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Design, development and synthesis of a novel labeled PNA monomer incorporated in DNA-hexamer to act as a hybridization probe by FRET

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A novel PNA monomer with adenine nucleobase and a modified backbone with charged $>\overset{+}{N}-H$ and a methylene substituted for $>C=O$ in the linker arm has been synthesised. This modified PNA monomer is further linked with rhodamine via an amide bond, converted to phosphoramidite after linking with a 2-carbon linker. This unit is used for the end coupling at 5′-prime of a hexamer oligonucleotide 5′-TCC ATT-3′ in solid phase. The complementary labeled heptamer has been prepared by coupling the phosphoramidite of fluorescein with 5′-AAT GGAT-3′ as the end coupling step at 5′-prime on solid phase. The two complementary heptamer oligos are hybridized and the FRET signal recorded. The Tm of the modified oligomer has been found to be higher as compared to the normal one. Such a probe can detect single base mismatch by FRET.

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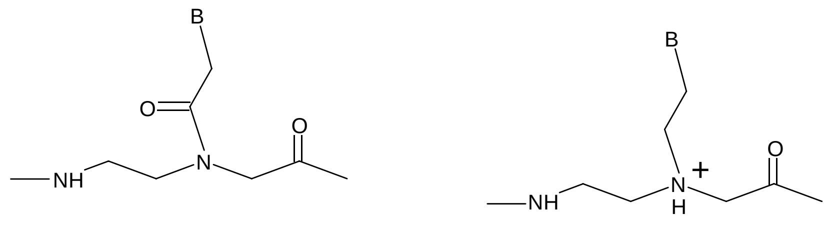
Oligonucleotides (both DNA and RNA) have been extensively used in molecular diagnostics and in antisense therapeutics[1-3]. However, the susceptibility of DNA and RNA to enzymatic degradation makes them undesirable for *in vivo* application. Therefore, there has been an intensive search for analogs of DNA or RNA. Several chemical modifications have been reported to give oligonucleotides with improved properties. Peptide-nucleic acid (PNA) was originally designed and developed as a DNA mimic in which, the negatively charged sugar-phosphate backbone was replaced by a neutral pseudo-peptide composed of *N*-(2-aminoethyl) glycine units[4] having resistance to DNase and RNase degradation. PNA's have been shown to have high binding affinity and hybridize to complementary single stranded DNA's, RNA's or PNA's to form stable duplexes with high sequence selectivity[5,6]. Fluorophore-labelled PNA's have therefore been widely used as diagnostic probes for nucleic acid sequences (e.g. PNA-FISH assay)[7]. PNA has been shown to be a promising candidate for antisense application. However, the cellular uptake of PNA is reported to be extremely low[5,8]. We report herein synthesis of a novel PNA monomer in which the amide linkage to the nucleobase is replaced with tertiary amine (**Figure 1**). This modification is opted to eliminate the conformational constraints of the amide bond and introduce a positive charge to the backbone. The conformational adoptability of the backbone is anticipated to assist in cellular uptake and the charge may result in stronger binding. Number of modifications are already reported where the linker chain length is mainly varied by substituting the amide bond with methylene groups and also by varying the length of linker[4,9-12]. The main objective of this study is to assess the effect of introducing a novel type of peptide monomer in a DNA sequence and study its hybridization, which has been studied by melting behaviour and also by FRET. This study is of great significance in DNA mimicking and molecular diagnostics.

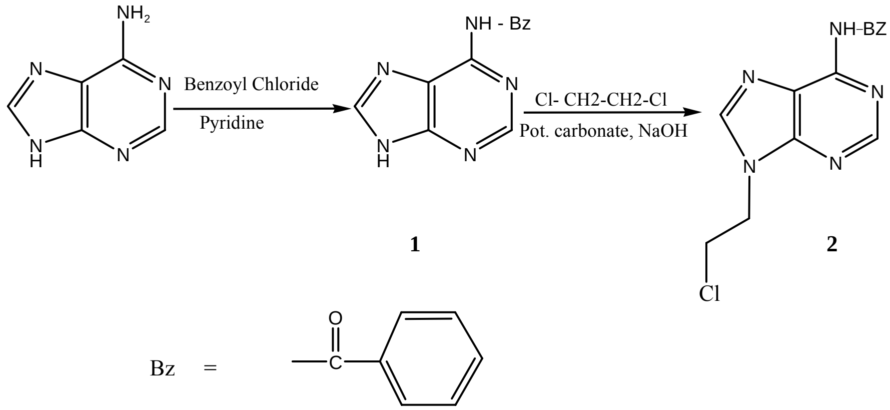
Results and Discussion

A PNA monomer 1-*N*-Boc,4-*N*,*N*-(6-*N*-benzoyl, 9-*N*-ethyladenyl), (glycyl)-ethylenediamine **5** (**Scheme II**) was synthesized in solution phase using Boc chemistry. The primary amino group of adenine was benzoylated **1** (**Scheme I**) and the *N*-protected nucleobase was condensed with dichloroethane to yield (6-*N*-benzoyl)-9-ethylchloro adenine **2** (**Scheme I**). The corresponding 7-substituted isomer was not detected. On the other hand, the backbone *N*-(2-*N*-Boc)



A B

Figure 1—Conventional PNA monomer (A) and present modified monomer (B)



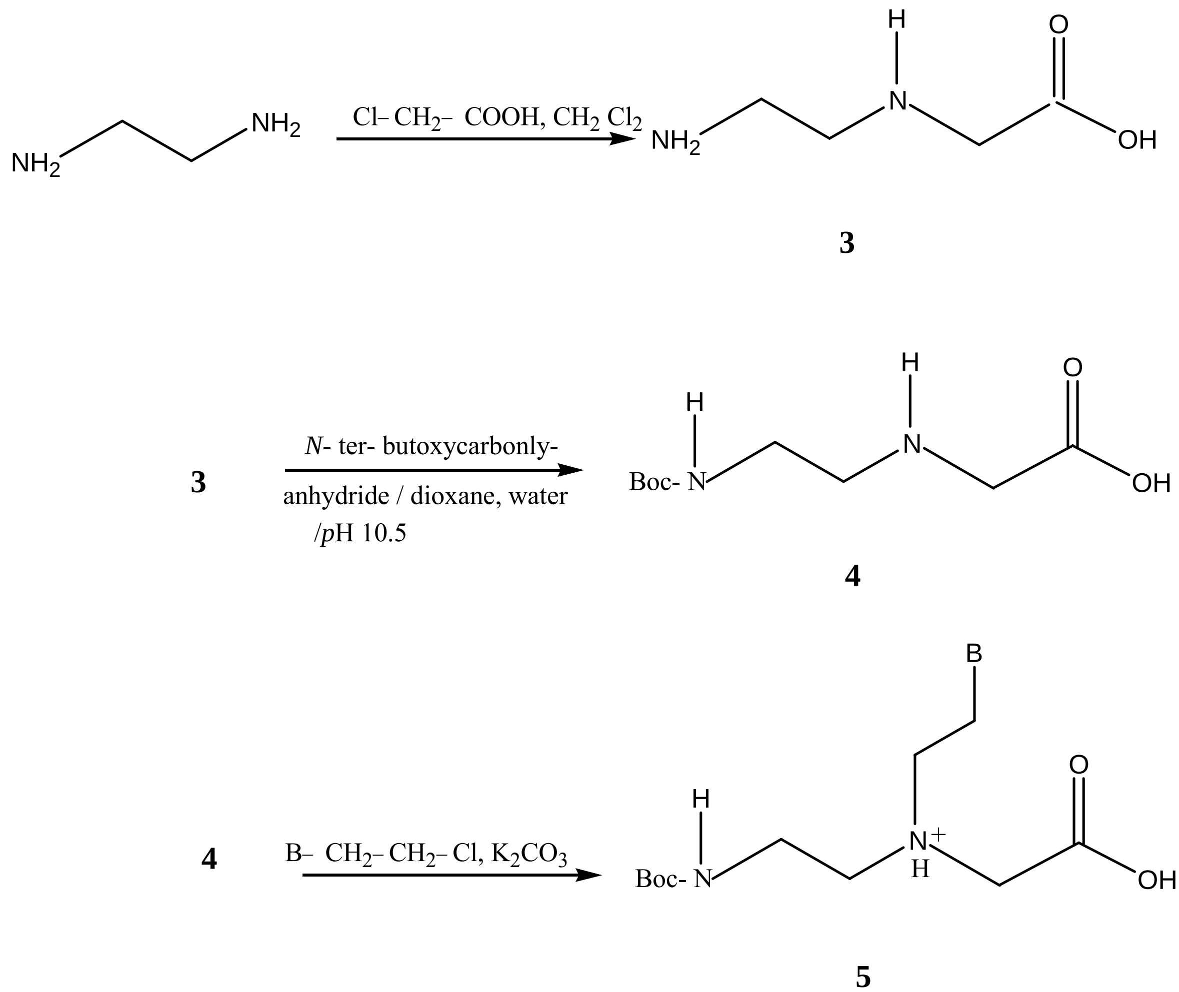
Scheme I—Synthesis of Linker arm attached to N^6-Benzoyl adenine at N^9

aminoethyl glycine **4** (**Scheme II**) was generated by condensing one primary amino functionality of ethylene diamine with chloroacetic acid and protecting the other with Boc. Finally the earlier synthesized nucleobase with linker arm **2** (**Scheme I**) was attached to secondary amino group present in backbone simply by base catalysed condensation (**Scheme II**). Subsequently two labeled heptamer DNA sequences i.e. 5′-*flu* AAT GGA T-3′ (*flu*=fluorescein) and its complementary sequence 5′-*rh*A* TCC ATT-3′ (*rh*=rhodamine, A* denotes PNA monomer with adenine as base) (**Scheme III**) were synthesised.

The fluorescein unit is attached at the 5′-end of the sequence (5′-AAT GGA T-3′) by using 5-aminopentanol as linker. The fluorescein was first attached with 5-aminopentanol and the free hydroxyl terminus was phosphitylated. In the last coupling cycle of DNA sequence synthesis amidite was coupled to give 5′-*flu*-AAT GGA T-3′ (**Scheme IV**).

5′-*rh*-A*TC CAT T-3′, the labeled and modified sequence was synthesized using phosphoramidite approach. The A* unit represents the modified PNA monomer having adenine as nucleobase (**Scheme III**). The rhodamine was first attached to the unit **5** via its amino group after deprotection of Boc group. This condensation was done by activating the COOH function of rhodamine with *p*-nitrophenol, resulting in amide group. The end carboxy function was again activated and coupled with ethanolamine resulting in another amide bond and an end hydroxy function. The free-OH terminus was further used to generate phosphoramidite by using the activator pyridinium trifluoroacetate (Py-TFA) and 2-cyanoethyl *N*, *N*, *N′*, *N′*-tetraisopropyl phosphoramidite (bis-reagent) in 1,2 dichloroethane. Since, *N*,*N*,*N′*,*N′*-tetra isopropyl-phosphoramidite (bis-reagent) is fairly stable and less expensive, therefore, it has been used in the present work. Pyridinium trifluoroacetate was used in place 1*H*-tetrazole since it is an excellent activator, stable cheaper less toxic and has higher *p*Ka (5.23) then *p*Ka of other activators to avoid depurination and detritylation and is more eco-friendly.

The melting profile of the two duplexes i.e. one with modified PNA monomer (5′-*rh*A* TCC ATT-3′) and the other unmodified (5′-*rh*A TCC ATT-3′) with the same complementary oligo (3′-T AGG TAA-*flu*-5′) were recorded by measuring absorbance (OD) at 260 nm on heating at rate of 0.5°C per minute. The melting temperature of the modified duplex $\left(\begin{array}{c}\text{5'-rh-A* TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{array}\right)$ was 19°C, which was 1.5°C



B= Adenine

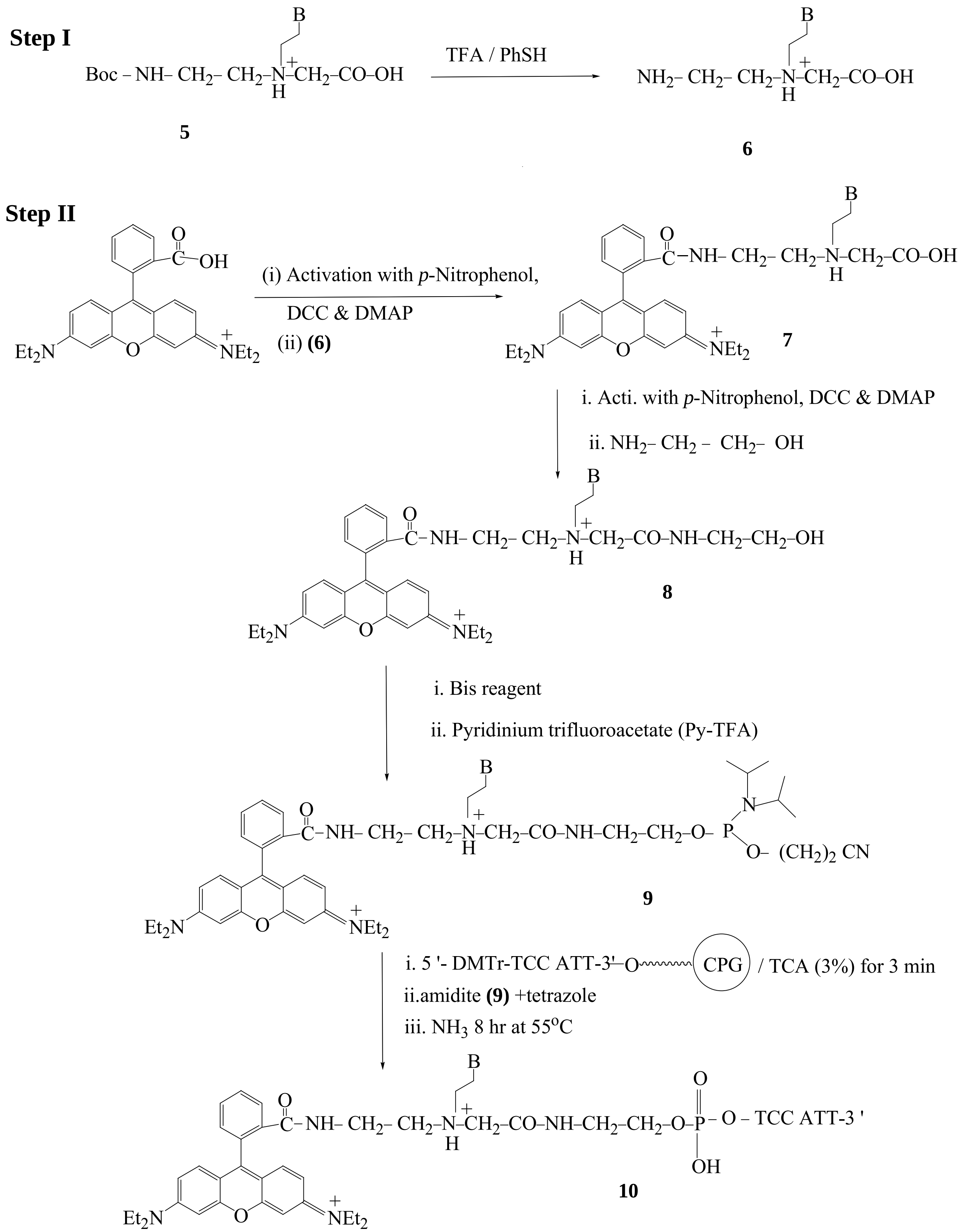
Scheme II — Synthesis of backbone with nucleobase attachment

higher than that of the unmodified duplex $\left(\begin{array}{l}\text{5'-rh-A TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{array}\right)$(**Figure 2**).

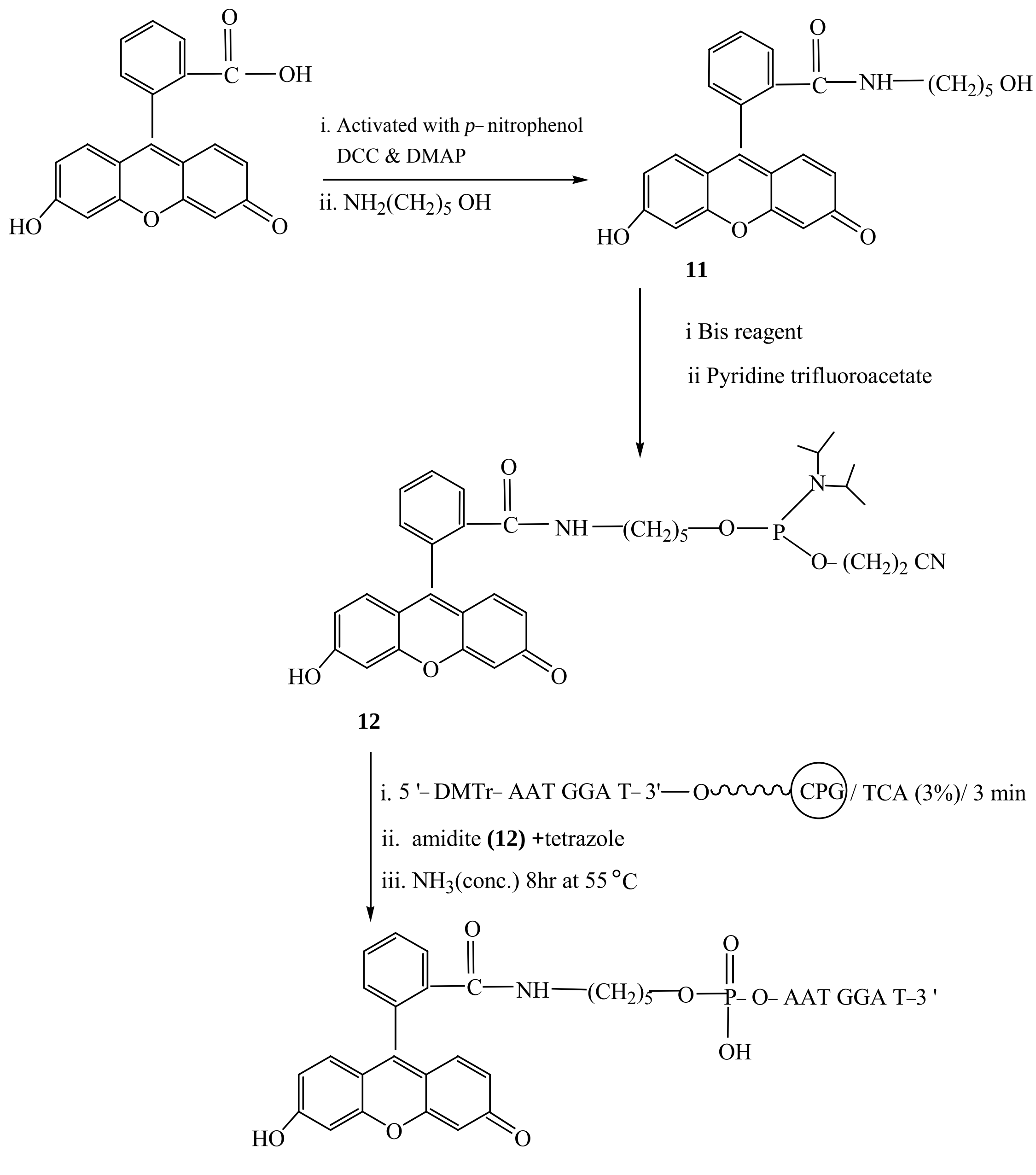
The enhanced melting temperature (19°C) of the duplex with PNA modified monomer i.e. $\left(\begin{array}{l}\text{5'-rh-A* TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{array}\right)$ where A* is the PNA monomer with adenine nucleobase as compared to the unmodified one i.e. $\left(\begin{array}{l}\text{5'-rh-A TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{array}\right)$ (17.5°C) is clear identification of the positive contribution of PNA monomer to stronger hybridisation, thereby showing higher affinity and more stability. Although it would have been better if the melting temperature would have been recorded with the strands without the attached dyes i.e. rhodamine and fluorescence but the contribution of the two is supposed to be counterbalanced and therefore ignored.

The main characteristic of the modified oligonucleotide to be used as a antisense drug is its strong hybridisation with target. The present molecule was designed, so that the charged backbone assists in stronger hybridisation with the normal sequence. The absence of the $>$C=O group in the linker must enhance its flexibility and therefore cellular uptake.

As tabulated in **Table I**, fluorescein and its amidite have excitation wavelength at 496/492 nm respectively, while rhodamine and its amidite have excitation at 543/540 nm respectively. Their respective emission values are 525/520 nm and 580/565 nm. When excited at the excitation wavelength of fluorescein i.e. at 496 nm the fluorescein tagged heptamer sequence shows emission at 516 nm. The rhodamine tagged heptamer with the PNA monomer has excitation wavelength at 542 nm



Scheme III—Synthesis of phosphoramidite of rhodamine labeled PNA monomer and its covalent linking to hexamer 5′-TCC ATT-3′ at 5′-prime.



Scheme IV—Synthesis of phosphoramidite of Fluorescein and its coupling with the heptamer 5′-AAT GGA T-3′ at 5′-prime

with emission at 565 nm (**Figure 3a**). On hybridization the two pairs of complementary heptamers, one with PNA monomer and the other the normal one when excited at 490 nm show only one emission peak i.e. 560 nm (**Figure 3b**). This is the FRET signal peak, which has same value for both, the modified and unmodified duplexes. Since FRET is an extremely sensitive technique and even one base mismatch effects it, it can be concluded that the modified PNA backbone does not effect energy transfer which remains unaffected as long as the base is present. Such a modified oligo sequence can act as a probe for molecular diagnostics.

Material and Methods

All solvent used were of Qualigens analytical grade, which were further purified and dried prior to use. 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetrasopropylphosphoramidite (bis reagent) and pyridinium trifluoroacetate

Table I — Values of Excitation and Emission wave lengths

Dye, Primer	Excitation	Emission
Fluorescein	496 nm	525 nm
Fluorescein amidite	492 nm	520 nm
5×-*flu*-AAT GGA T-3×)	490 nm	516 nm
Rhodamine	543 nm	580 nm
Rhodamine amidite	540 nm	565 nm
(5×-*rh*-A*TC CAT T-3×)	542 nm	565 nm

* A unit represents the modified PNA monomer having adenine as nucleobase

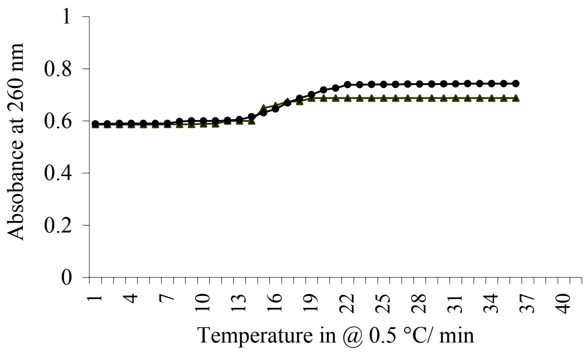


Figure 2 — Comparative melting curves of modified duplex $\begin{pmatrix}\text{5'-rh-A* TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{pmatrix}$ (•, 19°C) and unmodified duplex $\begin{pmatrix}\text{5'-rh-A TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{pmatrix}$ (▲, 17.5°C) sequences.

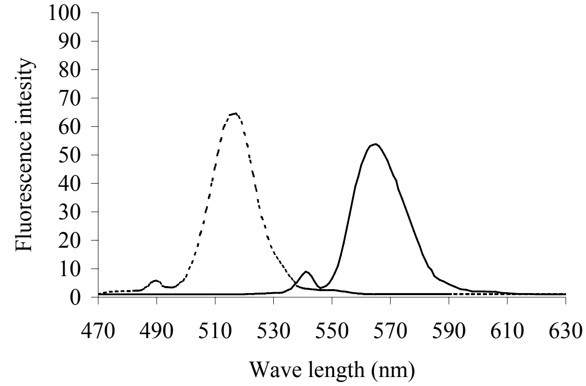


Figure 3a — Overlapping fluorescence spectra of 5'-*flu*-AATGGAT-3' (----) (λ_{ex} 490 nm; λ_{em} 516) and 5'-rh-A*TCCATT-3' (—) (λ_{ex} 542 nm; λ_{em} 565nm)

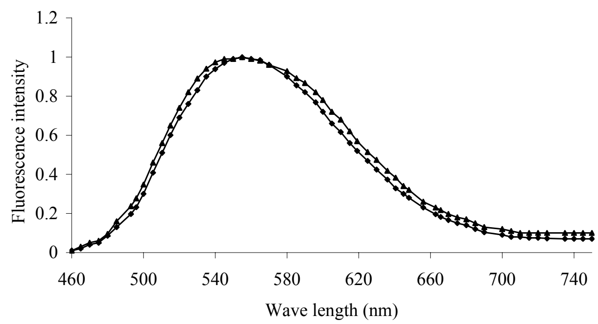


Figure 3b — Florescence Spectra (FRET signal) of duplex $\begin{pmatrix}\text{5'-rh-A TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{pmatrix}$ (▲) and $\begin{pmatrix}\text{5'-rh-A* TCC ATT-3'}\\ \text{3'- T AGG TAA-flu-5'}\end{pmatrix}$ (■)

(Py.TFA) were obtained from ISIS pharmaceuticals. Synthetic oligonucleotide was obtained from Hybridon Inc. USA. UV was recorded on a Hitachi 220S UV-Visible Spectrophotometer. Purification of labeled oligo's was carried out by Pharmacia HPLC using C_{18} coloumn employing UV-VIS detector Hybridization studies were carried out on a HITACHI 220 S, UV-visible spectrophotometer attached with HAAKE DC-5 refrigerated circulatory bath for temperature programming. Fluorescence spectra were recorded on a Kontron. Elemental data were recorded on a Shimadzu 34-408 analyser; ^{1}H NMR and ^{31}P NMR spectra were obtained using Brookers DRX 300.

Experimental Section

6-*N*-(Benzoyl)-adenine 1. To a suspension of adenine (1.351g, 10 mmole) in about 100 mL of pyridine, 10 equivalent of benzoyl chloride (11.6 mL) was added dropwise. Reaction mixture stirred at r.t., reaction was monitored by TLC. After completion of the reaction (after 2 hr) reaction mixture was poured in water, and extracted with ethyl acetate (200 mL), ethyl acetate layer was concentrated *in vacuo* and finally crystallized in EtOH: yield 70% (1.743g). Anal. Calcd for C_{12} H_9 N_5 O: C, 61.84; H, 3.63; N, 28.10. Found: C, 61.74; H, 3.65; N, 28.16%. ^{1}H NMR ($CDCl_3$): δ 8.87 (s, 1H, O=C-NH), 8.73 (s, 1H, H-8), 8.51(s, 1H, H-2), 7.7(s, 5H, Bz), 3.5 (d, 1H, NH).

6-*N*-(Benzoyl)9-(2-chloroethyl) adenine 2. To a suspension of **1** (478 mg, 2 mmole) in dry DMF (5 mL) was added anhydrous K_2CO_3 (276 mg, 2 mmole), after stirring for 5 min, dichloroethane (0.156 mL, 2 mmole) was added dropwise. After stirring for 2-3 hr the reaction mixture was evaporated *in vacuo* and the residue was partitioned between ethyl acetate (35 mL) and water (10 mL). The organic phase was washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. Purified by column chromatography, ethyl acetate-hexane, (40:60, gradient elution), as white solid, yield 50% (301 mg). Anal. Calcd for $C_{14}H_{12}N_5OCl$: C, 55.71; H, 4.00; N, 23.20. Found: C, 54.10; H, 4.8; N, 23.30%. ^{1}H NMR ($CDCl_3$): δ 8.75 (s, 1H, H-8), 8.52 (s, 1H, H-2), 8.87 (s, 1H, NH-C=O), 7.8 (5H, s), 2.8 (t, 2H), 3.0 (t, 2H).

***N*-(2-Aminoethyl) glycine 3**. To a vigorously stirred solution of ethylenediamine (3mL, 4.5 mole) in dichloromethane (20 mL) at 0°C, was added chloroacetic acid (470 mg, 0.5 mmole) in dichloromethane (4 mL). The resulting reaction mixture was washed with water (3 × 50 mL) and extracted with dichloromethane (50 mL). The combined organic layers were dried over Na_2SO_4 and filtered and further dried *in vacuo*, yield 75% (398mg). Anal. Calcd for $C_{14}H_{10}N_2 O_2$: C, 40.67; H, 8.54; N, 23.71. Found: C, 40.82; H, 8.58; N, 23.72%. ^{1}H NMR ($CDCl_3$): δ 10.68 (s, 1H, COOH), 3.0 (s, 1H, NH), 2.75 (t, 2H, H_2N-CH_2-CH_2), 2.67 (t, 2H; H_2N-CH_2-CH_2), 2.32 (s, 2H), 2.12 (t, 2H; NH_2).

***N*-(2-*N*-Boc)aminoethyl glycine 4**. To a suspension of **3** (202 mg, 2 mmole) in a mixture of 6 mL of dioxane and water, Boc-anhydride (406 mg, 2 mmole) was added. The mixture was allowed to cool at room temperature and the *p*H was maintained at 10.5 by the addition of aqueous NaOH (2N). The solution was concentrated to gum and was triturated with DCM (50 mL). The suspension was filtered and organic phase was dried over $MgSO_4$ and evaporated to give the product, yield 90% (392mg). Anal. Calcd for $C_9H_{18}N_2 O_4$: C, 49.52; H, 8.31; N, 12.83. Found: C, 49.50; H, 8.30; N, 12.55%. ^{1}H NMR ($CDCl_3$) : δ 10.65 (s, 1H, COOH), 7.82(t, 2H, O=C-NH-CH_2), 3.40 (m, unresolved, 2H, Boc-HN-CH_2-CH_2), 3.29 (m, unresolved, 2H, Boc-HN-CH_2-CH_2), 2.32 (s, 2H, CH_2), 2.51 (d, 1H, NH), 2.42(m, 4H, NH-CH_2-CH_2-NH), 1.42 (s, 9H, $(CH_3)_3$).

1-*N*-Boc, 4-*N*,*N*-(6-*N*-benzoyl, 9-*N*-ethyl adenyl), (glycyl)-ethylenediamine 5. To a suspension of **4** (438 mg, 2 mmole) in dry DMF (5 mL) was added anhydrous K_2CO_3 (276 mg, 2 mmole), after stirring for 5 min, 6-*N*-(benzoyl) 9-(2-chloroethyl) adenine **2** (1.56 mL, 2 mmole) was added dropwise. After stirring for 2-3 hr the reaction mixture was neutralized and evaporated *in vacuo* and the residue was partitioned between ethyl acetate (35 mL) and water (10 mL). The organic phase washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. Purified by column using ethyl acetate-hexane (40: 60), as eluent to give a white solid, yield 65% (628 mg). Anal. Calcd for $C_{23}H_{29}N_7 O_5$: C, 57.13; H, 6.04; N, 20.27. Found: C, 57.41; H, 6.04; N, 20.28%. ^{1}H NMR ($CDCl_3$): δ 10.68 (s, 1H, COOH), 8.57 (s, 1H, NH-C=O), 8.70 (s, 1H, H-8), 8.56 (s, 1H, H-2), 7.48-8.2 (s, 5H, benzoyl), 7.47 (t, 1H, C=O-NH-CH_2), 3.5 (m, 4H, N-CH_2-CH_2-N), 3.45 (m unresolved, 2H, Boc-HN-CH_2-CH_2), 3.21 (m, unresolved, 2H, Boc $HNCH_2CH_2$), 2.32 (s, 2H, CH_2), 2.9 (d, 2H), 2.2 (d, 2H), 1.41 (s, 9H, $(CH_3)_3$).

Synthesis of 1-*N*-rhodamine-4-*N*, *N*-(6-benzoyl-9-ethyl adenyl), (glycyl)-ethylenediamine 7. Boc group of PNA monomer **5** was deblocked with TFA and thiophenol at 20°C for 1 hr. Rhodamine (800mg, 2 mmole) was dissolved in dioxane (15mL) and to its stirred solution *p*-nitrophenol (333.9 mg, 2.4 mmole) was added followed by DCC (2 eq) and DMAP (0.05 eq). After 2 hr 4-*N*,*N*-(6-*N*-benzoyl, 9-*N*-ethyl adenyl), (glycyl)-ethylenediamine was added in equimolar ratio with further addition of DCC (2eq). The reaction mixture was stirred for another 5 hr at r.t. Completion of reaction was monitored on tlc with a new spot (R_f = 0.6, compared to rhodamine; R_f = 0.4 in DCM). The reaction mixture was evaporated *in vacuo* and then loaded on a short silica column (80-120 mesh; 6 × 2 cm). The desired product was eluted with hexane-ethyl acetate (40 : 60) DCM, yield 65%. Anal. Calcd for $C_{46}H_{51}N_9 O_5$: C, 68.21; H, 6.34; N, 15.56. Found: C, 68.21; H, 6.51; N, 15.59%. ^{1}H NMR ($CDCl_3$): δ 10.9 (s, 1H, COOH), 8.87 (1H, s, NH-C=O), 8.65 (s, 1H, H-8), 8.52 (s, 1H, H-2), 7.5-7.9 (m, 4H, rhodamine), 6.10-7.15 (m, 6H, rhodamine), 7.48-8.2 (s, 5H, benzoyl), 3.5 (m, 4H, N-CH_2-CH_2-N), 3.4 (s, 2H, N-CH_2-CO), 3.39 (q, 8H, rhodamine), 2.6 (m, 2H, CH_2), 2.3 (t, 2H, NH-CH_2), 1.3 (q, 12H, rhodamine).

Synthesis of rhodamine labeled peptide monomer 8. Compound **7** (2 mmole) was dissolved in dioxane (15 mL). To its stirred solution *p*-nitrophenol (333.9 mg, 2.4mmole) was added followed by DCC (2 eq) and DMAP (0.05 eq). After 2 hr ethanolamine was added in equimolar ratio with further addition of 2 eq of DCC. The reaction mixture was stirred for another 5 hr at r.t. Completion of reaction was monitored on tlc with a new spot [R_f = 0.8, 1-*N*-rhodamine-4-*N*, *N*-(6-benzoyl-9-ethyl adenyl), (glycyl)-ethylenediamine] has R_f of 0.6 in pure DCM). The reaction mixture was evaporated *in vacuo* and then loaded on a short silica column (80-120 mesh; 6 × 2 cm). The desired product was eluted with hexane-DCM (40 : 60), yield 60% (1.023g). Anal. Calcd for $C_{48}H_{56}N_{10}O_5$: C, 67.58; H, 6.61; N, 16.42. Found: C, 67.41; H, 6.61; N, 16.45%.

Synthesis of rhodamine labeled phosphoramidite of peptide monomer with adenine base 9. Compound **8** (1.5 mM) was suspended in DCM (15 mL). To it bis reagent (3 mM, 1 mL) was added followed by pyridinium trifluoroacetate (3 mM, 600 mg) at ambient temperature under argon. After 4 hr the reaction mixture was checked on tlc (DCM, irrigating solvent). The product spot was found at (R_f = 0.6). The reaction mixture was concentrated *in vacuo* and purified on silica by column chromatography, yield 80% (1.265g). ^{31}P NMR (CD_3CN): 150.2 and 149.8 ppm (diasteriomers).

Covalent attachment of 9 to 5′-A*TC CAT T-3′ 10. The CPG loaded thymidine was taken in a functionalization vessel having frit and a supply for uninterrupted argon and the sequence was synthesized with standard phosphoramidite approach. The CPG was washed with dry acetonitrile and the 5′-O-DMTr-group was deprotected by 3% trichloroacetic acid for 3 min. The column was washed twice with dry acetonitrile. The rhodamine amidite was dissolved in dry acetonitrile and was mixed with equimolar amount of 1*H*-tetrazole and added to the vessel. A coupling time of 20 min was allowed, followed by washing with acetonitrile. The sequence is oxidized using I_2 in THF-Pyridine-water (90: 5:5) for 15 min. The column was again washed with acetonitrile and the resin was transferred in 30% aqueous ammonia and incubated at 55 °C for 8 hr. The ammonia solution was filtered again through frit and evaporated *in vacuo*. The desired sequence was precipitated and desalted using isopropanol. The pellet obtained after centrifugation was stored in deep freeze at-20 °C.

5-(Fluorescenylamido)-pentanol-1, 11. Fluorescein (664.62 mg, 2mmole) was dissolved in dioxane (15 mL) and to its stirred solution *p*-nitrophenol (333.9 mg, 2.4 mmole) was added followed by 2 eq of DCC and 0.05 eq. of DMAP. After 2 hr, 5-aminopentanol (217.42 mg, 2 mmole) was added in equimolar ratio with further addition of 2 eq of DCC. The reaction mixture was stirred for another 5 hr at room temperature. Completion of reaction was checked on tlc with a new spot (R_f 0.4 as compared to R_f of fluorescein i.e. 0.3 in pure DCM). The reaction mixture was evaporated *in vacuo* and then loaded on a short silica column (80-120 mesh; 6 × 2 cm). The desired product was eluted with hexane-DCM (40: 60), yield 58% (483 mg). Anal. Calcd for $C_{25}H_{23}NO_5$: C, 71.92; H, 5.55; N, 3.35. Found: C, 71.41; H, 5.61; N, 3.34%. ^{1}H NMR ($CDCl_3$): δ 7.89-7.70 (m, 4H, fluorescein), 6.75-6.61 (m, 6H, fluorescein), 5.0 (s, 1H, OH), 4.8 (s, 1H, NH), 3.5 (m, 2H, NH-CH_2), 3.0 (m, 4H, NH-$CH_2$$CH_2CH_2$), 2.9 (m, 2H, NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$), 2.5 (t, 2H, NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-OH).

5-(Fluorescenylamido)-pentane-1-*O*-phosphoramidite 12. Fluorescein amide-1-pentanol was suspended (626.3 mg, 1.5 mmole) in DCM (15 mL). To it bis reagent (1mL, 3 mmole) was added followed by pyridinium trifluoroacetate (600 mg, 3 mmole) at ambient temperature under argon. After 4 hr the reaction mixture was checked on tlc (DCM irrigating solvent; R_f = 0.52). The reaction mixture was concentrated *in vacuo* and purified on silica by column chromatography, yield 70% (649 mg). ^{31}P NMR (CD_3CN), 148.3 ppm.

Covalent attachment of phosphoroamidite of fluorescein 12 to 5′-AAT GGA T-3′ 13. The polymer-attached sequence was taken in a functionalization vessel having frit and a supply for uninterrupted argon. The CPG was washed with dry acetonitrile and the 5′-O-DMTr group was deprotected by 3% trichloroacetic acid for 3 min. The column was washed twice with dry acetonitrile. The fluorescein amidite was dissolved in dry acetonitrile and was mixed with tetrazole and added to the vessel. A coupling time of 20 min was allowed, followed by washing with acetonitrile. Oxidation of the sequence was done using I_2 in THF-pyridine-water (90:5:5) for 15 min. The column was again washed with acetonitrile and the resin was transferred in 30% aqueous ammonia and incubated at 55 °C for 8 hr. The ammonia solution was filtered again through frit to remove the polymer and evaporated *in vacuo*. The desired sequence was precipitated and desalted using isopropanol. The pellet obtained after centrifugation was stored at-20 °C.

Hybridisation studies. The hybridization of labeled modified sequence (5′-*rh*-A*TC CAT T-3′) with the complementary sequence (3′-T AGG TAA-*flu*-5′) was effected at total oligonucleotide conc. of 6×10^{-5} mole/L in buffer containing 0.1 M sodium chloride, 0.01 M sodium hydrogen phosphate and 0.01 M potassium dihydrogen phosphate with *p*H adjusted to 7.0.

(i) *Melting temperature (Tm)*

The melting curves were studied by recording the change in O.D. concentration at 260nm wavelength. The variation in temperature was done at the rate of

0.5°C/min. The same experiment was repeated with labelled unmodified heptamer sequence (5′-*rh*-ATC CAT T-3′) with the complementary (3′-T AGG TAA-*flu*-5′) sequence. The melting profiles of the two heptamer sequences are shown in **Figure 2**. The modified heptamer shows higher melting temperature Tm 19 °C than the unmodified duplex with Tm 17.5°C.

(ii) *FRET study*

The excitation and emission wavelength were recorded for fluorescein and rhodamine, their respective amidites and for the two tagged heptamers, (3′-T AGG TAA-*fl*-5′) and 5′-*rh*-A*TC CAT T-3′) **(Table I).** The two heptamer sequences were mixed at r. t. (25 °C) in equimolar ratio i.e. equal O.D.'s of the two were mixed and allowed to hybridise. The resulting mixture on excitation at 490 nm gave emission at 560 nm. This emission band at 560 nm (FRET signal) **Figure 3** is indication of fluorescence energy transfer. Similar result was obtained with the unmodified sequence i.e. 5′-*rh*-ATC CAT T-3′ with complementary sequence (3′-T AGG TAA-*fl*-5′) also.

Conclusion

In the present work a modified PNA monomer has been designed and synthesised. It has been incorporated in a heptamer DNA sequence and its effect on melting behaviour of the corresponding duplex has been studied. The enhanced melting temperature of this DNA hybrid with PNA monomer clearly indicates that the monomer can be exploited by making PNA analogs which can not only mimic DNA but also hybridise more tightly to the target resulting in a better antisense PNA analog.

The probable explanation for the enhanced hybridisation intensity is that the modified PNA unit is supposed to change both the conformational flexibility and the charge on PNA. The amide bond connecting the backbone and the linker to the nucleobase in conventional PNA is a rigid unit due to partial double bond character of C-N bond whereas its replacement by 4-*N*,*N*-(6-*N*-benzoyl, 9-*N*-ethyl-adenyl), (glycyl)-ethylenediamine, a tertiary amine is significantly more flexible. It is likely to enhance its cellular uptake. The tertiary amine is expected to be protonated at neutral *p*H and might therefore contribute towards an electrostatic attraction between PNA/DNA. The magnitude of attraction should be dependent on the distance between amine and phosphate of the DNA backbone. Furthermore, it will be sensitive to changes in *p*H as well as changes in ionic environment. The higher Tm recorded in the case of modified duplexes vis-a-vis the unmodified duplex corroborates this conclusion. A number of synthetically prepared oligonucleotides and peptide oligomers are recently undergoing human clinical trials for the treatment of variety of cancerous, viral disorders and host of inflammatory disorders. With growing success of clinical programs and potential market demands for oligonucleotide based drugs, demand of synthetic oligomers/PNA oligomers and consequently fluorescently labeled oligomers is increasing.

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