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REGULAR ARTICLE

# A facile route to synthesize N-(Boc-Aminoethylglycin)thymine Ethyl Ester, application to the synthesis of PNA-oligonucleotide conjugates

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**Abstract.** Peptide nucleic acid oligonucleotide conjugates are attracting immense interest currently because of their use in the biomedical and diagnostic field as antigene and molecular sensors. The efficient PNA synthesis methods can reduce their cost and may increase availability for their wider use. Here we described a facile synthesis of the peptide nucleic acid monomer N-(Boc-Aminoethylglycin)thymine Ethyl Ester [Ethyl 2-(N-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)acetamido)acetate]. The monomer N-(Boc-Aeg)thymine Ethyl Ester has been prepared in a good yield (96%) by highly efficient procedure involving direct coupling of nucleobase thymine with the backbone Ethyl N-(Boc-aminoethyl)-N-(chloroacetyl)glycinate, which was prepared from the reaction of Ethyl N-[(2-Boc-amino)-ethyl]glycinate with chlroacetylchloride. The key intermediate Ethyl N-[(2-Boc-amino)-ethyl]glycinate involved in the synthesis has been prepared *via* a scalable and cost-effective route with a yield of (98%). The thyminyl PNA monomer was reported to be used in various synthetic applications, and our cost-effective, highly scalable method of synthesis will expand its wider use.

**Keywords.** Ethylenediamine; peptide nucleic acid synthesis; nucleic acid analogs; nucleobase.

## 1. Introduction

Peptide Nucleic Acids (PNA) have generated great interest in the scientific community for nucleic acid mimic as well as for potential application as a molecular biology tool. PNAs were first reported in the year 1991 by Nielsen *et al*.[1] They used computer modelling to develop a new concept to design a new nucleic acid analogous to natural nucleic acid, which is composed of (2-aminoethyl)glycine repeat units, a polyamide backbone with the four nucleobases as side chains. The concept was to retain the nucleobases of DNA and replace the deoxyribose phosphodiester backbone by a pseudo-peptide backbone which according to computer modeling was homomorphous with the DNA backbone. The different bases purines (Adenine, Guanine) and pyrimidines (Cytosine, Thymine) are joined to the backbone *via* a methylene or carbonyl linkages. The intramolecular spacing between pyrimidine or purine side chains in a PNA matches the spacing between the bases in DNA or RNA; as a consequence of which, PNA can form a normal hydrogen bond with a DNA or RNA strand.[2] A large number of chemical modification of the original aminoethyl glycine PNA backbone into cyclohexylpna,[3] prolinepna,[4] ethylamine,[5] aminoacids[6] has been reported.[7] Although a considerable number of PNA derivatives have been reported by modifying the PNA backbone, nucleobase and the linker attaching a nucleobase to the backbone, there is a ubiquitous requirement to develop new PNA analogues with superior properties to unmodified PNA. The backbone of Peptide nucleic acid (PNA) is achiral, uncharged as a consequence of which it shows a stronger binding affinity towards complementary PNA/DNA strands than complementary DNA/DNA strands.[8] It is chemically stable and resists hydrolytic (enzymatic) cleavage and is un-degraded

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inside a living cell. PNA is a very potent molecule in sequence-specific recognition of DNA and RNA strictly obeying the Watson-Crick hydrogen-bonding rule, and the hybrid complexes exhibit extraordinary thermal stability.[9] Owing to this superior hybridization properties and improved chemical and enzymatic stability relative to nucleic acid, PNA has attracted a major attention for a wide variety of applications in the diagnostic and pharmaceutical field, such as PNA is capable of inhibiting trasncscription[10–12] as well as translation,[13] so it acts as an active component for antisense therapy[13] and gene diagnostics.[14] PNA can break up DNA duplex and form PNA/DNA triplex[15] or double duplexes without denaturing the DNA duplex, and thus are capable of working as antigene agents.[14] PNAs do not contain any (pentose) sugar moieties or phosphate groups like DNA, but it mimics the behavior of DNA in many respects and studies also reveal that PNA in place of DNA can be used as a molecular probe for many investigations purposes.[7,16] Owing to these superior characteristics of PNA compared to DNA oligos, development of convenient methodologies for the synthesis of PNA monomers is highly desirable and much in focus in recent years.[17–19] PNA can be synthesized by using Fmoc or Boc solid-phase peptide synthesis.[20–22] The development of PNA with modified nucleobases shows unique physiochemical properties and due to the modular nature of PNA, many backbone-modified PNA analogs attached to modified nucleobases have been synthesized.[23–25] All these endeavors rely on accessible methods of PNA monomer synthesis and especially on a scalable and cost-effective synthesis of the PNA monomer bearing additional functional groups. In the field of peptide nucleic acid research to develop unusual or modified monomers, there is a requirement to prepare N-(2-aminoethyl)glycine derivatives. This compound is a key intermediate in the synthesis of all standard PNA monomers. Several synthetic methods for preparation of such molecules N-[2-(Boc)aminoethyl]glycine esters have been reported in the literature.[21,26,27] The N-[2-(Boc)aminoethyl]glycine Ester has previously been synthesised[2,21] by alkylation of monobocethylenediamine with halo acetic acid derivative; commonly ethyl bromoacetate but the procedure affords to lower reaction conversion and is inconvenient to scale up as it involves tedious chromatographic separation, giving the product in an overall moderate to low yield. This compound is also synthesized by the reductive alkylation of Boc-ethylenediamine using ethyl glyoxylate hydrate in a quantitative yield and high purity without chromatography[27] but this strategy involves hazardous conditions and is time-consuming. Previously suitable methods have been reported for the preparation of Ethyl-N-(Boc-aminoethyl)-N-Chloroacetyl)glycinate backbone and coupling to the nucleobase thymine.[2,11,21] The approach described by Egholm *et al.*, for the synthesis of thyminyl PNA monomer has a number of experimental difficulties. Meltzer *et al.*, developed an alternative route to the coupling of Ethyl-N-(Boc-aminoethyl)-N-Chloroacetyl)glycinate with base thymine that provides directly the desired product. However, the reactions involve tedious workup procedure and coupling reaction of nucleobase thymine to backbone provides monomer in low yield. We, therefore, developed an efficient and more general route to the coupling of Ethyl-N-(Boc-aminoethyl)-N-Chloroacetyl)glycinate with base thymine that provides the desired end product in higher yield. Herein we have explored the methodology that has drawn significant advantages over the traditional methods. A major benefit of this method is the higher conversion of reaction, minor byproducts, and minimum purification requirements. We afford a brief description of experimental condition for a facile synthesis of N-(Boc-Aeg)thymine Ethyl Ester monomer that proceeds via 4 steps with an overall yield of 72%. The synthesis of target monomer aeg-PNA-T was performed starting from the monoboc protection of 1,2-diaminoethane. Bocethylene diamine (**1**) was prepared following the method described in the literature.[21] The protected form of ethylenediamine is normally in a good physical state to be handled for the next step. Bocethylene diamine was alkylated at $N_2$ with ethyl bromoacetate to form aminoethyl glycine ethyl ester (Scheme 1, molecule **2a**) in good yield (98%). The molecule **2a** was then acylated with a more reactive compound chloroacetylchloride to get the corresponding N-(chloroacetyl) derivative (Scheme 2, molecule **3**) in 85% yield which was subsequently used for alkylation with nucleobase thymine to obtain N-(Boc-Aminoethylglycin)thymine Ethyl Ester (Scheme 3, molecule **4**), the desired PNA monomer in excellent yield (96%) with high purity. This compound will find use in the synthesis of modified reporter groups or modified nucleobases and also can be used for the oligomerization of PNA monomer to give PNA dimer, trimer and tetramer and also for synthesis of peptide nucleic acid conjugates.[28,29]

## 2. Experimental

### 2.1 *Ethyl-N-(2-Boc-aminoethyl) glycinate*

Ethyl bromoacetate (9.6 mL, 0.06 mol) was added dropwise (0.2 mL/min) over a period of 1 h to a solution of **1** (9.204 g, 0.0573 mol) and triethylamine (13.5 g, 0.09 mol) dissolved in acetonitrile (250 mL) under vigorous stirring. The reaction mixture was stirred for 1 h at room temperature. The resulting reaction mixture was then concentrated

under vacuum followed by dilution with 300 mL of water. The aqueous solution was extracted with dichloromethane at pH 10.5 (3 times) and 11.2 (3 times). The pH of the solution was maintained by adding 30% sodium hydroxide solution. The combined organic layer was passed through $K_2CO_3$ and concentrated on a rotary evaporator. The crude product contains the mixture of desired monoalkylated product **2a** (13.87 g, 98%) and a minor bis-alkylated product **2b** (0.38 g, 2%). Finally, the residue was purified by flash chromatography with simultaneous monitoring by TLC (ethylacetate:hexane, 4:1). After purification and concentration in a vacuum (98%) of the product **2a**, a light-yellow viscous oil was obtained (Scheme 1).

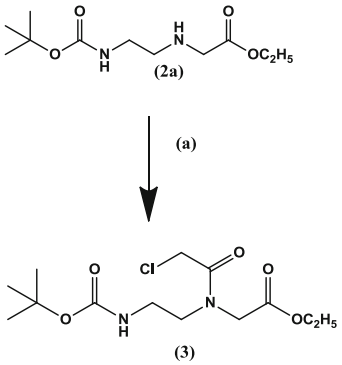
**2a**: $^1$H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta$-5.15 (s, br, 1H), 4.11 (q, $J$ = 7.2 Hz, 2H), 3.33 (s, 2H), 3.15 (t, $J$ = 5.6 Hz, 2H), 2.67 (t, $J$ = 4 Hz, 2H), 1.93 (s, 1H), 1.37 (s, 9H), 1.21 (t, $J$ = 7 Hz, 3H).

**2b**: $^1$H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta$-5.50 (s, br, 1H), 4.05 (q, $J$ = 7.2 Hz, 4H), 3.42 (s, 4H), 3.04 (t, $J$ = 6 Hz, 2H), 2.7 (t, $J$ = 4 Hz, 2H), 1.33 (s, 9H), 1.16 (t, $J$ = 4 Hz, 6H).

## 2.2 *Ethyl-N-(Boc-aminoethyl)-N-(chloroacetyl)glycinate*

In a two neck, round bottom flask **2a** (20 g, 0.08 mol) was dissolved in a mixture of triethylamine (12 mL) and dichloromethane (500 mL) and stirred at 0 °C. After 15 min (the solution became uniform at 0 °C) 20 mL of chloroacetyl chloride was added dropwise (0.4 mL/min) over a period of 60 min under vigorous stirring. The reaction mixture was allowed to come to room temperature and stirred for 1 h. About 1000 mL of water was then added to the reaction mixture. The organic layer was separated by separating funnel. The remaining aqueous layer was extracted with dichloromethane. The organic layers were combined and washed with saturated sodium chloride solution. It was dried over $K_2CO_3$ and filtered through celite powder. Concentrated and evaporation on rotary evaporator yielded a yellowish viscous oil. Finally, the oil was purified by flash chromatography (eluent: EtOAc:hexane, 1:1) with simultaneous monitoring by TLC to form (22.28 g, 85%) of molecule **3** as a yellow viscous oil.

**Molecule 3** (Scheme 2). $^1$H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta$-5.4 (br, s, 1H), 4.18 (q, $J$ = 5.2 Hz, 2H), 4.14 (s, 2H), 4.00 (s, 2H), 3.45 (t, $J$ = 5.6 Hz, 2H), 3.28 (q, $J$ = 5.7 Hz, 2H), 1.46 (s, 9H), 1.2 (t, $J$ = 5.2 Hz, 3H).

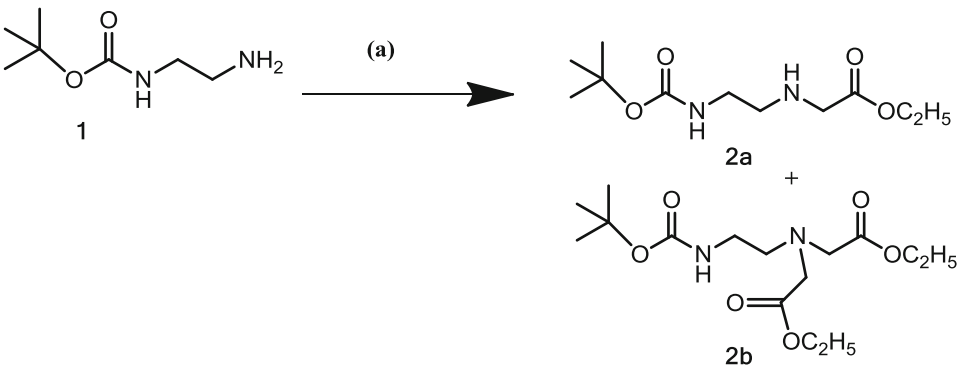



Reagents and conditions: (a) Chloroacetyl chloride, $NEt_3$, $CH_2Cl_2$, $K_2CO_3$, rt, 85%

**Scheme 2.** Ethyl-N-(Boc-aminoethyl)-N-(chloroacetyl)-glycinate

## 2.3 *Synthesis of N-(Boc-Aeg)thymine Ethyl Ester*

A mixture of thymine (5.22 g, 0.04 mol) and anhydrous $K_2CO_3$ (5.32 g, 0.02 mol) dissolved in dry DMF (400 mL) was stirred for 1h under a nitrogen atmosphere. To this stirred mixture, molecule **3** (12.26 g, 0.036 mol) dissolved in dry DMF was added slowly dropwise (0.1 mL/min) under a nitrogen atmosphere. The reaction mixture was stirred vigorously at room temperature for 12 h. The completion of the reaction was monitored by TLC $R_f$ 0.39 (EtOAC). The DMF was then removed under reduced pressure and 500 mL of water was then added to it. After




Reagents and conditions: (a) Ethylbromoacetate, $NEt_3$, $CH_3CN$, rt, 98%

**Scheme 1.** Synthesis of Ethyl-N-(2-Boc-aminoethyl)glycinate

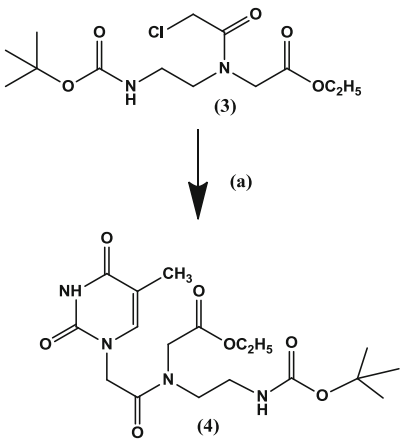
aqueous workup and column chromatographic purification N-(Boc-Aeg)thymine Ethyl Ester was obtained as a white solid with (15.04 g, 96%) yield. Melting point was found to be 162–164 °C.

**Molecule 4** (Scheme 3). $^1$H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta$-9.30 (s, 1H), 6.96 (s, 1H), 5.62–5.64 (m, 1H), 4.56 (s, 2H), 4.23 (q, $J$ =7.2 Hz, 2H), 4.03 (s, 2H), 3.51 (t, $J$ = 5.6 Hz, 2H), 3.30 (q, $J$ = 6 Hz, 2H), 1.89 (s, 3H), 1.42 (s, 9H), 1.25 (t, $J$ = 7.2 Hz, 3H).

HRMS (ESI-TOF) m/z: $[M+H]^+$ calculated for $C_{18}H_{28}N_4O_7$ (Molecule **4**) was found to be 413.2034.

## 3. Results and Discussion

Our initial attempt to synthesize N-(Boc-Aeg)thymine Ethyl Ester began with the stepwise functionalization of Ethylenediamine. Monobocethylenediamine was prepared in 90% yield according to the method described in the literature.[21] Once the pure compound monobocethylenediamine (**1**) was achieved the next step of the reaction was easily carried out. The alkylation of monobocethylenediamine with ethyl bromoacetate in the presence of triethylamine was successfully performed under simple experimental condition. The reported method[2,21] for the reaction of monobocethylenediamine with ethyl bromoacetate as described previously leads to the formation of monoalkylated product and also minor dialkylated product often containing the unreacted starting material. The unreacted starting material was to be recover again. This procedure is therefore inefficient to scale up. As an alternative approach, this compound is also synthesized by the reductive alkylation of Boc-ethylenediamine using ethyl glyoxylate hydrate.[27] Although this method gives the product in a quantitative yield and high purity without chromatography, it involves hazardous condition and is time-consuming. We report a convenient synthetic procedure to obtain the desired product (Scheme 1, molecule **2a**) in 98% yield. This procedure is more efficient because of the higher conversion of reaction, minor byproducts, and minimum purification requirements. This procedure is based on the quantitative addition of reagent ethyl bromoacetate. Ethyl bromoacetate dissolved in the dichloromethane is added dropwise quantitatively 0.2 mL/min over a period of 1h to the reaction mixture containing monobocethylenediamine, by reducing the possibility of overalkylation. The crude product contained bis-alkylated monobocethylenediamine as a minor byproduct (Scheme 1, molecule **2b**) that was removed by chromatography using silica gel-60. The requirement of excess monobocethylenediamine as described in the literature procedure for the reaction is avoided here. NMR analysis after purification of the product showed almost no sign of the undesired dialkylated product and starting material. The product, light yellow viscous oil liquid is stable to storage for extended periods and in a good physical condition to be handled for next reaction. To demonstrate the scalability of this route the reaction has been performed on 3 g, 9.204 g (monobocethylenediamine) scales. On each scale, the product was obtained in high yield. Our second approach was to react the backbone (Scheme 1, molecule **2a**) with chloroacetylchloride for chloroacylation in methylene chloride using the base triethylamine to give the desired molecule **3** in 85% yield as described in the Scheme 2. The procedure involved the controlled addition of the reagent chloroacetylchloride. The crude product was purified by column chromatography using silica gel-60. The product is strongly viscous light-yellow oil and can be stored for a longer period. Thus, molecule **3** has been synthesized quantitatively and the analytical data confirm its purity. The next approach was to couple the backbone (Scheme 2, molecule **3**) with the nucleobase thymine. The coupling to nucleobase thymine with the Boc protected Ethyl-N-(Boc-aminoethyl)-N-Chloroacetyl)glycinate backbone has been previously described in the literature.[2,11] The method developed by Egholm *et al.*, for the synthesis of N-(Boc-Aeg)thymine Ethyl Ester involves the coupling of Boc protected N-(2-aminoethyl)glycine (Aeg) side chain with the N-carboxy-methylated base. However, this synthetic route suffers from a number of experimental difficulties, involves tedious chromatographic separation and affords a moderate to low yield of monomer (Table 1). As an alternative approach, Meltzer *et al.*, described a method for the synthesis of




Reagents and condition: (a) Thymine, $K_2CO_3$, DMF, rt, 96%

**Scheme 3.** Synthesis of N-(Boc-Aeg)thymine Ethyl Ester

**Table 1.** Comparison of the % yield of compound N-(Boc-Aeg)thymine Ethyl Ester in the present study with previous methods.

| Entry | Reaction scale (g) | Reaction condition | Temperature | Time of reaction (h) | % Yield | References |
|---|---|---|---|---|---|---|
| a | 12.26 | Thymine, $K_2CO_3$/DMF | Room temperature (RT) | 12 h | 96% | This work |
| b | 13.5 | Dhbt OH, Thymin-1-ylacetic acid, DCC | RT | 3 h | 70% | Egholm *et al.* |
| c | 11.7 | Thymine, NAH/DMF | 75 °C | 1 h | 55% | Meltzer *et al.* |

PNA thyminyl monomer which is based on the direct coupling of a suitably protected N-(chloroacetyl)-Aeg with thymine nucleobase, but the reaction affords the desired monomer in 55% yield (Table 1). We have anticipated a convenient and easy route to the coupling of backbone with nucleobase thymine. The nucleobase thymine is coupled to the backbone (**3**) in DMF at room temperature with the use of $K_2CO_3$ as a base instead of the usual NaH. However, we found that with the use of base $K_2CO_3$ the coupling occurs predominantly giving the product N-(Boc-Aeg)thymine Ethyl Ester in a yield of 96% (Table 1). Use of the coupling reagent is avoided here. To demonstrate the scalability of this route the reaction was performed at much lower and higher scales e.g., 0.613 g, 12.26 g of Ethyl-N-(Boc-amino ethyl)-N-(chloroacetyl)glycinate. On each scale, the product was obtained in quantitative yield. The alkylation of thymine is regiospecific.[30] A single spot was observed in TLC indicating the formation of a single product. The $^1$H-NMR spectra obtained for compound **4** shows substitution at the 1-position only. However, there is an appearance of solvent peaks at the chemical shift of 2.88, 2.96, and 8.02 which is because of the presence of traces of DMF in compound **4** as an impurity. We have observed two sets of peaks in the $^1$H NMR of compound **4** with 3:1 ratio. Since the thymine system at the tertiary amine position of the compound **4** can exist in half chair conformation, the isomers resulted from the pseudo axial and equatorial substitutions may present in 3:1 ratio. However, it will be premature to assign the peaks for the isomers from the $^1$H NMR data of compound **4**. The ESI mass spectrum of compound **4** showed a peak at m/z 413.2034 corresponding to $[M+H]^+$.

## 4. Conclusions

We have developed a new synthetic route to Boc protected PNA monomer N-(Boc-Aeg)thymine Ethyl Ester and have described that this route can be used for the large-scale synthesis of PNA monomers. We have improved synthesis of the intermediate Ethyl-N-[(2-Boc-amino)-ethyl]glycinate which is a ubiquitous requirement in the preparation of PNA backbone. The backbone (**3**) Ethyl-N-(Boc-aminoethyl)-N-(chloroacetyl)glycinate can also be used to prepare a variety of other PNA monomers by the introduction of other suitable n-protecting groups. Due to the wide variety of medicinal and diagnostic applications, the development of new methods for the synthesis of PNA has drawn much more intention recently in the scientific community. This report provides useful improvements in the synthesis of standard PNA monomers to support such endeavours.

## Supplementary Information (SI)

Figures S1-S6 are available at www.ias.ac.in/chemsci.

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