion in the PNA-DNA duplex. That is supported by the experimental results that the thermal stability of PNA-DNA and DNA-DNA duplexes is equal to each other at ionic strength about 1M Na^+, while the changes in ionic strength have little effect on the stability of PNA –DNA hybrids. Therefore, the consequence of the polyamide backbone is that PNAs hybridize virtually independently of salt concentration. Thus, the Tm of PNA-DNA duplex is barely affected by low ionic strength [35].

The properties of PNA-DNA/RNA duplexes have been investigated by utilizing the pentadecamer H-TGTACGTCACAACTA-NH_2. This oligomer comes into being a duplex by binding anitparallel complementary DNA at a Tm of 69.5°C. The corresponding DNA-DNA only has a Tm of 53.3°C. PNA is also able to bind parallel complementary DNA with lower affinity with a Tm of 56.1°C. Moreover, the kinetic binding studies by applying capillary gel electrophoresis reported the formation of PNA-DNA duplexes is faster for antiparallel complexes than the parallel hybrids [37].

Some PNAs oligomers with pyrimidine are able to bind the complementary DNA by the formation of the triplexes, $(PNA)_2$-DNA [38]. These complexes are very stable. The Tm is more than 70°C for decamer hybrids, which was observed as monophasic, well-defined melting curves. And the binding of these complexes might be controlled by the Hoogsteen base pairing and Watson-Crick. Moreover, the stability of the complexes is dependent on the length of the oligomers regularly with the Tm increasing by 10°C per base pair [20]. Triplex formation with cytosine, such as

C+GC triplets, is slightly pH dependent. The lower pH is able to increase the hybrid stability [39, 40].

The high stability of $(PNA)_2$-DNA triplexes induces the strand displacement to take place on the target of dsDNA (double stranded DNA) [41-43] (Figure 13). In this unique binding mode, homopyrimidine PNA oligomers displace the pyrimidine strand of complementary ds DNA targets upon formation of a $(PNA)_2$-DNA triplex with the homopurine strand. And in this binding, the parallel orientation is slightly preferred to the antiparallel. This formation of strand displacement complexes is dependent on the ionic strength in a salt concentration.

Surprisingly, it has been shown that a strand displacement complex is mostly formed when homopyrimidine PNA is targeting dsDNA, however, the expected triple helix complex was not formed. This binding mode of PNA has inspired the study of a series of techniques developing the property of PNA. It should be suggested that very high stability of $(PNA)_2$-DNA triplexes is important for strand displacement because strand displacement has only been observed with PNAs to take part in triplex formation. Therefore, increasing the stability of PNA-DNA duplexes might allow targeting of dsDNA.

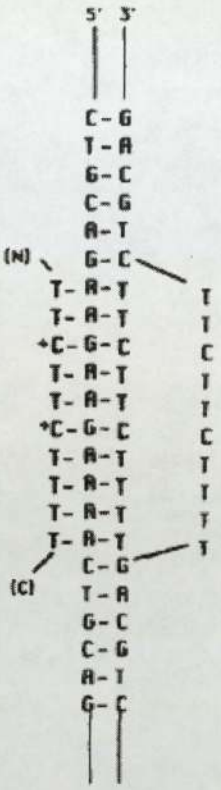


Figure 13. Strand displacement of dsDNA by homopyrimidine PNA

Bis-PNAs have consequently been obtained by connecting two PNAs accompanied by partial hybridization of PNA to target in a continuous synthesis with one strand antiparallel to the target DNA (designed for Watson-Crick recognition) and the second strand parallel to DNA target (designed for Hoogsteen hydrogen bonding). This success can be attributed to the increased stability of $(PNA)_2$/DNA triplexes when PNA and DNA [44, 45] interact.

1.5.2 Membrane penetrability

At present, the property of poor cellular uptake is the main reason why the PNA only has a limited use in the antisense and antigene. The uptake properties of PNA have been examined in cell cultures and *in vivo*. Phospholipid vesicles as a model system for biological membranes have been used for this purpose [46]. It was found that the permeability of PNA is relatively poor to such membranes compared with same magnitude of DNA oligonucleotides. When this issue was studied in cell cultures, neither higher PNA concentrations nor extended incubation time increased the permeability of PNA [47, 48].

However, researchers have been addressing several issues to overcome poor cellular uptake of PNA [49, 50]. The recent work illustrated that several new techniques for increasing cellular delivery have been developed in the past few years, such as PNA conjugating with peptide, binding with antibody and PNA modification. Although these systems are not completely satisfactory for PNA cellular delivery, they provide a promising look for future PNA application in antisense and antigene [51, 52].

The peptides were reported to be able to increase delivery of PNA oligomers across the plasma membrane into eukaryotic cells [53-55]. PNA oligomers can be easy to equip with a wide variety of peptides, because the synthesis of PNA is by conventional peptide chemistry. Such peptide–PNA constructs can penetrate into the cells more easily than PNA itself. In the recent research, the nuclear localisation signal (NLS) has been shown to help PNA oligomers penetrate through the plasma membrane into the cytosol and further convey into nuclei of Burkitt's lymphoma derived cell-lines. At the same time, the PNA oligomers are found to be delivered into cells by other peptides with a same NLS sequence. However, the nuclear transport of PNA was achieved only with a particular peptide that corresponds to the active/wild type NLS [56, 57].

To develop PNA as an antisense or antigene agent, the cell permeability of PNA based drugs also required to be conferred by the PNA molecule itself, so several modifications of PNA have been introduced to meet these requirements. It is reported that attachment of lipophilic groups such as adamantyl or liposome to PNA backbone chain or the nucleobases be able to increase the affinity of PNA to cell membrane and cellular uptake in applications. In some cell types, the liposome-mediated PNA delivery has the positive action on the cellular uptake of PNA [58].

Similarly, it is reported the PNA transport over the rat blood-brain barrier can be increased by conjugating PNA to an antibody recognizing the rat receptor [57]. In a receptor-mediated endocytosis experiment, PNA was conjugated with a D-aminoacid analog of insulin-like growth factor. This displayed a significantly higher uptake of PNA. The uptake of these carriers is believed to be dependent on their physical properties and not rely on ATP and receptor [53-55]. It has been thought that such approaches could facilitate cell- or tissue-specific administration of PNA.

However, it is illustrated by recent work that the uptake properties may vary greatly in different cell types, which indicates that PNA may enter certain cell types much faster and more efficiently than others. It was found that neuronal cells in culture appear to internalize PNA relatively efficiently. In general, passive diffusion is not considered as the mechanism of PNA intracellular delivery.

1.5.3 Biological stability

The major concern for applications of antisense and antigene compounds is their biostability. PNA is a pseudopeptide, so it is relevant to study its stability to proteases and peptidases. However, it has not been determined how PNA disappeared in human serum. There are two possible mechanisms: degradation and aggregation with cellular components. In some experiments, PNA was exposed to proteolytic activity in the media when it was incubated in the presence of a control peptide [59].

An ideal antisense agent should be resistant to enzymatic degradation considering pharmacokinetic issues. It is reasonable to assume that PNA can be used as an antisense drug based on sufficient biostability. For instance, it was reported that PNA has not undergone significant degradation when incubated with human serum, bacterial cells and tumor cells, or even high concentration of isolated proteinase or peptidase. Thus, PNA appears to have ample biological stability for drug development.

1.5.4 Transcription modulation

Protein expression in the cell can be modulated on different levels. Down regulation of biosynthesis is achieved, either by targeting DNA — an antigene approach — or targeting mRNA — an antisense approach. Acting on the DNA level is usually carried out by inhibiting replication or transcription (Figure 14). PNA-mediated inhibition of gene transcription is mainly due to the formation of strand-invaded complexes or strand displacement in DNA targets.

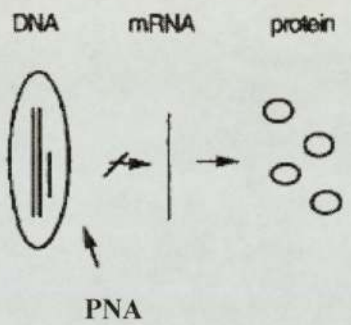


Figure 14. Antigene inhibition

It was reported by different laboratories that transcription was arrested by PNA *in vitro* [60, 61]. PNA targeted against the promoter region of gene can form stable PNA-DNA complexes that restrict the DNA access of polymerase. At the same time, PNA-DNA complexes located far from the promoter can block the polymerase progression and lead to the production of truncated RNA transcripts.

Additionally, incubation of homopyrimidine PNA with DNA containing the appropriate target sequence resulted in the formation of highly stable triplexes [81]. Concentration dependent transcription elongation arrest was observed when 10-mer PNA triplexes were bound to the template strand. But when PNA triplexes binding with the non-template strand, there was no same effect acquired [62, 63].

The binding of a few DNA recognizing proteins has been impaired by strand displacement between homopyrimidine PNA and dsDNA targets. For example, when PNA was bound proximal to the cleavage sites, cleavage by the restriction enzymes was fully inhibited [34]. The inhibition of cleavage was also observed when a single PNA mismatch was present; however, there was no effect on the cleavage with two mismatches present. According to these results, it should be feasible to inhibit transcription by PNA to promoter targeting [62, 63].

The inhibition of replication was found in a primer extension experiment in that PNA was incubated with a single stranded DNA target and a primer oligonucleotide [50]. There was a formation of a truncated product by DNA polymerase at the PNA binding site [60].

However, there is an ability to activate transcription of PNA. It usually not considered an antigene effect. Activation of transcription might turn out to be a valuable tool in medicinal chemistry as well. In a recent study, the transcription initiation took place when strand invasion was performed [61]. And also, it is suggested that transcription was initiated at several positions in the single- strand DNA loop via reverse transcriptase primer extension experiments on the produce transcripts. These findings emphasize that PNA promoter is very strong and might have the potential to act as a gene activator *in vivo* if the strand invasion is accessible inside cells.

1.5.5 Translation arrest

The potential application of PNA as an antisense agent has been evaluated by examining the ability of PNA to inhibit translation. The antisense effect of conventional oligonucleotides is generally believed to depend on the activation of RNase H which cleaves the RNA portion of the heteroduplex. It is notable that PNA/RNA hybrids are not substrates for RNase H [62, 63]. Thus, antisense effects mediated by PNA are thought to rely on steric interference of either RNA processing, transport into cytoplasm or translation caused by binding to the mRNA (Figure 15). The efficiency of PNA should therefore rely on the stability of the resulting RNA/PNA hybrid.

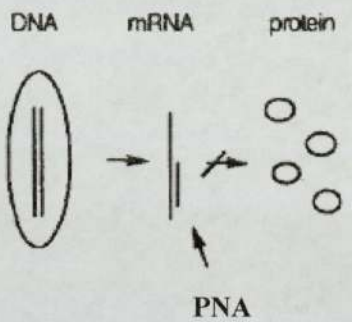


Figure 15. Antisense inhibition

It is reported that several researchers have studied the antisense properties of PNA *in vitro* using a rabbit cell free translation system. In the translation reaction, the presence of a triplex-forming PNA complementary to the mRNA actually results in translation arrest, and also results in a truncated protein product corresponding to the position of the PNA target in many cases. It appears that translation arrest may be obtained using homopyrimidine. In general, it is thought that duplex-forming PNAs can inhibit translation *in vitro* when they are targeted towards the ribosome binding site. However, triplex-forming PNAs are effective when targeted against polypurine targets downstream of the translation initiation site [61].

A few recent papers have reported on efficient antisense effects of PNA in living cells and whole animals. Two duplex-forming PNAs were found to recognize and down regulate the mRNA of the human galanin receptor which mediates the effect(s) of the neuropeptide galanin. Additionally, the PNA conjugates were tested in melanoma cells and were shown capable of down regulating the level of galanin binding capacity in cellular membranes [51].

The good stability of PNA oligomers, their strong binding efficiency as well as their lack of toxicity at even relatively high concentrations suggested that PNAs could constitute highly efficient compounds for effective antisense/antigene application.

1.6 Applications of PNA

PNA has a number of advantages such as high sequence specificity, biological stability and ability to obtain the desired effects with lower concentrations. Thus, PNAs are used in a variety of applications including purification of chromosomal DNA, induction of gene expression, clamping PCR and gene array analysis. Full exploitation of potential by PNA requires knowledge of methods for PNA synthesis, purification, characterization and delivery into cells.

1.6.1 PNA as a delivery vehicle

PNA has a number of characteristics: high binding affinity and specificity to DNA, RNA and peptide, ability to covalently link other molecules to its polyamide backbone, so it can be used for a vehicle as adapters, linking plasmid vectors to peptides, proteins, drugs, and molecular tracers.

One approach to act as a cell delivery tool is modification of PNA by adding targeting molecules or peptides to the polyamide backbone. It was reported that a number of ligands could be attached to PNA and used to selectively target the molecules to the desired cell types or tissues. Some groups developed cancer-targeting liposomes to PNA for delivery of plasmids specifically to certain cells by modification [64, 65].

But the major problem of this application is the low efficiency in transferring to target cells of PNA. Many barriers exist, such as the extracellular matrix, the endosomal/lysosomal environment, the endosomal membrane, and the nuclear envelope [66]. However, several research groups found certain cells amenable to PNA. This suggests specific transport mechanisms for the molecules.

1.6.2 PNA as a PCR strategies tool

PNAs can inhibit the elongation of oligonucleotide primers by binding to the template or competing with the primers although there is no direct interaction between PNA probes and DNA polymerase. At the same time, PNA-DNA chimeras can be recognized by the DNA polymerase and can thus be used primers for PCR reactions.

It is reported that the high affinity binding of PNAs has also been used for detecting single base pair mutations by PCR. This strategy is termed PNA directed PCR clamping. This method uses PNAs to prevent the amplification of a specific target by direct competition of the PNA targeted against one of the PCR primer sites and the conventional PCR primer. This PNA-DNA complex formed at one of the primer sites effectively blocks the formation of the PCR product. Because the procedure is so powerful, it can be used to detect single base-pair gene variants in the applications for mutation screening and gene isolation [67].

More recently, PNAs have been developed to use in novel automated real-time PCR. In a study, named Q-PNA PCR, a generic quencher labeled PNA (Q-PNA) is hybridized to the 5'tag sequence of a fluorescent dye-labelled DNA primer for the purpose of quenching the fluorescence of the primer. During PCR, the Q-PNA is displaced into amplicons by incorporation of the primer and the fluorescence of the dye label is liberated [68].

This technology is now gaining wider acceptance in the studies of tumor mutations. It is a means of suppressing the amplification of the wild-type gene. And also, this method was recently used in clinical diagnostic studies of genetic variations. It is not surprising that PNAs may be constructed as detection beacons for real time monitoring of PCR reactions [69].

1.6.3 PNA as a probe for chromosomal analysis

Because of the added advantages of tighter binding and higher specificity, PNAs can be used in many of the same hybridization applications as natural or synthetic DNA probes. During the last few years, research of PNAs has focused on their applications as probes for *in situ* hybridization assays. Considering its high binding specificity, a single 15-mer PNA probe can replace a set of longer DNA probe [70].

And also, PNAs can bind to DNA or RNA under low ionic strength conditions for the neutral backbone of PNA. This is particularly advantageous for *in situ* targeting of repeat sequences because both the length and the repetitive nature can favor renaturation over hybridization with probes. Additional benefits of using PNAs are lower background signals, mild washing procedure and unlimited stability of the probe mixture. However, PNAs are compatible with a wide range of reporter molecules as well as cyanine dyes available in a large variety of colors.

In a recent research of chromosomal analysis, by comparing fluoresceine-labelled DNA, RNA and PNA probes for the detection of telomeric repeat sequences on human metaphase chromosomes, it was shown that PNA probes yielded superior staining of telomeres. The PNA-FISH (Fluorescence in situ hybridization) approach was used in the distinction of fluorescence of individual sister chromatid ends and the accurate estimate of individual and global telomere length in metaphase chromosomes of various cultured human cells [71].

It was reported that an experiment of the PNA technology in human sperm has been developed [72]. Because of the particularities of sperm nucleus in term of genomic compaction and accessibility of DNA sequences, there is an interesting challenge in the adaptation of PNA technology to human sperm. In this experiment, it was found that the hybridization timing of PNA probes was greatly shortened compared to FISH reaction. FISH requires an overnight reaction to efficiently complete sperm preparations, however, PNA probes only need 45 minutes and similar to the PRINS

reaction (about 30 minutes). The reason for this similarity might be the small size of both PNA probes and PRINS (primed in situ) primers. This result turned out that the probe size is important for *in situ* sperm labeling and pointed out PNA probes have great potential for chromosomal screening on difficult biological material.

Further developments of PNA technology were focused on the improvement of the specificity of PNA probes and the *in situ* detection of numerical chromosome abnormalities. It has been reported that PNA probes could discriminate between two centromeric DNA repeat sequences that differ by only a single base pair. Identical results were obtained with PRINS primers and oligonucleotide probes, but never with standard DNA probes. The identification of chromosomal variation and the analysis of polymorphisms could greatly benefit from the discrimination power of PNAs. The procedure of PNA synthesis allows considering the further production of allele-specific probes. This will constitute an evident improvement over the current labeling techniques [73, 74].

All these preliminary studies showed that PNAs have multiple advantages for the *in situ* analysis of nucleic acid sequences. The high binding efficiency, the discrimination and the fast hybridization kinetics of PNA probes make then valuable tools for chromosomal investigations. The PNA hybridization method might develop quickly within the field of *in situ* labeling methodology and become a powerful complement to FISH and PRINS for *in situ* chromosomal investigations.

1.6.4 PNA as a tool in solid-phase hybridization techniques

The neutral backbone of PNAs significantly increases the rate of hybridization in assays where either the target or the probe is immobilized. Therefore, PNAs can be used for sequence specific capture of single stranded nucleic acids taking advantage of the tight complex formation at low ionic strength which destabilizes nucleic acid secondary structure. A system for capture of double stranded DNA was also experimented using $(PNA)_2$–DNA openers creating a large single strand DNA loop to which a biotinylated oligonucleotide can hybridize. [75].

The high-affinity binding of PNA oligomers has led to faster and easier procedures in most standard hybridization techniques such as Southern and Northern blotting [76]. The PNA pre-gel hybridization process is also an alternative to Southern analysis, which can significantly simplify the procedure of Southern hybridization. Additionally, labeled PNAs are then used as probes, allowing hybridization to a denatured dsDNA sample at low ionic strength prior to loading on the gel.

This is different from conventional Southern blotting where hybridization occurs after gel electrophoresis and membrane transfer. Here, the mixture is directly subjected to electrophoresis for separation of bound and unbound PNA probes. Because of their neutral charge, excess unbound PNA probes do not migrate in an electrical field. The PNA–DNA hybrids are then blotted onto a nylon membrane and detected using standard chemiluminescent techniques. The method is sensitive enough to detect a single mismatch in a DNA sample [77].

The hybridization PNA-based biosensor procedures have been developed in which a single-stranded PNA probe is immobilized onto optical or mass-sensitive transducers in order to detect the complementary strand or corresponding mismatch in a DNA sample solution. The hybridization events are converted into electric signals by the transducers [78].

1.6.5 PNA as a therapeutic agent

The structural and hybridization properties of PNAs make it an attractive candidate for developing effective antisense and antigene agents and drugs. This is also supported by relative ease of synthesis and very high chemical and most importantly biological stability of PNAs. It was reported to be very promising from the results obtained with PNA using *in vitro* cell-free systems and celluar microinjection. Although PNA-RNA duplexes are not substrates for RNase H, efficient antisense inhibition of translation could be demonstrated [79-81].

Also, PNA strand displacement complexes were bound to double-stranded DNA and resulted in blocking of transcription initiation and even elongation by RNA polymerase. At the same time, PNA can be bound to template RNA and targeted to the RNA part of telomerase. These binding can efficiently arrest reverse transcriptase by inhibiting telomerase activity [82].

In another study, PNAs were targeted to rRNA in order to find out whether PNAs would be bacteriostatic by preventing ribosome function (like many natural antibiotics). It was found that two bis-PNAs targeted to homopurine stretches did indeed inhibit translation at submicromolar concertrations in a cell-free system. These results had the promise that PNAs may be used to develop novel genetic designer "antibiotics" under the condition of better ways of delivering them to bacteria [83].

It has been identified that several PNAs potently inhibited the human telomerase at nanomolar concentrations. Such PNAs may be candidates for anticancer drugs. It has also been shown that PNAs targeted to the RNA template effectively inhibited HIV reverse transcriptase. It suggested that this mechanism may be a means of developing novel drugs to treat HIV infections (AIDS).

However, before we can even get close to evaluating PNA's real drug potential, there are still very much more to learn about the biological, pharmacological properties and behavior of PNAs. But, it is safe to conclude that present research about PNAs allows one to look to the future application of PNA with optimism.

1.6.7 Other applications

It is obvious that PNA has great potential to be used as a tool in molecular biology, an agent in diagnostics and a drug in disease therapy. And also, PNA itself is a valuable tool in elucidating the mechanism and binding forces involved in DNA or RNA processes.

The isolation of specific active genes can be achieved by PNA strand invasion. In a study, a biotinylated PNA has been used to isolate active chromatin fragments. In a low ionic strength, excess PNA oligomers were bound to the chromatin fragments of interest. And then, the PNA-DNA hybrids were separated from the free PNA by density gradient centrifugation and DNA is isolated with the use of streptavidin coated magnetic particles. However, it is unclear for this molecular binding mechanism, because PNA usually would not have the role in stable Waston-Crick-Hoogsteen type triplex strand displacement.

It is very similar in structure between PNA-DNA and DNA-DNA duplexes. This similarity gives an opportunity to study the interactions of PNA-DNA complexes with DNA binding ligands [84]. It is assumed that there were two binding forces divided into electrostatic and hydrophobic contributions. If the negatively charged deoxyribose backbone is replaced by a neutral pseudopeptide backbone, it might indicate the role of the phosphate groups in the binding of a given ligand. In recent research, it was found that the binding affinity between ligands and PNA-DNA duplex was decreased relative to the binding affinity between ligands and DNA-DNA duplex [85]. That might be a result of the reduced negative charge in the minor groove of PNA-DNA duplex.

Finally, it has been published that PNAs were used for nucleic acid capture in sample preparation protocols. It means PNAs can be applied for sequence-specific capture of single stranded nucleic acids. A method for capture of dsDNA with extreme sequence specificity has been devised. Two bis-PNA DNA openers were used to capture a biotinylated oligonucleotide in this method. And also, the approaches for predicting the thermal stability of PNA-DNA duplexes are available now, which is helping the design of suitable PNA in terms of length and sequence.

1.7 Aims

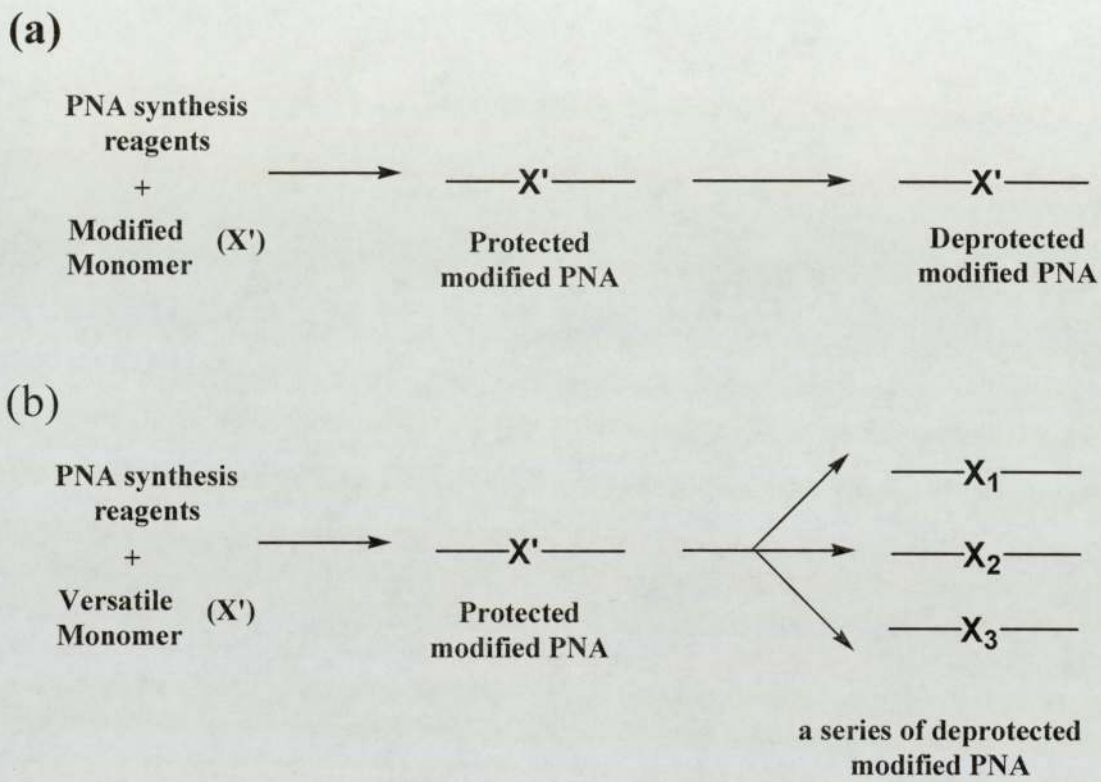


Figure 16. Different strategies for modified PNA synthesis.

As discussed above, a large number of PNA analogues (or modified PNA) have been prepared with aims of improving cellular uptake of PNAs and their use as diagnostic and biological tools. However most methods for modified PNA synthesis follow the strategy presented in Figure 16 (a). A modified monomer is synthesized in a protected form. This modified monomer is then incorporated at a desired position during the PNA oligomer synthesis. The resin-bound, fully protected modified PNA oligomer thus obtained is then deprotected to yield the final product. The modified monomer must be stable to the chemical treatment involved in assembly, in particular, the repeated treatment of strong acids.

This strategy has several limitations: (1) each new modified monomer requires the synthesis of a modified monomer because these are normally not commercially available and even a minor change in the structure of modified base, needs multistep synthesis of a new monomer, followed by synthesis and purification of the new modified PNA; (2) a desirable modified monomer, which is commonly made expensively and labour intensively, is sometimes not stable under the conditions used in PNA synthesis.

So we make an alternative strategy for the synthesis of modified PNA, which is to incorporate a versatile monomer into a PNA oligomer which combines the properties of stability to the normal procedure of PNA synthesis with sufficient chemical reactivity to allow one to convert it into a number of desirable products after synthesis of the oligomers. The strategy has at least the following potential advantages: (1) a single synthesis of an oligomer containing the versatile base could provide a source of PNA oligomers each containing a different modified base; (2) it offers the possibility of making PNA containing a labile or chemically reactive base.

In the above case, the modified monomer is a base derivative which is sufficiently stable to withstand the conditions of synthesis but can be converted into various different modified bases after the synthesis. It is termed the postsynthetic substitution strategy and summarized in Figure 16 (b) just as in the previous method. This also employs a modified monomer in addition to conventional synthetic reagents. Versatile monomers and modified monomers are necessary in the beginning of this strategy of hypothesis. So, to develop a versatile method for the synthesis of monomers and modified PNA is first aim of this project.

However, the reagent of ethyl N-[(2-Boc-amino) ethyl] glycinate is a key intermediate for the synthesis of all standard PNA monomers. Although it is commercially available, it is very costly and of limited availability. Some research groups have developed a number of synthetic procedures for it [27.38]. However, there are drawbacks in those approaches, such as long synthetic route, or harsh reaction conditions. To develop a new convenient and economical method for the synthesis of ethyl N- [2-Boc-amino] ethyl] glycinate is another aim of this project.

Chapter II Experimental Section

2.1 Materials

1. 1,2-diaminoethane (Sigma-Aldrich Company LTD, United Kingdom)
2. dichloromethane (Sigma-Aldrich Company LTD, United Kingdom)
3. di-tert-butyl dicarbonate (Sigma-Aldrich Company LTD, United Kingdom)
4. NaCl (Sigma-Aldrich Company LTD, United Kingdom)
5. Na_2SO_4 (Sigma-Aldrich Company LTD, United Kingdom)
6. Triethylamine (Sigma-Aldrich Company LTD, United Kingdom)
7. Potassium iodide (Sigma-Aldrich Company LTD, United Kingdom)
8. Ethyl chloroacetate (Sigma-Aldrich Company LTD, United Kingdom)
9. EtOAc(Sigma-Aldrich Company LTD, United Kingdom)
10. Methanol (Sigma-Aldrich Company LTD, United Kingdom)
11. Et_2O (Sigma-Aldrich Company LTD, United Kingdom)
12. HCl (Sigma-Aldrich Company LTD, United Kingdom)
13. Thymine (Sigma-Aldrich Company LTD, United Kingdom)
14. K_2CO_3 (Sigma-Aldrich Company LTD, United Kingdom)
15. NaOH (Sigma-Aldrich Company LTD, United Kingdom)
16. P_2O_5 (Sigma-Aldrich Company LTD, United Kingdom)
17. Ethyl N- (2-Boc-aminoethyl) glycinate. HCl (Sigma-Aldrich Company LTD, United Kingdom)
18. DhbtOH (Sigma-Aldrich Company LTD, United Kingdom)
19. N,N- Diisopropylethylamine (Sigma-Aldrich Company LTD, United Kingdom)
20. DMF (Sigma-Aldrich Company LTD, United Kingdom)
21. DCC (Sigma-Aldrich Company LTD, United Kingdom)
22. $NaHCO_3$ (Sigma-Aldrich Company LTD, United Kingdom)
23. $KHSO_4$ (Sigma-Aldrich Company LTD, United Kingdom)
24. THF (Sigma-Aldrich Company LTD, United Kingdom)
25. LiOH (Sigma-Aldrich Company LTD, United Kingdom)
26. Iodine monochloride (Sigma-Aldrich Company LTD, United Kingdom)
27. Uracil (Sigma-Aldrich Company LTD, United Kingdom)
28. Chloroacetic acid (Sigma-Aldrich Company LTD, United Kingdom)

29. DMSO (Sigma-Aldrich Company LTD, United Kingdom)

30. $CDCl_3$ (Sigma-Aldrich Company LTD, United Kingdom)

2.2 Instruments

1. Heated stage microscope melting point apparatus
2. Proton NMR spectra were recorded at 250 MHz on a Bruker AC-250 instrument
3. Thin-layer-chromatography (TLC) was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and viewed under UV light at 254nm
4. Mass spectra were obtained on an HP G1034C GC/LC-MS Chemstation using atmospheric chemical ionisation (ACPI) method

2.3 Synthesis of ethyl N-[(2-Boc-amino)-ethyl] glycinate (C) and its hydrochloride (D)

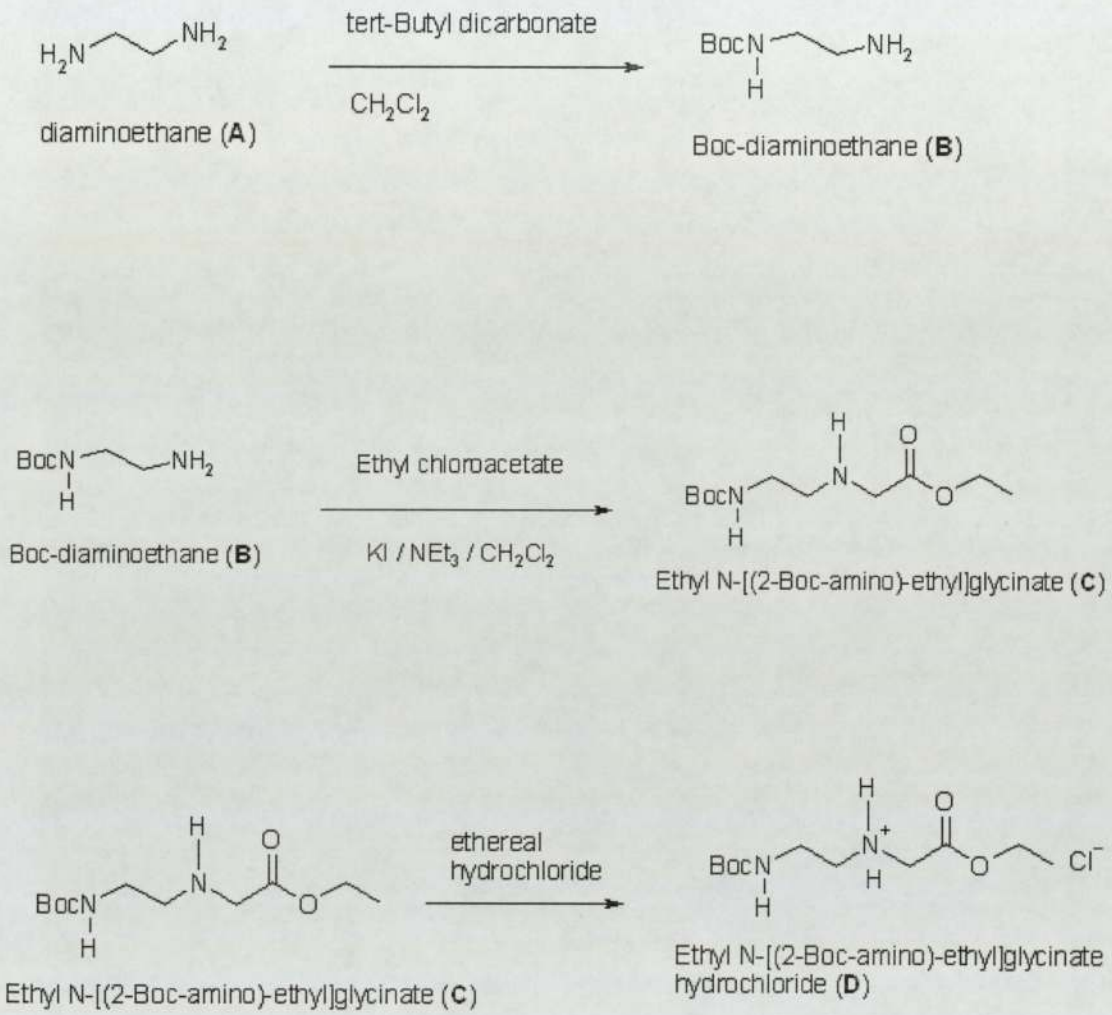


Figure 17. Synthesis of ethyl N-[(2-Boc-amino)-ethyl]glycinate and its hydrochloride

2.3.1 Synthesis of Boc-diaminoethane (B)

1, 2-diaminoethane (**A**, 12 ml; 0.18 mmol) was dissolved in dichloromethane (45 ml). With stirring and cooling a solution of di-tert-butyl dicarbonate (5.0 g; 23 mmol) in dichloromethane (12 ml) was added dropwise over 2 h. The mixture was stirred for a further 20 h. Water (23 ml) was added and the resulting mixture was agitated vigorously. Following phase separation the aqueous phase was extracted twice with dichloromethane (10 ml). The combined organic phases were washed modified with half-saturated NaCl (aq), dried (Na_2SO_4) and filtered. The organic phase was concentrated to dryness on a rotary evaporator to afford title product as green oil (**B**). ^{1}H NMR ($CDCl_3$): 5.2 (s, 1H, -NH), 3.22 (t, 2H, -CH_2), 2.7 (t, 2H, -CH_2), 1.38 (s, 9H, -CH_3), 1.20 (s, 2H, NH_2); HR-MS: calcd 160.2156 for $C_7H_{16}N_2O_2$, found: 160.1654. The crude product could be used directly for the next step of the synthesis without further purification.

2.3.2 Synthesis of ethyl N-[(2-Boc-amino)-ethyl] glycinate (C)

The organic phase from the above was concentrated to a volume of approx. 20 ml, NEt_3 (3.1 ml; 23 mmol) and potassium iodide (0.0125 g; 0.075 mmol) were then added. A solution of ethyl chloroacetate (5.0 ml, 22 mmol) in dichloromethane (12 ml) was added dropwise with stirring and the mixture was stirred at rt for 5 h. Water (12.5 ml) was added and the mixture was agitated vigorously. The organic phase was separated, dried (Na_2SO_4), filtered and the solvent was removed in vacuo. The resulting crude product was chromatographically purified on silica gel with EtOAC/methanol (96:4). Yield: 1.52 g (52%) ^{1}H NMR ($CDCl_3$): 5.20 (s, 2H, -NH), 4.15(q, 2H, -CH_2), 3.36(t, 2H, -CH_2), 3.16 (t, 2H, -CH_2), 2.71 (t, 2H, -CH_2), 1.38 (s, 9H, -CH_3), 1.22 (t, 3H, -CH_3); HR-MS: calcd 248.3216 for $C_{11}H_{24}N_2O_4$, found: 248.8736.

2.3.3 Synthesis of ethyl N-[(2-Boc-amino)-ethyl] glycinate hydrochloride (D)

To an ice-bath cooled solution of ethyl N- [(2-Boc-amino)-ethyl]glycinate (**C**) (1.0 g, 4 mmol) in Et_2O (18 ml) was added ethereal HCl (1.00 M, 5 ml, 4.3 mmol) dropwise over 30 min. After the precipitate was filtered, the mixture was stirred at 0°C for 1 h, then filtered, rinsed with Et_2O, and dried in vacuo to afford 0.7 g of ethyl N-[(2-Boc-amino)-ethyl] glycinate.HCl (yield 0.48g 47%), an air-stable, non-hygroscopic white solid. MP: 118-119°C (MP: 121°C [15]), ^{1}H NMR (DMSO): 4.20 (q, 2H, $-CH_2$), 3.97 (s, 2H, $-CH_2$), 3.22 (t, 2H, $-CH_2$), 2.97 (t, 2H, $-CH_2$), 1.37 (s, 9H, $-CH_3$), 1.25 (t, 3H, $-CH_3$). HR-MS: calcd 282.7697 for $C_{11}H_{23}ClN_2O_4$, found: 282.0431.

2.4 Synthesis of peptide nucleic acid monomer containing Thymine

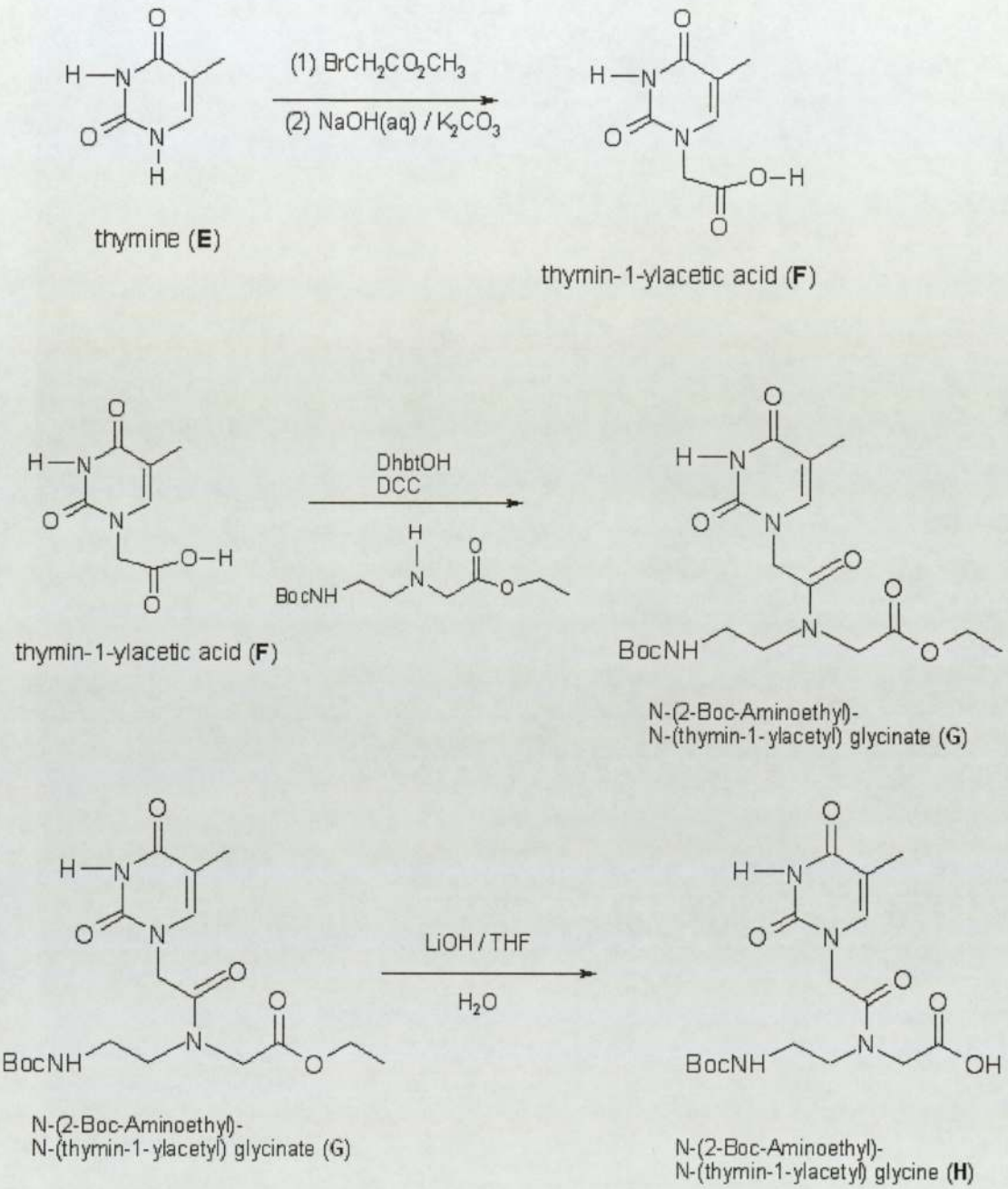


Figure 18. Synthesis of peptide nucleic acid monomer containing Thymine

2.4.1 Synthesis of thymin-1-ylacetic acid (F)

To a suspension of thymine (2.0 g, 15.9 mmol) and K_2CO_3 (2.2 g, 15.9 mmol) in dry DMF (48 ml) was added methyl bromoacetate (1.5 ml, 5.9 mmol), and the mixture was stirred vigorously for 25 h under argon. The mixture was filtered and evaporated to dryness in vacuo. The solid residue was cooled to 0°C, treated with water (15.0 ml) and 4M HCl (aqueous, 0.7 ml), and stirred for 30 min. The precipitate was collected by filtration and washed with water (3 × 8.0 ml). The precipitate was treated with water (16.0 ml) and 2M NaOH (aqueous, 8.0 ml) and boiled for 10 min. The mixture was cooled to 0 °C, treated with 4M HCl (aqueous, 5.4ml, and stirred for 30 min. The title compound was collected by filtration, washed with water (3 × 8.0 ml), and dried over P_2O_5 yielding 0.82 g (45%). ^{1}H NMR ($CDCl_3$): 11.37 (s, 1H, -NH), 7.49 (s, 1H, -CH), 4.35 (s, 2H, $-CH_2$), 1.74(s, 3H, $-CH_2$). HR-MS: calcd 184.1512 for $C_7H_8N_2O_4$, found: 183.8863.

2.4.2 Synthesis of ethyl N- (2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl) glycinate (G)

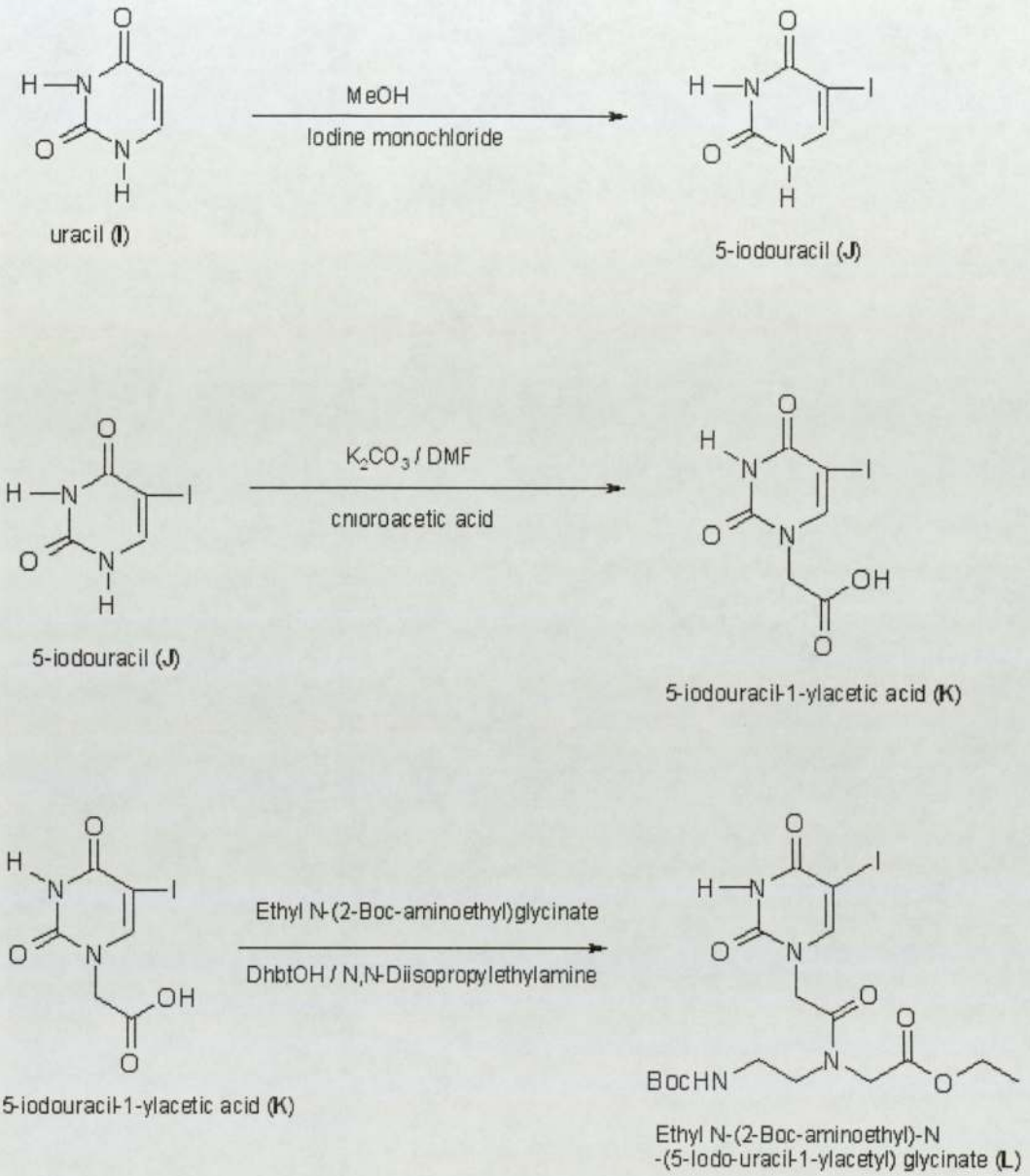
Ethyl N-(2-Boc-aminoethyl)glycinate.HCl (0.2135 g, 1 mmol), DhbtOH (0.1625 g, 99 mmol), N,N-Diisopropylethylamine (0.6 ml, 1 mmol) and thymin-1-ylacetic acid (0.2109 g, 1 mmol) were dissolved in DMF (7 ml) and DCM (7 ml) was added. The solution was cooled to 0°C and DCC (0.2172 g, 1.2 mmol) was added. The ice bath was removed after 1 h and the stirring was continued for another 4 h at rt. The precipitated DCU was removed by filtration and washed with DCM (2 × 2.0 ml). To the combined filtrates was added more DCM (10 ml) and the solution was washed successively with dilute aqueous $NaHCO_3$ (3 × 8 ml), dilute aqueous $KHSO_4$ (2 × 8 ml), and finally brine (1 × 8 ml).The precipitate in the organic phase was removed by filtration, whereupon the organic phase was dried and evaporated to dryness, in vacuo. The oily residue was dissolved in DCM (3 ml) and filtered, and the title

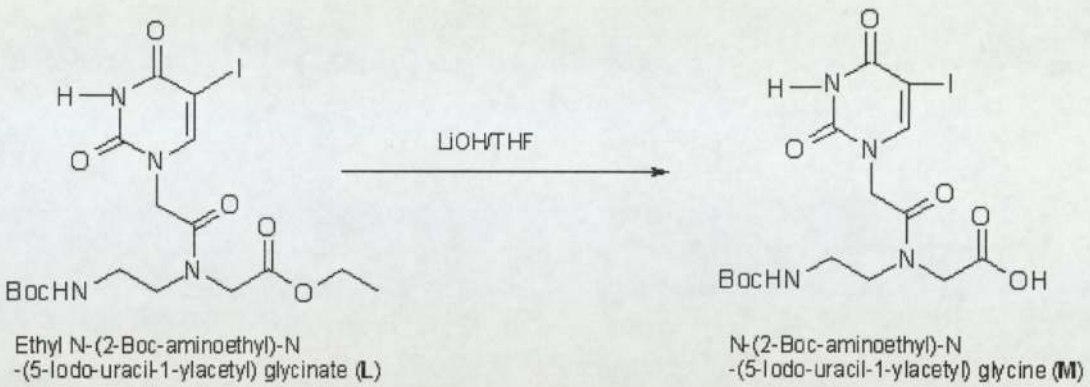
compound was precipitated by the addition of petroleum ether (4.5 ml) at 0°C. The precipitate was re-dissolved in DCM (3 ml) and precipitated once more with petroleum ether (4.5 ml). This afforded 0.15g of **G** (yield: 0.28 g 37%). ^{1}H NMR ($CDCl_3$): 11.25 (s, 1H, -NH), 7.32 (d, 1H, -CH), 4.63 (s, 2H, $-CH_2$), 4.02 (d, 2H, $-CH_2$), 3.33 (d, 2H, $-CH_2$), 3.14 (d, 2H, $-CH_2$), 3.11 (s, 3H, $-CH_3$), 1.40 (s, 9H, $-CH_3$); HR-MS: Calcd 412.4418 for $C_{18}H_{28}N_4O_7$, found: 412.5437.

2.4.3 Synthesis of N- (2-Boc-Aminoethyl)-N- (thymin-1-ylacetyl) glycine (H)

Compound **G** (0.1 g) was suspended in THF (1 ml) and 1M LiOH (aqueous,1 ml) was added. The mixture was stirred for 45 min at room temperature and was then filtered to remove residual DCU. Water (2 ml) was added to the solution and was washed with DCM (4 ml). Additional water (1.5 ml) was added and the solution was washed once more with DCM (3 ml). The aqueous solution was cooled to 0 °C and the pH was adjusted to 2 by the dropwise addition of 1 M HCl. The title compound was extracted with EtOAC (9 × 4 ml). The combined extracts were dried and then evaporated to dryness, in vacuo. The residue was evaporated once from MeOH and afforded Boc-T-OH as a colorless glassy solid after drying overnight. Yield 0.03g (21%). ^{1}H NMR ($CDCl_3$) 11.33 (s, 1H, -NH), 7.29 (d, 1H, -CH), 4.60 (s, 2H, $-CH_2$), 3.95 (d, 2H, $-CH_2$), 3.36 (d, 2H, $-CH_2$), 3.15 (d, 2H, $-CH_2$), 3.01 (s, 3H, $-CH_3$), 1.35 (s, 9H, $-CH_3$). MP: 122-123 °C, HR-MS: Calcd 384.2232 for $C_{16}H_{24}N_4O_7$, found: 384.5437.

2.5 Synthesis of modified PNA monomer containing 5-iodouracil





Ethyl N-(2-Boc-aminoethyl)-N -(5-Iodo-uracil-1-ylacetyl) glycinate (**L**)

N-(2-Boc-aminoethyl)-N -(5-Iodo-uracil-1-ylacetyl) glycine (**M**)

Figure 19. Synthesis of PNA monomer containing 5-iodouracil

2.5.1 Synthesis of 5-iodouracil (J)

Iodine monochloride (3.25 g, 0.02 mol) was added to a stirred suspension of uracil (1.12 g, 0.01 mol) in 80 ml of MeOH. The resulting solution was heated at 50°C for 2 h and separation of a crystalline mass occurred and TLC indicated complete conversion of uracil to 5-iodouracil. The mixture was filtered and the crystals were washed with Et_2O. The combined filtrate and washings were chilled at 0°C overnight and a second crop of crystals was collected to give 0.7g after drying. (Yield: 0.78 g, 54%), ^1H NMR ($CDCl_3$): 11.38 (d, 2H, -NH); 7.87 (s, 1H, CH); HR-MS: Calcd 174.9485 for $C_4H_3N_2O_2I$, found: 174.7647.

2.5.2 Synthesis of 5-iodourail-1-yalacetic acid (K)

To a suspension of 5-iodouracil (0.2 g, 0.8 mmol) and K_2CO_3 (0.1 g) in dry DMF (20 ml) was added chloroacetic acid (0.08 g, 0.8 mmol).The mixture was stirred vigorously for 4 h under Argon. The mixture was filtered and evaporated to dryness in vacuo. The solid residue was cooled to 0 °C, treated with water (20 ml) and 4M HCl (aqueous, 1 ml) and stirred for 30 min. The precipitate was collected by filtration and washed with water (3 × 2.0 ml) and title compound was collected. (Yield: 0.38g 47%) ^{1}H NMR ($CDCl_3$): 11.77(1H, 1 N-H); 8.19(1H, -CH); 4.39(2H, -CH2); HR-MS: Calcd 233.0216 for $C_6H_5N_2O_4I$, found: 233.4431.

2.5.3 Synthesis of N-(2-Boc-aminoethyl)-N-(5-iodouracil-1-ylacetyl) glycine (M)

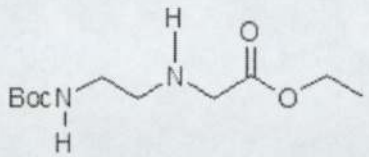
Ethyl N-(2-Boc-aminoethyl)glycinate.HCl (0.21 g, 1 mmol), DhbtOH (0.16 g, 99 mmol), N,N-Diisopropylethylamine (0.6 ml, 1 mmol) and 5-iodouracil-1-ylacetic acid (0.29 g, 1 mmol) were dissolved in DMF (7 ml) and DCM (7 ml) was added. The solution was cooled to 0 °C and DCC (0.22 g, 1.2 mmol) was added. The ice bath was removed after 1h and the stirring was continued for another 4h at rt. The precipitated DCU was removed by filtration and washed with DCM (2 × 2.0 ml). To the combined filtrates was added more DCM (10 ml) and the solution was washed successively with dilute aqueous $NaHCO_3$ (3×8ml), dilute aqueous $KHSO_4$ (2 × 8 ml), and finally brine (1 × 8 ml).The precipitate in the organic phase was removed by filtration, whereupon the organic phase was dried and evaporated to dryness, in vacuo. The oily residue was dissolved in DCM (3 ml) and filtered, and the title compound was precipitated by the addition of petroleum ether (4.5 ml) at 0 °C. The precipitated was colleted and redissolved in DCM (3ml) and precipitated once more with petroleum ether (4.5 ml). This afforded Compound **L** (see Figure 3.3). (Yield: 0.08 g 38%) ^{1}H NMR, $(CD_3)_2CO$: 7.91 (s, 1H, -NH), 6.03 (s, 1H, -CH), 4.85 (s, 2H, CH_2), 4.30 (s, 2H, -CH_2), 4.08 (q, 2H, -CH_2), 3.50 (t, 2H, -CH_2), 3.25 (m, 2H, -CH_2),

1.21 (s, 9H, -CH_3), 1.14 (t, 3H, -CH_3); HR-MS calcd for $C_{17}H_{25}N_4O_7I$, 524.0768, found: 523.8927.

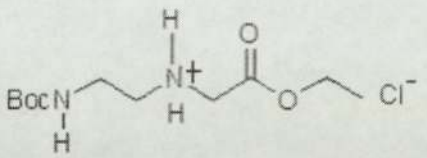
Compound **L** (0.1g) was suspended in THF (1 ml) and 1M LiOH (aqueous, 1 ml) was added. The mixture was stirred for 45 min at room temperature and was then filtered to remove residual DCU. Water (2 ml) was added to the solution, and it was washed with DCM (4 ml). Additional water (1.5 ml) was added and the solution was washed once more with DCM (3 ml). The aqueous solution was cooled to 0 °C and the pH was adjusted to 2 by the dropwise addition of 1 M HCl (aqueous, 1.0 ml). The title compound was extracted with EtOAC (9×4ml). The combined extracts were dried and then evaporated to dryness, in vacuo. The residue was evaporated once from MeOH and afforded title product (M) as a solid after drying overnight (Yield: 0.04g 27%) ^{1}H NMR: 7.87 (s, 1H, -NH), 6.24 (s, 1H, -CH), 4.76 (m, 2H, -CH_2), 4.14 (s, 2H, -CH_2), 3.58 (q, 2H, -CH_2), 3.37 (t, 2H, -CH_2), 1.35 (s, 9H, -CH_3), 1.21 (t, 3H, -CH_3); HR-MS calcd 496.0412 for $C_{15}H_{21}N_4O_7I$, found: 496.7582.

Chapter III Results and Discussion

3.1 Synthesis of ethyl N-[(2-Boc-amino)-ethyl]glycinate (C) and its hydrochloride (D)



Ethyl N-[(2-Boc-amino)-ethyl]glycinate (**C**)



Ethyl N-[(2-Boc-amino)-ethyl]glycinate hydrochloride (**D**)

Figure 20. Ethyl N- [(2-Boc-amino)-ethyl]glycinate and its hydrochloride

In order to prepare PNA oligomers, it is an important and ubiquitous need to synthesize PNA monomers. N-[(2-Boc-amino)-ethyl] glycinate is an essential reagent for the synthesis of PNA monomers. Although it is commercially available it is very costly and of limited availability. Therefore my project started with the development of a convenient and economic synthetic method for this reagent.

Ethyl N-[(2-Boc-amino)-ethyl]glycinate and its hydrochloride salt were synthesized with an improved method. This method is just a two-step reaction as shown below, and the title compound was acquired after flash column chromatography. It was found that this compound was suitable for the synthesis of peptide nucleic acid monomers as it had been used for the preparation of the thymine monomer and iodouracil monomers in this project as described in Section 2.2 and 2.3. The glycinate was easily converted into its hydrochloride. The benefit associated with the hydrochloride salt is that it is a more stable and convenient form and easy to handle and store.

The backbone polymer of PNA is comprised of 2-aminoethylglycine units. This structure is normally prepared by the reaction of Boc-ethylenediamine with a haloacetic acid derivative, most often ethyl bromoacetate [14]. In this procedure, a mixture of the desired product and varying amounts of the undesired dialkylated amine is produced often containing unreacted Boc-ethylenediamine. It is inefficient and inconvenient to scale-up for use, therefore necessary to purify this product by chromatography.

After column chromatographic purification, ethyl N-[(2-Boc-amino)-ethyl]glycinate was obtained. The purity of the product was good enough for the subsequent reaction but high purity product could be obtained by converting it to hydrochloride salt that is a white solid and can be recrystallised from acetone. The salt can be more conveniently stored and is more stable than neutral form which is usually a viscous oil. We have prepared the thymine PNA monomer from this salt in this project and did not find any disadvantage over the use of the neutral form.

In our experiment, the most difficult part is to choose suitable temperature for the synthesis and best time point to stop the reaction. According to our observation, there were a few side products in this chemical reaction that influenced the final expected yield. We selected different temperature and time points to compare our product yield and purity. This procedure took me almost 4 months to find a best reaction condition. However, finally, it turned out it was worthwhile to do it.

According to Table 1, the highest product percentage, 52%, could be achieved, when the reaction time was 5 hours and reaction temperature was 25°C. At the same reaction temperature and the different reaction time, longer reaction time could not make the product percentage continuously higher and higher. The reason is that the longer reaction time increased the side products in the reaction, so the percentage of desired product decreased. Moreover, at the same reaction time and the different reaction temperature, the higher temperature could not make the product percentage

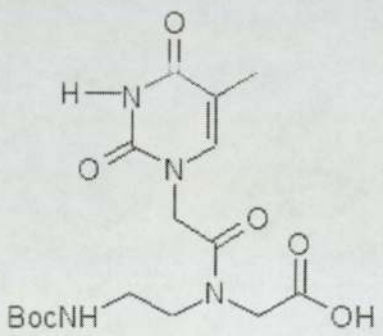
higher, either. The reason is higher temperature also possibly increases the side products of the reaction and causes the product percentage to be lower.

Table 1. Percentage yields of product for different reaction times and reaction temperatures

Product Percentage	2 hours	5 hours	8 hours	10 hours
15 °C	31%	41%	42%	40%
25 °C	44%	52%	37%	32%
30 °C	42%	38%	31%	--
40 °C	--	36%	25%	--

There are some other methods for synthesizing this product, but compared to them, our method simplifies the reaction to two steps and the satisfying product is obtained although purification by chromatography is needed. This is the advantage. In summary, when the procedure is carried out as described above, an improved synthetic method for ethyl N-[(2-Boc-amino)-ethyl] glycinate, the PNA backbone monomer is obtained in highly pure form; and its hydrochloride salt was developed in this project. These reagents were used for the synthesis of PNA monomers in this project.

3.2 Synthesis of N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl) glycine (H)



N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl) glycine (H)

Figure 21. N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl)glycine

Peptide nucleic acid monomers containing the four natural nucleobases are commercially available. However, they are expensive and of limited availability (to our knowledge only Applied Biosystems supplies these monomers). To gain some experience on the synthesis of PNA monomers and, to a lesser extent, save some expenses on the purchase of the PNA monomers, the synthesis of N-(2-Boc-aminoethyl)-N-(thymin-1-ylacetyl)glycine (**H**) was carried out. The synthesis of **H** essentially followed a reported procedure.

PNAs are DNA analogs in which 2-amonoethylglycine linkages replace the normal phosphodiester backbone. There is a methylene carbonyl linker connecting standard nucleotide bases to the backbone at the amino nitrogens. This chemistry has three important results: firstly, PNAs are neutral molecules having influence of absence of charge; secondly, PNAs are achiral, there is no need for PNAs to develop a stereoselective synthesis; and thirdly, PNAs can use standard protocol for solid-phase peptide synthesis.

The synthesis of the thymine monomer (Figure 21) involved alkylation of thymine (**E**) with methyl bromoacetate followed by the hydrolysis of the resultant methyl ester to give thymin-1-ylacetic acid (**F**). For the attachment to the backbone moiety ethyl N-(2-Boc-aminoethyl) glycinate was used. The resulting T monomer ethyl ester (**G**) was hydrolysed with LiOH in water/THF to afford the monomer (**H**).

In this experiment, ethyl N- (2-Boc-aminoethyl) glycinate was used to attach to the backbone moiety rather than N-(2-Boc-aminoethyl) glycine as previously reported. This may improve the workup and purification. It is found that thymine can be easily alkylated with methyl bromoacetate in an appropriate condition.

Because the ethyl N-(2-Boc-aminoethyl) glycinate.HCl was used in this synthesis and it was a salt, N, N-diisopropylethylamine was added into the reaction mixture to

neutralize hydrochloride. Due to the restriction of stirring condition, the reaction time was prolonged from overnight to 25 h so that the thymine can be completely converted into the desired product.

In our experiment, thymin-1-ylacetic acid was coupled to the Boc-protected backbone N-(2-Boc-aminoethyl) glycine. This reaction was activated by pentafluorophenyl ester. The methyl or ethyl ester backbone is usually used. However, this reduces the nucleophilicity of the secondary amino group of the backbone; we need more potent activation of nucleobase-acetic acid. The combined use of DhbtOH and DCC was beneficial for introduction of thymin-1-yl-acetic acid in situ activation. It can improve work up and purification by esterification of the backbone. It is reported that the Boc-protected backbone esters can be obtained by a reductive amination of Boc-aminoacetaldehyde with glycine methyl or ethyl ester.

It is essential to keep the reagents used as anhydrous as possible for PNA synthesis. If the reagents were contaminated by water, it will result in incomplete reaction. To avoid this problem, reagents should be unpacked on arrival and kept in a sealed container at -20°C. To ensure proper synthesis, it is necessary to dry the powdered reagents in *vacuo* overnight before use. We found this always a worthwhile precaution during humid weather. At the same time, reagents had better be warmed to room temperature before solubilization in order to prevent water from condensing in the container.

After successful synthesis of thymine-monomer, it is very possible to get other PNA-monomers such as cytosine-monomer, adenine-monomer and guanine monomer by similar chemical reaction. However, there will be differences in use of reagents and protective groups because of the difference of actual structure in every base. For example, Cbz group has to be used to protect the exocyclic amino group before alkylation in preparation of cytosine or adenine monomer.

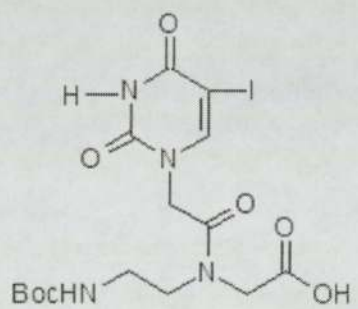
And also, by using such PNA monomers, chemical functional groups can be

introduced into the backbone and the nucleotide bases of PNAs. The physical properties of the PNAs such as hydrophobicity, hydrophilicity and ionic character, etc. can be finely tuned and controlled. This method maybe greatly facilitates the optimisation of pharmacokinetic behaviour of PNA.

3.3 Synthesis of modified PNA monomer containing 5-iodouracil (M)

The recognition of nucleic acid sequences by oligonucleotides is an important feature in many molecular biological techniques and PNAs have been used in such applications because of their improved hybridization characteristics. It is important to use labeled PNA in antisense and ribonucleoprotein inhibition studies by tracking cellular compartmentalization or localization.

The radio labeling of PNA has been done by an appended peptide or oligonucleotide. But these methods have some potential drawbacks, for example, labeling PNA with smaller reporter groups has generally been done through the C-terminus or N-terminus. This is limited to only one or two labels. It is therefore necessary to introduce substituents to label or polylabel PNA with a modified peptide nucleic acid monomer.



N-(2-Boc-aminoethyl)-N-(5-Iodo-uracil-1-ylacetyl) glycine (M)

Figure 22. N- (2-Boc-aminoethyl)-N-(5-Iodo-uracil-1-ylacetyl) glycine

The synthesis of PNA monomer containing 5-iodouracil (**M**) followed the synthetic

procedures as shown before. It was found that the treatment of uracil (**I**) with iodine monochloride under appropriate conditions gave clean conversion to the 5-iodo derivative (**J**). It has been reported previously that chlorination of uracil compounds can be effected using elemental chlorine or N-chlorosuccinimide in acetic acid and provide the 5-chlorouracils in moderate yields. Some researchers have described recently a new procedure that utilizes hydrogen chloride and m-chloroperoxybenzoic acid.

For the successful synthesis of 5-iodouracil, it is crucial to choose the solvent. Other common solvents for this chemistry such as acetonitrile may result in an emulsion, so this makes the product isolation more difficult and produces lower yield.

The next reaction is a one-step alkylation which is accomplished by the use of chloroacetic acid in aqueous alkali. It has been reported that in aqueous base iodouracil is not stable or dialkylation could occur; we also found these problems with the traditional approach of alkylation with ethyl bromoacetate followed by hydrolysis. The 5-iodouracil-1-yl acetic acid was then condensed with ethyl (N-Boc-aminoethyl) glycinate followed by hydrolysis to yield the target compound (**M**).

It is important that the derivatization at the C_5 position of uracil does not modify the Watson-Crick base pairing face of the nucleobase, and a propynyl-substituent at this position should not cause problems. The duplex formation may be stabilized as seen in DNA. When incorporating a label to the nucleobase, the introduction of a stereocenter along the polyamide backbone is possible but should preferably be avoided; for instance, an amino acid may be used other than glycine.

The C- and N-terminus in PNAs are available for other forms of derivatization by internal labeling. It is notable that cyclic bisPNA could form with the possibility of intramolecular cyclization. If the labels incorporated in the oligomers, it may be possible to constructs new types of PNA-based molecular beacons with appended labels on C- or N- terminus.

Finally, it is desired to modify PNAs with other methods for future application. Those modifications can be developed by attaching compounds such as carboxylic acid groups to the N-terminal amine or by coupling amino acids during solid-phase synthesis. Once these modified PNAs have been synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry.

PNA synthesis can be made straightforward either by Boc or Fmoc protocols with using manual or automated protocols. From our research, we found that manual synthesis was simple, inexpensive and efficient. It is readily adaptable to the production of chemically modified PNAs or closely related PNAs. Manual synthesis is especially advantageous for laboratories with small budgets for PNA synthesis. Moreover, a pilot series of PNAs can be developed by manual synthesis to test the feasibility of a project. Finally, this laboratory's experience may support automated synthesis and have the advantage of foreseeing the need of synthesis of a large numbers of PNAs.

In the procedure of PNA synthesis, researchers should expect that some preparation be required to achieve experimental aims. PNA properties such as solubility and target recognition are not always predictable in laboratories lacking experience with PNAs. In general, we usually have to redesign our initial PNAs in order to get desired properties in obtaining oligomers. If laboratories cannot make the commitment of resources, they should re-examine plans for PNAs and balance the potential complication with the potential for future useful insights.

Chapter IV Conclusion and future work

Based on our research, we successfully got the ethyl N-[(2-Boc-amino) ethyl] glycinate, which is a key intermediate for the synthesis of all standard PNA monomers. We developed a new convenient and economical method for the synthesis of ethyl N-[2-Boc-amino] ethyl] glycinate.

We used the ethyl N-[2-Boc-amino] ethyl] glycinate made by our lab to get the PNA monomer including thymine. In this experiment, ethyl N-(2-Boc-aminoethyl) glycinate was used to attach to the backbone moiety rather than N-(2-Boc-aminoethyl)glycine as previously reported. This may improve the workup and purification. It is found that thymine can be easily alkylated with methyl bromoacetate in an appropriate condition.

Finally, a modified uracil PNA monomer was also synthesized based on methods reported, which can be used for the synthesis of modified peptide nucleic acid by post-synthetic modification.

In the future, the other PNA monomers can be developed by improved reaction routes. The modified uracil PNA monomer will be developed into other modified monomers by replacing uracil to other groups or molecules, which can be used in the synthesis of PNA oligomers including modified bases.

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