# Synthesis, Incorporation and Photophysical Characterization of Base-Modified Fluorescent Peptide Nucleic Acid Analogues


**A thesis submitted towards partial fulfillment of**

**5 year integrated BS-MS programme**

**by**

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**(20091021)**

**Under the guidance of**

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# Certificate

This is to certify that this dissertation entitled *"Synthesis, incorporation and photophysical characterization of base-modified fluorescent peptide nucleic acid analogues"* towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Anurag Agrawal at IISER Pune under the supervision of Dr. S. G. Srivatsan, Assistant Professor, Department of Chemistry, IISER Pune during the academic year 2013-2014.

**Supervisor: Dr. S. G. Srivatsan**

**Date: 02/04/2014**

**Place: IISER, Pune**

# Declaration

I hereby declare that the matter embodied in the report entitled *"Synthesis, incorporation and photophysical characterization of base-modified fluorescent peptide nucleic acid analogues"* are the results of the investigation carried out by me at the Department of Chemistry, IISER Pune under the supervision of Dr. S. G. Srivatsan and the same has not been submitted elsewhere for any other degree.

**Name of the student: Anurag Agrawal**

**(20091021)**

**Date: 02/04/2014**

**Place: IISER, Pune**

*Dedicated to class of ‘09*

-We started as tourists, but we became travelers**.**

# Acknowledgments

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## Abstract

Fluorescent nucleoside analogue probes incorporated into oligonucleotides have provided effective biophysical systems to study nucleic acid structure and function and in devising nucleic acid-based diagnostic tools. However, due to low fluorescence efficiency exhibited by the majority fluorescent nucleoside analogues within oligonucleotides and inherent instability of oligonucleotides in nuclease environment, the utility of nucleoside-modified oligonucleotides has been limited to in vitro systems only. In this regard, we envision that a nucleic acid mimic, peptide nucleic acid (PNA), which is resistant to nucleases and base-pairs strongly with complementary DNA and RNA oligonucleotides, would be a suitable candidate for labelling with fluorescent nucleobase analogue. Here, we report the synthesis and incorporation of fluorescent 5-benzofuran- and 5-benzothiophene-conjugated uracil PNA base monomers into PNA sequences. The base analogues incorporated into PNA oligomers and hybridized to complementary DNA oligonucleotides have marginal impact on the duplex stability. Furthermore, 5-benzofuran-conjugated uracil is highly sensitive to changes in its neighbouring base environment. Importantly, it displays significant enhancement in fluorescence intensity upon hybridization with complementary oligonucleotide, a property rarely exhibited by the majority of fluorophores. The straight forward synthesis, amicability to solid-phase PNA oligomer synthesis, structurally non-perturbing nature and sensitivity to changes in its microenvironment highlight the potential of benzofuran-conjugated pyrimidine PNA base analogue as an efficient fluorescent probe for nucleic acid diagnosis.

# Introduction

Nucleic acids along with proteins are the most important biological macromolecules essential for all forms of life known in nature. Nucleic acids which are polymeric macromolecules conventionally include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). These nucleic acids are made from monomers known as nucleotides. Each nucleotide has three components: a 5-carbon sugar, a phosphate group, and a nitrogenous base.[1] The sugar moiety distinguishes between DNA and RNA. If the sugar is 2'-deoxyribose, the polymer is DNA. If the sugar is ribose, the polymer is RNA (Figure 1). These two naturally occurring nucleic acids are vital, as they function in encoding, transmitting and expressing genetic information. Strings of nucleotides bound in a particular sequence are the mechanism for storing and transmitting hereditary or genetic information via protein synthesis.[2]

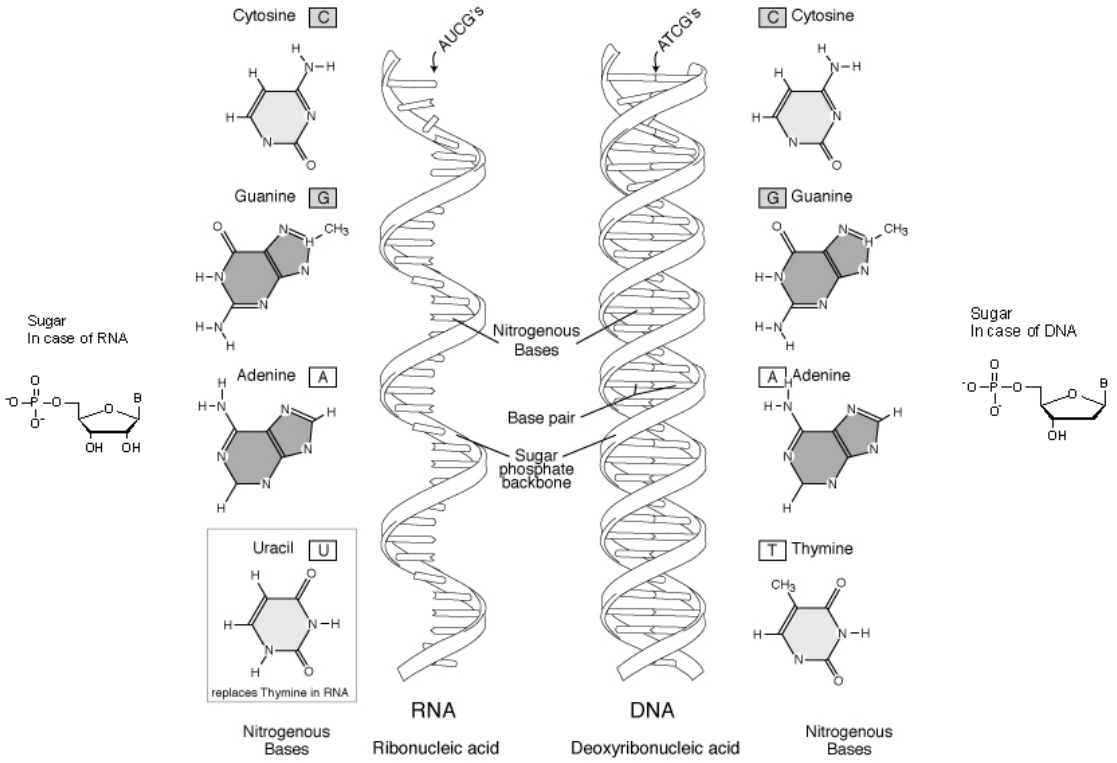



**Figure 1:** Structure of DNA and RNA.

Owing to such central utility, investigational studies of nucleic acids encompass a large part of contemporary biological and medical research, and structure the

foundation for research areas like genome and forensic science, biotechnology and pharmaceutical industries.[3] The research in these areas requires an aid from prominent tools such as spectroscopic measurements including X-ray, UV-Vis, fluorescence, EPR, NMR etc. Among the popular spectroscopic techniques, fluorescence spectroscopy remains one of the most popular and widely used technique. This can be credited to the fact that fluorescence technique is easily accessible, sensitive and gives information in real time. Unlike proteins, which contain fluorescent amino acids, nucleic acids are practically nonemissive as natural nucleobases exhibit very low quantum yields (Figure 2).[4]

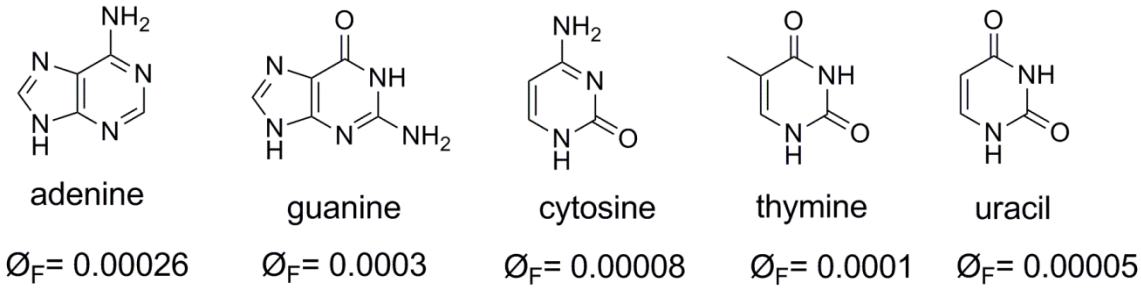



**Figure 2:** Natural nucleobases and their quantum yield in water.

In order to impart better photophysical properties to otherwise nonemissive natural nucleobases, most design principles rely on naturally occurring fluorescent heterocycles, polycyclic aromatic hydrocarbons or on extending the π-conjugation by appending heterocycles to the bases. The fluorescent nucleobase analogues thus synthesized can be broadly classified into the following categories (Figure 3).[5-6]

A. **<u>Polycyclic aromatic hydrocarbons</u>**: The category is dedicated to the cases where the natural nucleobases are completely replaced with known fluorophores mostly polycyclic aromatic hydrocarbons (PAH). This produces an assembly of fluorescent nucleoside where selective excitation and high quantum yields can be achieved on the cost of Watson- Crick hydrogen bonding.

B. **Pteridines**: This class of compounds involves utilization of naturally occurring heterocycles called pteridines, which are structurally similar to purines. Pterdines are desirable because of their intense quantum yields, visible fluorescence and relatively long excited state decay kinetics. However, these compounds suffer a sequence dependent quenching in fluorescence after incorporation into oligonucleotides.

**C. Nucleoside containing expanded nucleobases:** Fusing aromatic rings to natural nucleobases achieves certain beneficial properties. This system usually results in favorable photophysical properties such as emission bands near or in visible range and high quantum yields. The large surface area could however perturb the resulting oligonucleotides.

**D. Nucleoside containing extended nucleobases:** This is the class of compounds where a fluorescent moiety is attached to nucleobases through a linker. This gives these compounds favorable and unique characteristics in terms of photophysical properties compared to their parent moieties.

**E. Isomorphic nucleobases:** This is the most widely used and functional category of fluorescent nucleoside analogues. These nucleobases are heterocycles that strongly resemble the equivalent natural nucleobases with respect to their overall aspects such as hydrogen bonding patterns and ability to form WC base pairs. 2-Aminopurine (2AP) is one of the most widely used nucleoside from this category and displays remarkable photophysical properties desired. These molecules also suffer some setbacks such as fluorescence quenching and lack of emission in desired range (Figure 3).

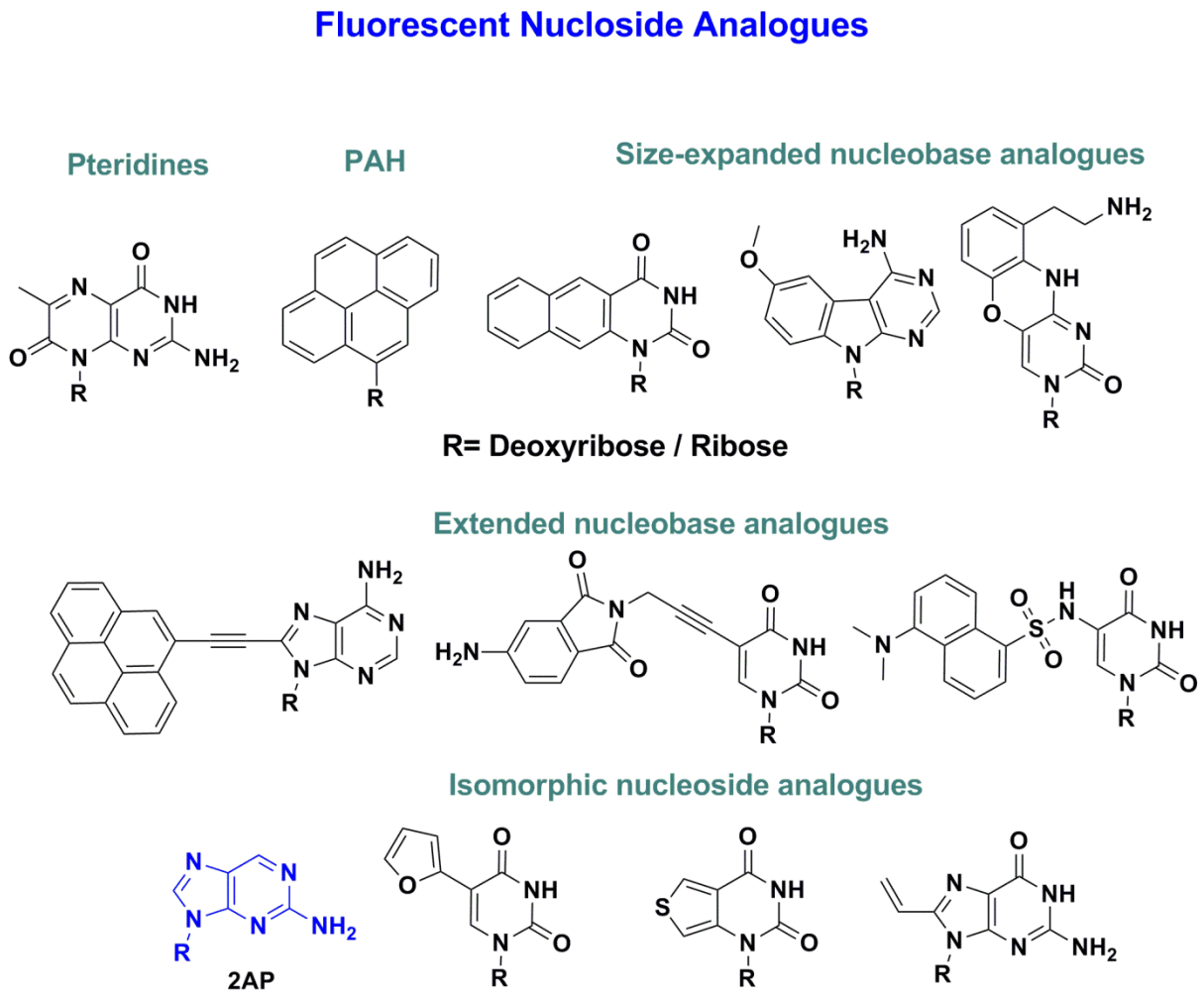



**Figure 3:** Family of fluorescently modified nucleoside analogues.

Several of the base-modified nucleoside analogues have been successfully implemented in assays to (i) monitor nucleic acid folding, cleavage, binding of drugs, small molecule metabolites and proteins to nucleic acids, and (ii) detect single nucleotide polymorphisms (SNPs) and mutation-induced variations in human genome.[7] In particular, 2AP, an adenosine analogue, which is structurally non-invasive and highly sensitive to its microenvironment, is the most extensively used nucleoside analogues in the study of nucleic acid conformation and function.[8] However, major drawbacks of many of these analogues including 2AP as mentioned earlier is that they exhibit drastically low quantum yields within oligonucleotides, which essentially prevents their use in certain fluorescence applications (e.g., anisotropy, single-molecule spectroscopy and cell microscopy).[9] Therefore, much of the recent efforts have been dedicated

towards the development of environment-sensitive nucleobase analogues that (i) are structurally less perturbing, (ii) have excitation and emission maxima in the visible region and (iii) importantly, retain reasonable fluorescence efficiency when introduced into oligonucleotides.[10]

Depending on the needs and specific applications such kind of modifications are performed and then they are usually incorporated into generally either DNA or RNA sequences. However, the trend is changing nowadays owing to the discovery of new kind of nucleic acid analogues called PNA i.e. peptide nucleic acid.[11] PNAs are nucleic acid structural mimics and offer certain advantage over conventional nucleic acids.

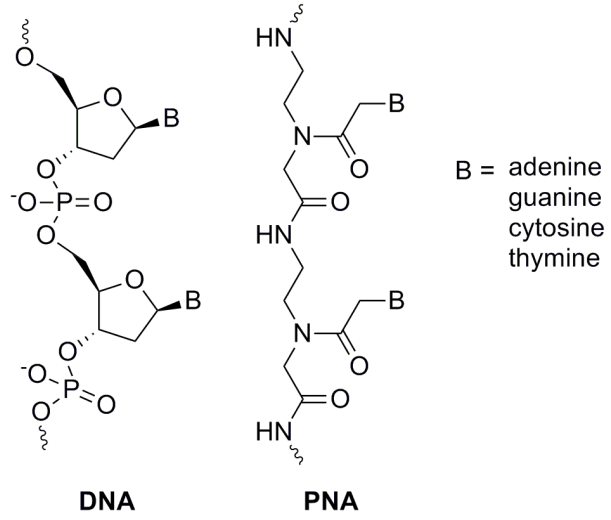



**Figure 4:** Chemical structure of DNA and PNA.

The original PNA (*aeg*-PNA) synthesized by Nielsen and coworkers consisted of repeating *N*-(2-aminoethyl)glycine (*aeg*) units linked through by amide bonds (Figure 4).[12] In the conventional form the nucleobases A, T, G and C are attached through methylene carbonyl linkages. PNA, synthesized as DNA mimic, evidently is made of repeating units of an uncharged pseudo-peptide backbone. This whole assembly of *aeg*-PNA has certain notable features. Most prominent feature of PNA is that it shows high binding affinity for complementary DNA and RNA oligonucleotides.[13] Secondly, PNAs are not recognized either by enzymes such as nucleases or proteases which makes them highly resistant to enzyme degradation.[14]

Due to such extraordinary properties, PNA oligomers are considered as excellent candidates in antisense and antigene therapy. Ability of PNA oligomers to bind strongly to their complementary regions of nucleic acids, along with capability to invade double stranded DNA makes PNA a suitable option for certain applications. These include protein blocking by sequence-specific binding of PNA to mRNA and retardation of RNA transcription by blocking the access of polymerases through PNA binding to promoter or coding regions.[16-17]

Furthermore, PNA has been gaining recognition over the last few years due to its utility as hybridization probe. Regulation of protein expression levels by targeting mRNAs has also been possible due to the inability of RNaseH to recognize PNA-RNA duplex. Further, labeled with appropriate affinity tags or fluorophores, PNAs have been demonstrated to be useful in the detection of heterogeneity in telomer length of human chromosomes, G-quadruplexes, single nucleotide polymorphism (SNP) and in blotting techniques.[18-19] PNA FIT (force intercalation)- probes have been designed to image two different viral mRNA molecules simultaneously which are expressed during replication cycle of the H1N1 influenza A virus.[20]

In addition to these advantages that PNA offers, there are certain shortcomings as well. The uncharged nature of PNA backbone results in poor water solubility. Then there are problems such as lack of cell permeability and self aggregation. To overcome such problems, different modifications are usually employed to conventional PNAs. Several design strategies have been adopted to improve the binding affinity and specificity of PNA by either modifying or substituting the backbone with other amino acids and cyclic systems.[21-24] Unfortunately, most of the attempts have resulted in PNA oligomers with lower affinity for complementary oligonucleotides as compared to the original *aeg*-PNA. Many unnatural and modified bases have been substituted in place of nucleobases in order to enhance the binding of PNA oligomers to complementary nucleic acids.[25] Use of pseudoisocytosine in place of conventional cytosine has proved to be useful in obtaining highly stable triplex-forming PNAs at physiological pH. Diaminopurine–thiouracil pair has been used in designing PNA oligomers for duplex-invasion of DNA or RNA.[26] A number of bi and tricyclic cytosine and thymine analogues that stabilize

duplexes via increased base-pair stacking have also been introduced into PNA oligomers.[27]

PNA oligomers containing large fluorophores such as fluorescein derivatives are mostly used for fluorescence hybridization assays. The shortcoming associated with such systems is that they reduce the hybridization efficiency of probe to the target sequence. To overcome this problem, incorporation of minimally perturbing modified monomers has started to grab attention. Incorporation of fluorescent base analogue, 8-vinylguanine resulting in fluorescent PNA hybridization probes capable of detecting quadruplex forming DNA oligonucleotides and incorporation of pyrene-labeled uracil into PNA oligomers are examples of the same.[19, 28]

However, except for a very few examples, the scope of nucleobase-modified fluorescent analogues has not been very well explored in designing PNA probes for applications in diagnosis. Keeping this in perspective, we believe that constructing PNA probes containing fluorescent base analogues as an integral part of the oligomer sequence would be appropriate as it would have structure and hybridization property similar to that of the unmodified oligomer. Therefore, we propose that constructing PNA probes containing microenvironment-sensitive fluorescent nucleobase analogues would provide opportunities in developing latest and potent diagnostic tools for studying the structure and functions of nucleic acids.

## Aim of the project

The aim of the project is to synthesize fluorescently modified PNA monomers, and incorporate them into PNA sequences through solid phase synthesis for photophysical studies and biophysical applications.

## Objectives

- To synthesize fluorescent nucleobase analogues and corresponding PNA monomers based on *N*-(2-aminoethyl)-glycine backbone.
- To incorporate fluorescent microenvironment responsive analogues site-specifically into model PNA oligomers.

- To characterize the fluorescently modified PNA oligomers by steady-state fluorescence spectroscopic techniques.
- To draw a correlation among PNA oligomers having different flanking bases and base-pair substitutions using fluorescent hybridization assay.
- To discover utility of multichromophoric systems by incorporating multiple fluorescent PNA analogues into oligomers and study their photophysical behavior.
- Based on the photophysical data obtained with model constructs, propose most suitable constructs such that PNA oligomers could be synthesized which can further be used to target therapeutically relevant nucleic acid motifs.

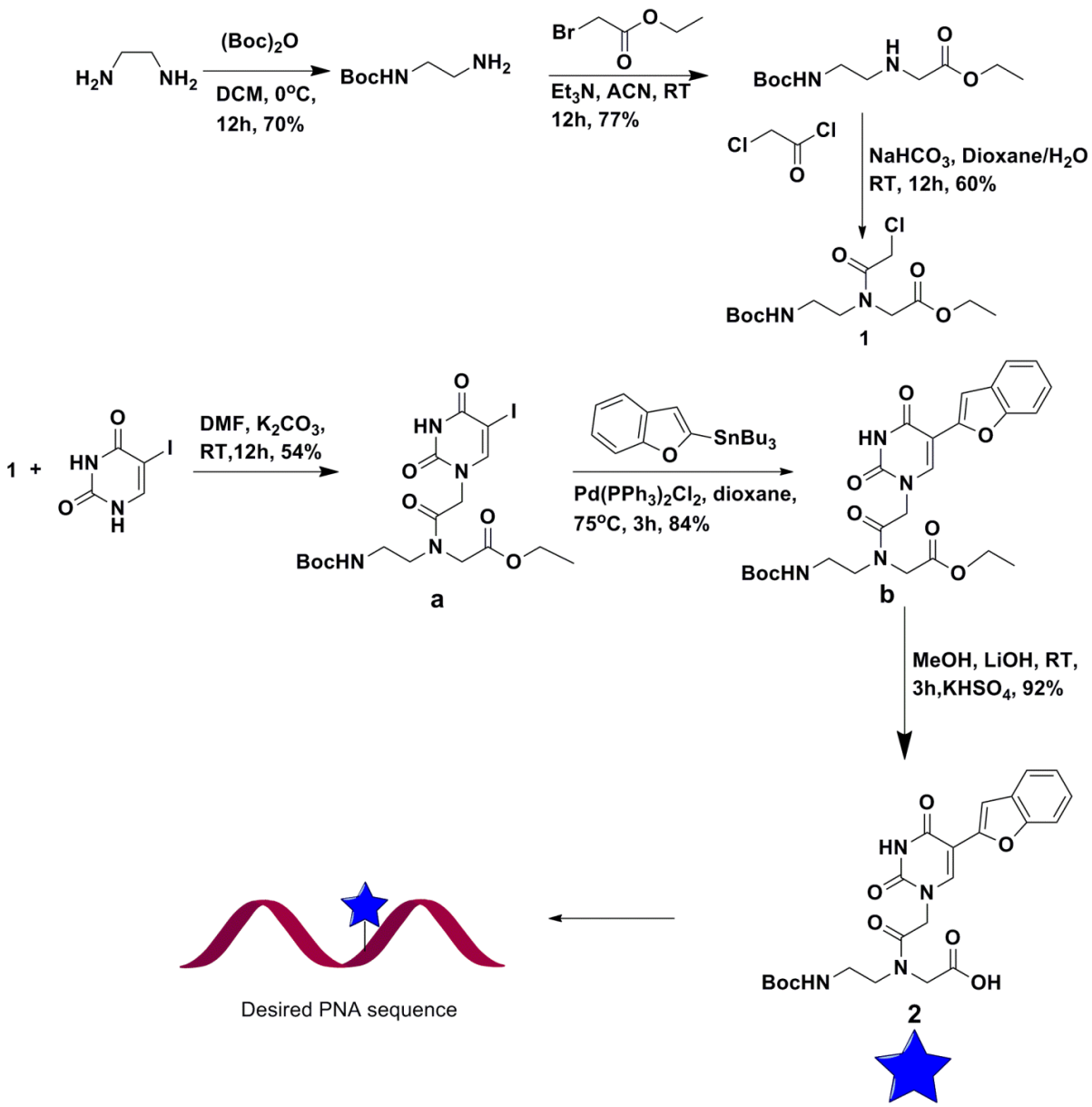
## Results and discussion

### Synthesis of PNA monomer

In an earlier report, fluorescent 5-benzofuran-conjugated uridine ribonucleoside analogue incorporated into oligonucleotides was found to be sensitive to changes in neighbouring bases.[29] When incorporated into oligonucleotides and hybridized to complementary oligonucleotides, the nucleoside is structurally noninvasive and uniquely retains appreciable fluorescence efficiency, a property rarely exhibited by the majority of fluorophores. These key observations, nuclease resistance and excellent base pairing properties of PNA inspired the development of the benzofuran-modified fluorescent PNA probe.

In accordance to our aim of developing non-perturbing fluorescent PNA base analogues, we synthesized first, a PNA base analogue by appending benzofuran at the 5-position of uracil (Scheme 1). The procedure for the synthesis of fluorescent PNA monomer **2** necessary for solid-phase PNA oligomer synthesis was established by one of my laboratory colleagues, and subsequently I re-synthesized the monomer for further evaluation. Compound **1** was obtained according to reported procedure.[30] Then compound **1** was reacted with 5-iodouracil to afford compound **a** using $K_2CO_3$ in DMF. Conversion of 5-iodouracil PNA **a** to fluorescent 5-benzofuran uracil PNA ester **b** was accomplished by Stille coupling reaction using a palladium catalyst. PNA ester **b** was

hydrolyzed using lithium hydroxide to yield 5-benzofuran uracil PNA acid **2** and compound **2** was used in the solid phase PNA synthesis.




**Scheme 1:** Synthesis of fluorescently modified PNA monomer **2.** See experimental section for details.

**Photophysical properties of fluorescent PNA monomer 2**

Photophysical studies after the Boc-deprotection of PNA monomer **2** was performed in a parallel work from our lab.[31] The results indicated that the benzofuran-modified PNA base analogue was found to be reasonably emissive ($\Phi$ = 0.27) in water with emission maximum in the visible region (447 nm). It was also reported that the excited-state properties such as emission maximum, quantum yield and lifetime were affected by solvent polarity changes. The trend indicated that as the polarity was decreased from water to dioxane, a blue-shifted emission band with reduced emission intensity and lifetime was observed in dioxane as compared to in water. Together, emission in the visible region with a reasonable quantum yield and ability to report polarity changes via changes in its fluorescence properties conferred sensor-like character to the emissive analogue.

**Solid-phase synthesis of PNA oligomers containing 2**

Using the fluorescent 5-benzofuran-cojugated uracil PNA monomer, a few model PNA sequences were constructed initially (Figure 6). These constructs were designed to serve as models for studying the effects of flanking bases as well as base pair substitution on the fluorescence properties of the emissive analogue within PNA sequences. These PNA sequences were prepared using standard solid phase PNA synthesis protocol under microwave conditions (Figure 5). PNA oligomers were synthesized on MBHA resin after attaching two lysine residues for increasing the solubility of desired PNA oligomers. Boc protected *aeg*-PNA monomers and fluorescent PNA monomer **2** were used for synthesizing primary PNA oligomers. The synthesis protocol consisted of deprotection of Boc group using TFA and then neutralization of residual acid using DIPEA solution in DCM. Washing steps using DMF/DCM were employed in between. It was followed by coupling step using a solution of PNA monomer in DMF. Followed by washing steps, the cycle continued till the final 15 mer PNAs were obtained. Base deprotection and cleavage of PNA from resin was performed using a solution of thioanisole, ethandithiol, TFA and TFMSA. Synthesized PNA oligomers were purified using RP-HPLC (Appendix). Finally the PNA oligomers were characterized using MALDI-TOF mass analysis (Table 1).

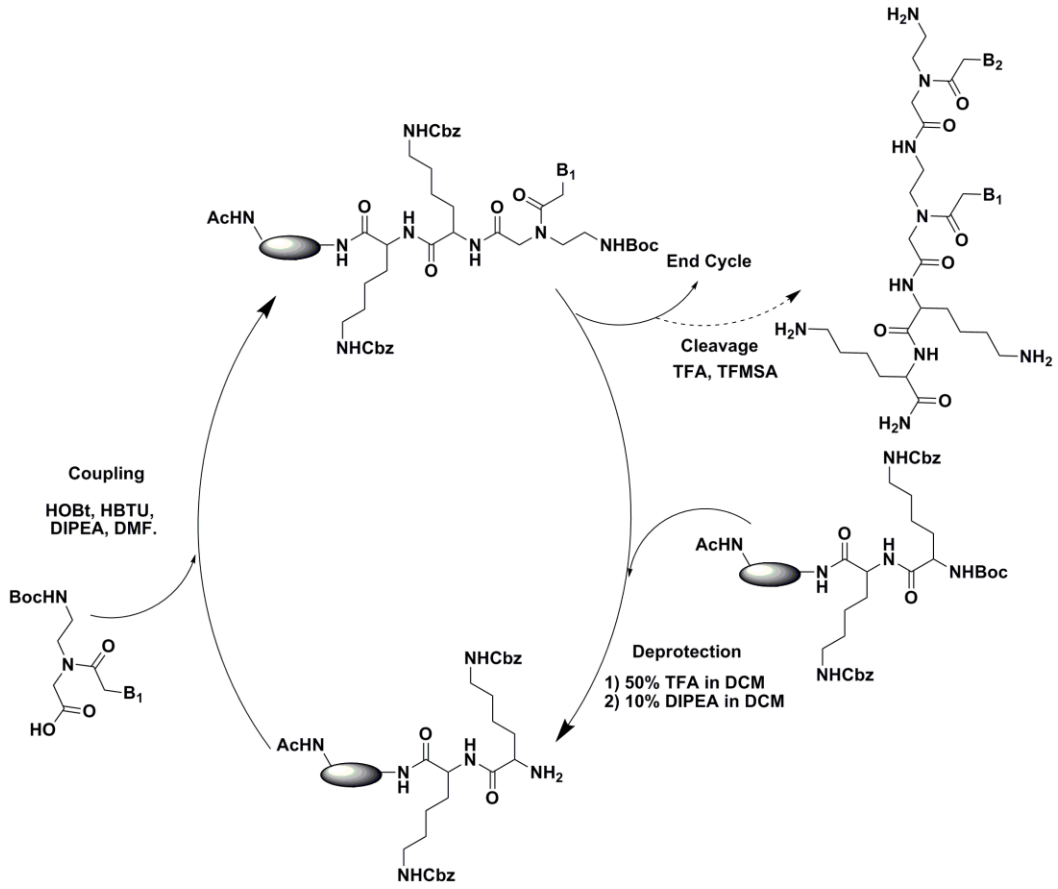



**Figure 5:** Solid-phase synthesis protocol for PNA oligomers. See experimental section for details.

**Table 1:** $\varepsilon_{260}$ and MALDI-TOF mass analysis of PNA oligomers

| PNA | Sequence[a] | $\varepsilon_{260}$ ($M^{-1}cm^{-1}$) | Calculated mass | Observed mass |
|---|---|---|---|---|
| 3 | CGATCAA**2**AACTAGCKK | 16.1 x $10^4$ | 4414.3 $[M+H]^+$ | 4414.9 |
| 4 | CGATCAT**2**TACTAGCKK | 15.1 x $10^4$ | 4365.2 $[M]^+$ | 4365.8 |
| 5 | CGATCAC**2**CACTAGCKK | 14.7 x $10^4$ | 4396.3 $[M+H]^+$ | 4396.1 |
| 6 | CGATCAG**2**GACTAGCKK | 15.7 x $10^4$ | 4446.3 $[M+H]^+$ | 4446.9 |
| 10 | CGATCA**22**AACTAGCKK | 15.9 x $10^4$ | 4507.4 $[M+H]^+$ | 4508.0 |
| 11 | CGATCA**92**AACTAGCKK | 15.6 x $10^4$ | 4523.5 $[M+H]^+$ | 4524.3 |
| 12 | CGATCA**2**A**2**ACTAGCKK | 15.9 x $10^4$ | 4506.4 $[M]^+$ | 4506.9 |
| 13 | CGATCA**9**A**2**ACTAGCKK | 15.6 x $10^4$ | 4561.6 $[M+H]^+$ | 4560.8 |

[a]PNA sequences are written from N to C terminus.

**Photophysical characterization of fluorescently modified PNA oligomers in different nucleobases environment**

The photophysical properties of fluorescent nucleosides incorporated into PNA sequences can be altered by a variety of mechanisms. It has been shown that factors such as stacking of the chromophore to flanking bases, collisional and hydrogen-bond interactions with neighbouring bases, the solvation-desolvation effect, conformation of the chromophore and excited-state processes with neighbouring bases can affect the fluorescence of nucleic acid analogues.[32-34] To study the effect of neighbouring bases on the fluorescence a series of PNA oligomers (**3–6**) were synthesized in which the fluorescence PNA monomer **2** was placed in-between different flanking bases (Figure 6).

**3** CGATCAA**2**AACTAGCKK
**4** CGATCAT**2**TACTAGCKK
**5** CGATCAC**2**CACTAGCKK
**6** CGATCAG**2**GACTAGCKK
**3a** 5' $_{d}$(CGCTAGTTATTGATCGC) 3'
**4a** 5' $_{d}$(CGCTAGTAAATGATCGC) 3'
**5a** 5' $_{d}$(CGCTAGTGAGTGATCGC) 3' **X**=
**6a** 5' $_{d}$(CGCTAGTCACTGATCGC) 3'
**3b** 5' $_{r}$(CGCUAGUUAUUGAUCGC) 3'
**4b** 5' $_{r}$(CGCUAGUAAAUGAUCGC) 3'
**5b** 5' $_{r}$(CGCUAGUGAGUGAUCGC) 3'
**6b** 5' $_{r}$(CGCUAGUCACUGAUCGC) 3'
**3c** 5' $_{r}$(CGCUAGUU**X**UUGAUCGC) 3'
**4c** 5' $_{r}$(CGCUAGUA**X**AUGAUCGC) 3'
**5c** 5' $_{r}$(CGCUAGUG**X**GUGAUCGC) 3'
**6c** 5' $_{r}$(CGCUAGUC**X**CUGAUCGC) 3'

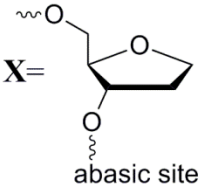



**Figure 6:** Sequence of fluorescently modified PNA (**3–6**, highlighted in bold) and custom oligonucleotides (**3a**–**6c**). Hybridization of **3**–**6** with **3a**–**6a** and **3b**–**6b** places the nucleoside **2** opposite to its complimentary bases in DNA and RNA ONs, respectively. Hybridization of **3–6** with **3c**–**6c** places **2** opposite to a chemically stable abasic site surrogate **X**.

The influence of neighbouring bases on the fluorescence properties of **2** was studied by performing steady-state fluorescence measurements with PNA oligomers **3–6** and duplexes constructed by hybridizing **3–6** with their respective complementary

ONs (DNA and RNA). PNA oligomers were also hybridized with their respective RNA compliments containing an abasic site, which gets placed opposite to **2**.

Interestingly for the PNA sequences where modification is placed in between adenine and thymine bases, significant increase in fluorescent intensity upon hybridization was observed compared to their corresponding single stranded sequences (Figure 7). The enhancement in fluorescence intensity was found to be more than 3-fold for perfect complementary PNA-DNA and PNA-RNA duplexes. This observation is particularly distinguished and remarkable, as the majority of emissive nucleoside analogues (e.g. 2AP, pyrroloC) display progressive fluorescence quenching upon incorporation into single-stranded and double-stranded ONs, a major hindrance to the use of many such analogues in certain fluorescence-based studies (anisotropy and single molecule fluorescence spectroscopy). However, PNA **5** in which the emissive base is flanked by cytosine, did not show any significant changes in fluorescence intensity upon hybridization to perfect complementary DNA and RNA ONs (Figure 7). Expectedly, when the base analogue was flanked by guanine residues in PNA **6**, it exhibited drastic fluorescence quenching as compared to other PNA sequences possibly due to electron transfer process between the guanine and fluorescent base. Among the nucleobases, guanine is known to quench the fluorescence of several fluorophore by photoinduced electron transfer process.[35-36]

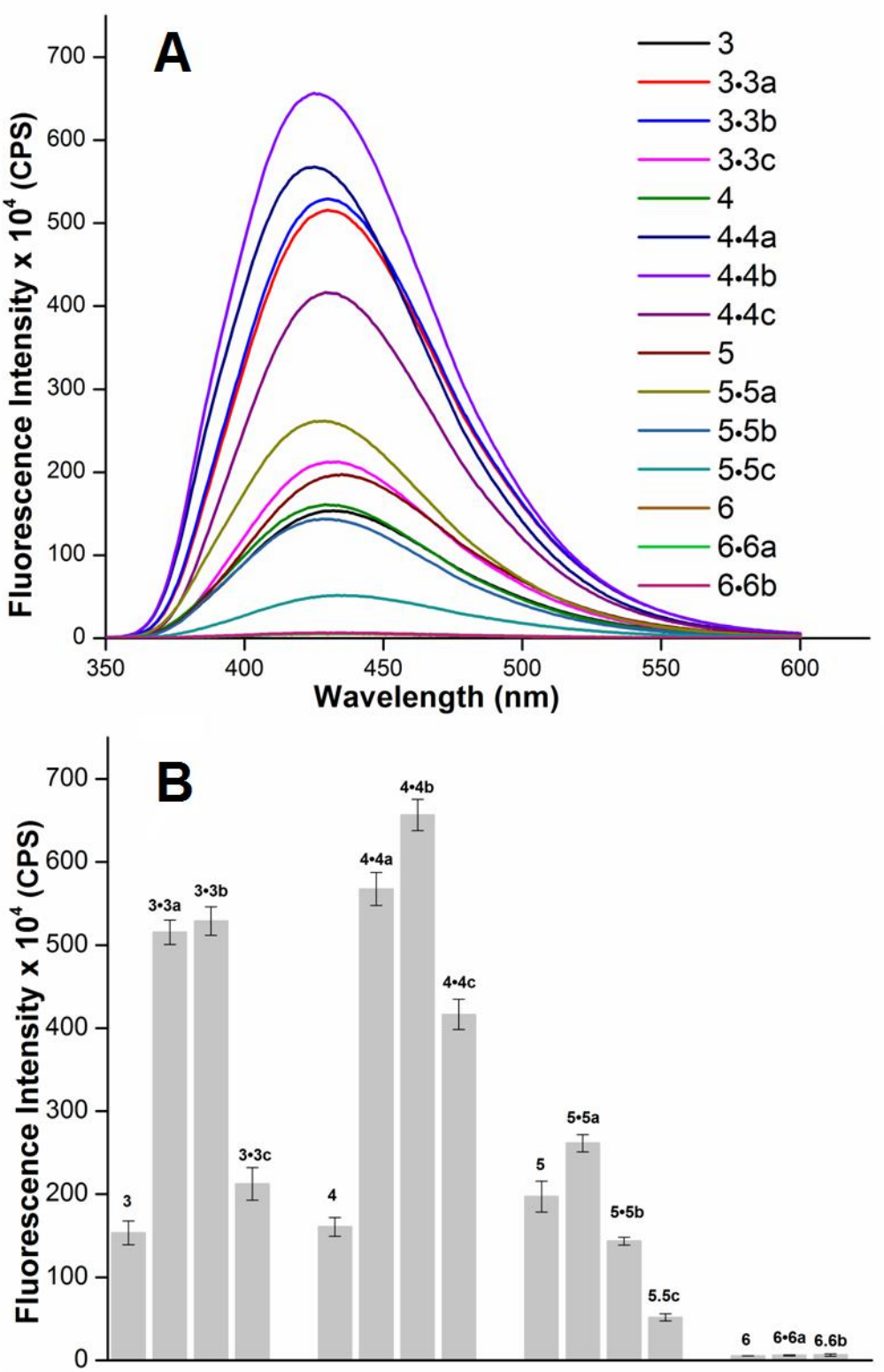



**Figure 7: (A)** Fluorescence spectra of PNA **3**–**6** and their duplexes formed by hybridization with their complementary DNA, RNA ONs and RNA ONs containing an abasic site. For DNA and RNA ONs sequences see Figure 6. **(B)** Fluorescence intensity of PNA **3**–**6** and their duplexes at respective emission maximum. See experimental section for details.

Mentioned earlier, a previous study from our research group demonstrated the potential of benzofuran-modified DNA ONs to detect RNA abasic sites.[29] These results prompted us to study the behavior the fluorescent PNA base analogue **2** opposite to an abasic site. A series of PNA–RNA heteroduplexes was assembled by annealing modified PNA oligomers **3**–**6** to the respective complementary RNA ONs **3c**–**6c** containing a chemically stable abasic site surrogate, tetrahydrofuran (Figure 6). Evidently, when nucleobase analogue **2** flanked by A, T and C was placed opposite to an abasic site in duplexes **3•3c, 4•4c** and **5•5c**, showed noticeable quenching in fluorescence intensity compared with the corresponding "perfect" duplexes **3•3b, 4•4b** or **5•5b** (Figure 7). Moreover, duplex **6•6b** and abasic-site-containing duplex **6•6c**, in which the modified monomer is flanked by guanine residues, exhibited very weak fluorescence (data not shown).

After the preliminary study, each PNA construct was used to examine the effect of base pair substitution. The study was designed to provide an insight to the ability of these PNA constructs to detect mismatches in the complementary nucleobases when placed opposite to fluorescently modified PNA monomer **2.** A series of duplexes were constructed by annealing fluorescent PNA oligomers **3**–**5** with custom DNA ONs **3d**-**5g** (Figure 6 and 8). The duplexes were designed in a fashion such that the emissive PNA base analogue was either placed opposite to perfect compliments or mismatched bases. Since PNA **6** containing **2** in-between guanine residues does not show significant fluorescence intensity under the same set of parameters, it was not used in further studies.

Steady state fluorescence measurements using these PNA-DNA duplexes were performed by hybridizing respective PNA and DNA sequences. The results for each individual experiment are illustrated in Figures 9–11. It is apparent from the graphs that the modified PNA base analogue is sensitive to base pair substitution.

**3d** 5' $_{d}$(GCTAGTTATTGATCG) 3'
**3e** 5' $_{d}$(GCTAGTTTTTGATCG) 3'
**3f** 5' $_{d}$(GCTAGTTGTTGATCG) 3'
**3g** 5' $_{d}$(GCTAGTTCTTGATCG) 3'
**4d** 5' $_{d}$(GCTAGTAAATGATCG) 3'
**4e** 5' $_{d}$(GCTAGTATATGATCG) 3'
**4f** 5' $_{d}$(GCTAGTAGATGATCG) 3'
**4g** 5' $_{d}$(GCTAGTACATGATCG) 3'
**5d** 5' $_{d}$(GCTAGTGAGTGATCG) 3'
**5e** 5' $_{d}$(GCTAGTGTGTGATCG) 3'
**5f** 5' $_{d}$(GCTAGTGGGTGATCG) 3'
**5g** 5' $_{d}$(GCTAGTGCGTGATCG) 3'

**Figure 8:** Sequence of custom DNA oligonucleotides (**3d**–**5g**). Hybridization of **3**, **4**, **5** with **3d**, **4d**, **5d** respectively places nucleoside **2** opposite to its perfect complimentary base. Hybridization of **3**, **4**, **5** with **3e**–**3g**, **4e**–**4g**, and **5e**–**5g**, respectively, places **2** opposite to corresponding mismatched bases (dT, dG, and dC, respectively).

PNA **3** upon hybridization with its perfect complimentary DNA ON **3d** shows almost a 4-fold enhancement in fluorescence intensity compared to single stranded PNA **3**. However, when PNA **3** was hybridized with DNA ONs **3e**–**3g** containing mismatched bases opposite to **2,** an enhancement of around 2-fold was observed. Noticeably, the enhancement in fluorescence intensity compared to single stranded PNA **3,** for duplexes having mismatched base is ~2-fold lower than perfect duplex, which indicates that PNA **3** is sensitive to base pair substitution (Figure 9). For PNA **4** after hybridization with perfect complementary DNA **4d** again a ~4-fold enhancement in fluorescence intensity compared to single stranded PNA **4** was observed. However, in this case even the duplexes having mismatched bases **4•4e, 4•4f, 4•4g** shows enhancement of ~3.5-, ~3- and ~2.5-fold, respectively (Figure 10). These enhancement although not equal but are almost of same magnitude of perfect duplex **4•4d,** which indicates that PNA **4** is not significantly sensitive to base pair substitution. Lastly, for PNA **5** the fluorescence intensity upon hybridization with perfect complementary DNA **5d** increases compared to single stranded PNA **5**. Interestingly, when PNA **5** is hybridized with DNA ONs **4e**–**4g** containing mismatched bases, decrease in fluorescent intensity compared to single stranded PNA **5** was observed (Figure 11). The trend

suggests that PNA **5** can sense the changes in its microenvironment in terms of base pair substitution.

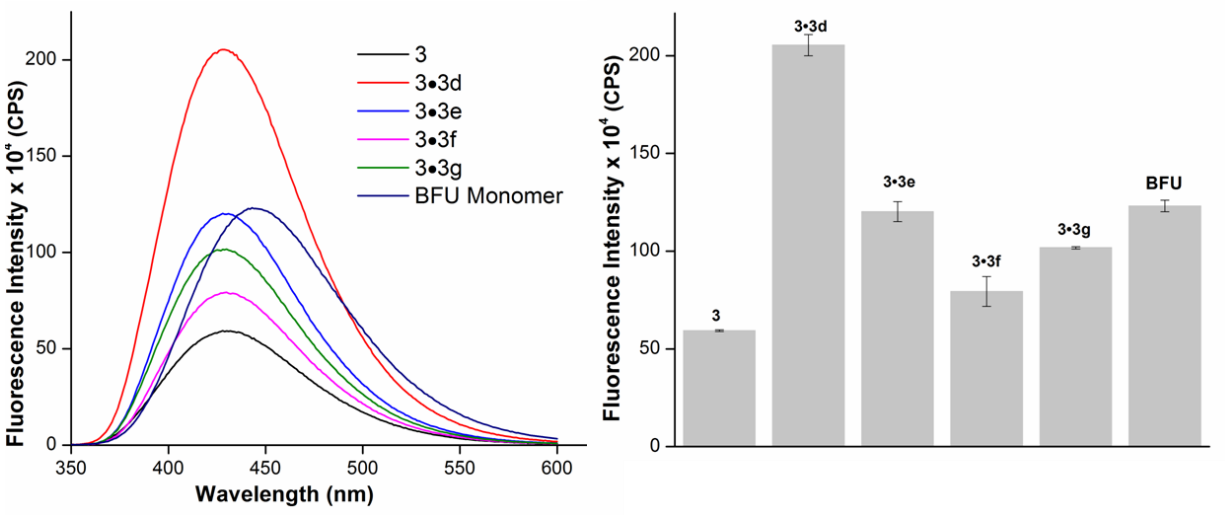



**Figure 9: (A)** Fluorescence spectra of PNA **3** and the duplexes formed by hybridization with its complementary DNA and mismatched DNA ONs. For DNA ONs sequences see Figure 8. **(B)** Fluorescence intensity of PNA **3** and their duplexes at respective emission maximum. BFU is acid form of **2**. See experimental section for details.

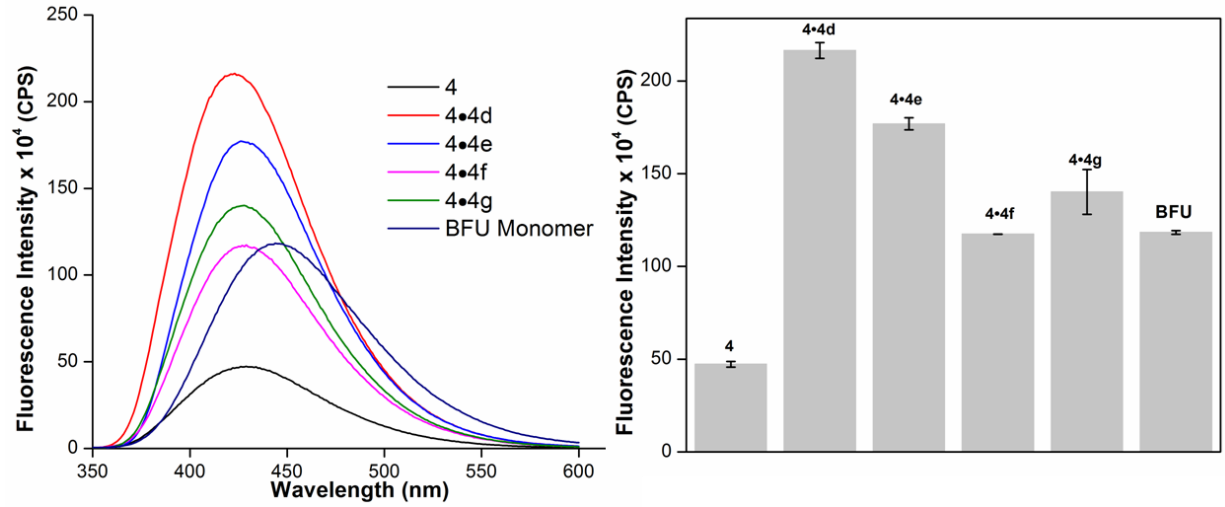



**Figure 10: (A)** Fluorescence spectra of PNA **4** and the duplexes formed by hybridization with its complementary DNA and mismatched DNA ONs. For DNA ONs sequences see Figure 8. **(B)** Fluorescence intensity of PNA **4** and their duplexes at respective emission maximum. BFU is acid form of **2**. See experimental section for details.

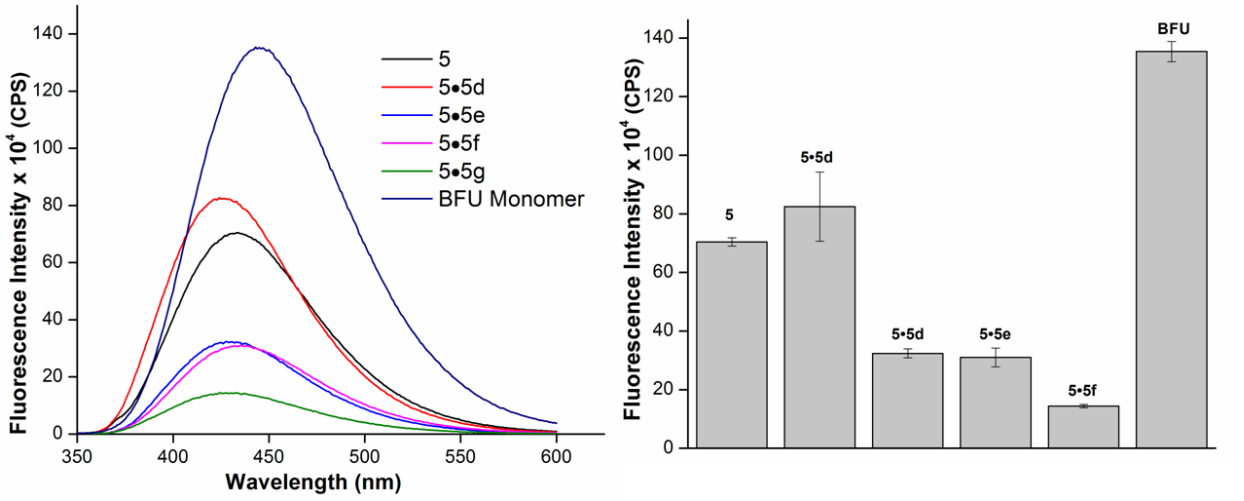
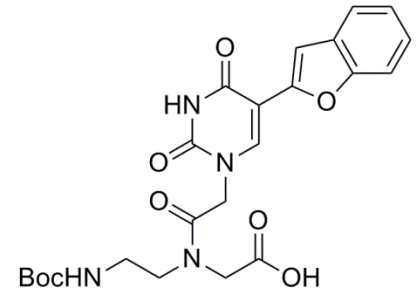



**Figure 11: (A)** Fluorescence spectra of PNA **5** and the duplexes formed by hybridization with its complementary DNA and mismatched DNA ONs. For DNA ONs sequences see Figure 8. **(B)** Fluorescence intensity of PNA **5** and their duplexes at respective emission maximum. BFU is acid form of **2**. See experimental section for details.

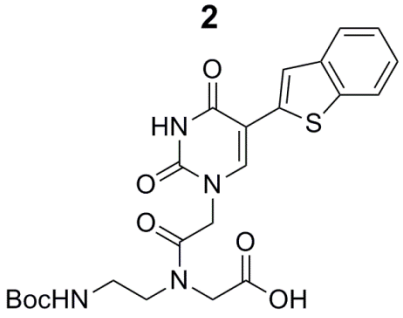
## Synthesis of multichromophoric PNA oligomers

Use of a single modification for constructing an appropriate biological probe has been widely practiced for imparting fluorescence properties to desired nucleic acid analogues. However, recently the utility of placing multiple modifications in a single oligomer-like chain has been demonstrated to result in interesting and desirable fluorescence properties.[37,38] From the result of previous study, it is evident that PNA **3** is capable of microenvironment sensitivity and shows enhancement in fluorescence intensity in case of duplex formation. Following on these properties we decided to test if multiple modifications can alter the photophysical properties of the construct significantly. PNA sequences **10**, **11**, **12**, **13**, **14** containing 5-benzofuran-conjugated uracil PNA monomer (**2)** and/or 5-benthiophene-conjugated uracil PNA monomer (**9)** were synthesized such that modifications are placed consecutively and alternately (Figure 12). 5-(benzo[b]thiophen-2-yl)pyrimidine PNA monomer **(9)** was synthesized by one of my colleagues in our laboratory and I incorporated it into PNA oligomers. PNAs **10**–**14** were synthesized by standard solid phase method (Figure 5). PNA oligomers synthesized such were purified by RP-HPLC and their integrity was confirmed by MALDI-TOF mass analysis (Table 1 and Appendix).

**10** CGATCA22AACTAGCKK
**11** CGATCA92TACTAGCKK
**12** CGATCA2A2ACTAGCKK
**13** CGATCA9A2ACTAGCKK
**14** CGATCA9A9ACTAGCKK
**10a** 5' $_{d}$(GCTAGTTAATGATCG) 3'




**2**




**9**

**Figure 12:** PNA sequences **10**–**14** having multiple modifications. **10** and **11** have consecutive modifications whereas **12**–**14** contain alternate modifications. Hybridization of **10** and **11** with **10a** places the modifications opposite to their complementary bases. Similarly hybridization of **12**–**14** with **4e** (Figure 8) places the modifications opposite to their complementary bases.

### Photophysical characterization of doubly modified PNA oligomers

Steady state fluorescence measurements were performed using PNA **10**–**14** containing two modifications. Each PNA sequence was hybridized to its complementary DNA ON. **10** and **11** were hybridized with **10a** and **12**–**14** were annealed with **4e** (Figure 8 and 12). The fluorescence intensity of perfect duplexes is compared with the single stranded PNAs (Figure 13). A distinctive trend can be deduced from the data of fluorescence intensity. Among all the constructs, PNA **3** and **12**, containing one and two benzofuran modifications, respectively, show significant enhancement in fluorescence intensity (~4-fold) as compared to respective single stranded PNA oligomers (Figure 13). However, other PNA sequences containing modifications at alternate as well as at consecutive positions did not show appreciable changes in fluorescence profile.

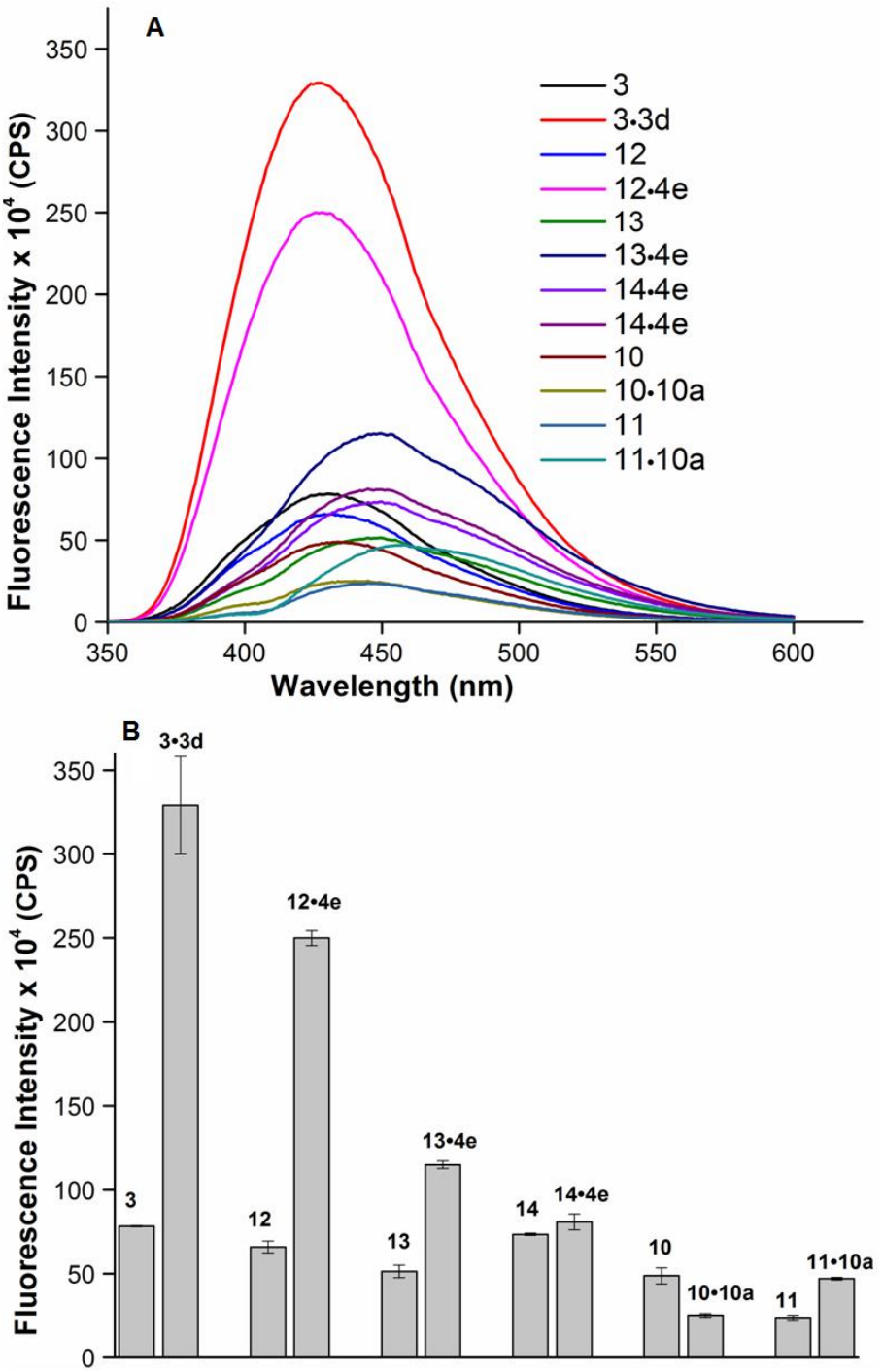



**Figure 13: (A)** Fluorescence spectra of PNA **3, 10, 11, 12, 13, 14** and their duplexes formed by hybridization with their complementary DNA ONs. For DNA ONs sequences see Figure 8 and 12. **(B)** Fluorescence intensity of PNA **3, 10, 11, 12, 13, 14** and their duplexes at respective emission maximum. See experimental section for details.

It can be observed that PNA ONs containing benzofuran-modified-conjugated-uracil monomer enhances the fluorescence intensity of the perfect duplex more than the PNA sequences, which have benzothiophene-modified-conjugated monomer incorporated. The diminutive change in fluorescence intensity for consecutively modified PNA sequences can be attributed to quenching mechanism due to effective stacking interaction between the adjacent fluorophores.[37]

**Effect of varying concentration of cDNA on photophysical characteristics of fluorescently modified PNA oligomers**

Generally for fluorescence hybridization studies the modified and complementary ONs are taken in 1:1 concentration ratio. PNA due to its neutral backbone is known to self aggregate. Hence, PNA oligomers show better solubility in aqueous buffer when hybridized with cDNA. Adventitiously, we found that the fluorescence of the PNA oligomer was dependent on concentration of cDNA. PNA **3** was mixed with increasing concentration of cDNA **3d** and samples were heated and cooled as before (Figure 8). Upon increasing the DNA-PNA ratio, an increase in fluorescence intensity was observed (Figure 14). This observation is intriguing as PNA is known to form stable hybrid with cDNA at 1:1 ratio. However, benzofuran-modified DNA oligonucleotide **7** did not show a similar trend upon increasing the concentration of cDNA **3a** (Figure 15). For DNA **7** upon hybridization to its complementary DNA **3a** an enhancement of ~2 fold is observed compared to single stranded DNA **7** (Figure 14). However, this enhancement remains constant even if the concentration of complementary DNA **3a** is increased, unlike the case of PNA **3**. In order to test if this increase in fluorescence is due to interaction between complementary PNA-DNA sequences, we measured the fluorescence of corresponding PNA **3** in the presence of increasing concentration of a random noncomplementary DNA **8**. In this case only marginal changes in fluorescence intensity were observed. Taken together, these results indicate that specific interaction exists between PNA **3** and its perfect complementary DNA ON. However, the explicit nature of interaction that leads to enhanced emission intensity needs to be proved, which is being considered as a future plan.

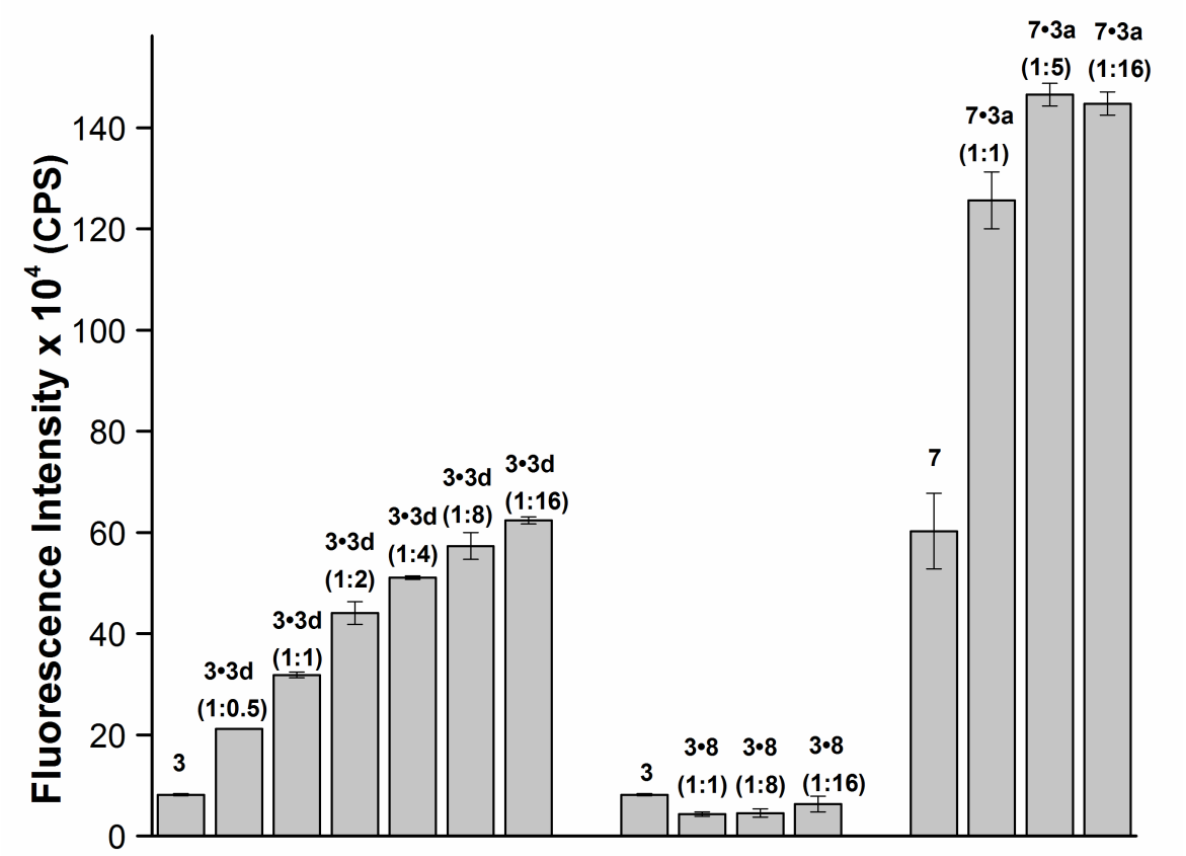



**Figure 14:** Fluorescence intensity at respective emission maximum of PNA **3** and DNA **7** and the duplexes formed by hybridization with their complementary DNA ONs in varying concentrations. PNA **3** was also conjugated with a random DNA sequence **8** (See Figure 14). Ratio of PNA/DNA and DNA/DNA is given in parenthesis. See experimental section for details.

**7** 5' $_d$(GCGATCAA**M**AACTAGCG) 3' **M**=

**8** 5' $_d$(GTGACCATTTGCAGTG) 3'

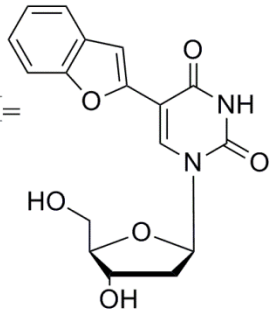



**Figure 15:** Control sequences for concentration dependent study. **7** is the DNA analogue of PNA **3** and **8** is a random DNA oligonucleotides used as control.

## Conclusions

A microenvironment-sensitive fluorescent PNA monomer that is based on a 5-(benzofuran-2-yl)pyrimidine core and that displays emission in the visible region has been incorporated into PNA oligomers. Evidently monomer **2** retains its fluorescence efficiency upon incorporation into ONs, a feature rarely displayed by majority of fluorescent nucleoside analogues. Further, the ability to show a huge enhancement in fluorescence intensity upon duplex formation specially in case of **2** flanked by A and T PNA monomers, opens up wide opportunities for these sequences to be a useful fluorescent probe. Also, the ability of multiple modifications to display significant changes compared to the characteristic fluorescence profile of singly modified PNA oligomer expands the scope of the fluorescent base analogues. Furthermore, the change in fluorescence intensity of PNA duplexes depending on concentration of complementary DNA ONs reveals an atypical trend. Taken together, the straight forward synthesis, amicability to solid-phase PNA oligomer synthesis, structurally non-perturbing nature and sensitivity to changes in its microenvironment highlight the potential of these flurescent pyrimidine PNA base analogues as efficient fluorescent probes for nucleic acid diagnosis.

## Experimental Section

### 1. Materials

Di-*tert*-butyl dicarbonate, ethylenediamine, ethyl bromoacetate, TFA and chloroacetyl chloride were purchased from Spectrochem. 5-Iodouracil, benzo[*b*]furan, benzo[*b*]thiophene, *bis*(triphenylphosphine)-palladium (II) chloride, HOBt, HBTU, *N*,*N*-diisopropylethylamine (DIPEA), 1,2-ethanedithiol, thioanisole and trifluoromethanesulfonic acid (TFMSA) were obtained from Sigma-Aldrich. Boc-protected *aeg*-PNA monomers were purchased from ASM Research Chemicals. 4-Methylbenzhydrylamine•HCl (MBHA) resin LL (100-200 mesh) was obtained from Novabiochem. DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Purity of Oligonucleotides were confirmed using HPLC on an Agilent 1260 Infinity instrument. All other chemicals supplied from commercial supplier were

used without any further purification. Autoclaved water was used in all biochemical reactions and fluorescence measurements.

## 2. Instrumentation

NMR spectra were recorded on a 400 MHz Jeol ECS-400. All mass measurements were recorded on an Applied Biosystems 4800 Plus MALDI-TOF/TOF analyzer and Water Synapt G2 high definition mass spectrometers. PNA oligomers were purified using Dionex ICS 3000 HPLC and Agilent 1260 Infinity. Purity was verified using Agilent 1260 Infinity instrument. Absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer. Steady-state fluorescence experiments were carried out in a micro fluorescence cuvette (Hellma, path length 1.0 cm) on either on a Fluoromax-4 spectrofluorometer (Horibha Scientific) or Fluorolog (Horibha Scientific).

## 3. Synthesis

### Ethyl 2-(N-(2-((tert-butoxycarbonyl)amino)ethyl)-2-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) acetamido) acetate a:

To a solution of ethyl 2-(*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-chloroacetamido)acetate **1** (2.00 g, 6.20 mmol, 1.0 equiv.) in DMF (20 mL) was added potassium carbonate (1.88 g, 13.63 mmol, 2.2 equiv.) and 5-iodouracil (1.62 g, 6.82 mmol, 1.1 equiv.). The reaction mixture was stirred for 12 h at room temperature (RT). Water (100 mL) was added and the product was extracted using EtOAc (2 × 100 mL). The organic layer was evaporated and residue was purified by silica gel column chromatography to afford the product **a** as a white solid (1.50 g, 46%). $R_f$ = 0.29 (Petroleum ether:EtOAc = 30:70); $^1$H-NMR (400 MHz, $CDCl_3$): δ = 9.66 (minor (mi.)) and 9.60 (major (ma.) (br, 1H), 7.65 (mi.) and 7.59 (ma.) (s,1H), 5.65 (ma.) and 5.10 (mi.) (t, *J* = 6.0 Hz, 1H), 4.63 (ma.) and 4.48 (mi.) (s, 2H), 4.26-4.05 (m, 4H), 3.51 ($_{app}$t, *J* = 5.6 Hz, 2H), 3.34-3.24 (m, 2H), 1.44 (ma.) and 1.42 (mi.) (s, 9H), 1.31 (mi.) and 1.27 (ma.) (t, *J* = 7.1 Hz, 3H) ppm; $^{13}$C-NMR (100 MHz, $CDCl_3$): δ = 169.7 (ma.) and 169.4 (mi.), 167.4 (mi.) and 167.1 (ma.), 160.8 (mi.) and 160.8 (ma.), 156.3, 150.9, 149.6, 80.2, 68.3 (ma.) and 68.2 (mi.), 62.5 (mi.) and 61.9 (ma.), 49.3, 48.9, 48.4, 38.8, 28.6 (ma.) and

28.5 (mi.), 14.2 (mi.) and 14.2 (ma.) ppm; HRMS: (*m/z*): Calc. for $C_{17}H_{25}IN_4O_7Na$: 547.0666 $[M+Na]^+$; found: 547.0666.

**Ethyl 2-(2-(5-(benzofuran-2-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)-*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)acetamido)acetate b**:

To a suspension of compound **a** (1.35 g, 2.57 mmol, 1.0 equiv.) and *bis*(triphenylphosphine)-palladium(II) chloride (0.09 g, 0.13 mmol, 0.05 equiv. ) in degassed anhydrous dioxane (40 mL) was added 2-(tri-*n*-butylstannyl)benzofuran[S3] (1.57 g, 3.87 mmol, 1.5 equiv.). The reaction mixture was heated at 75°C for 3 h, and filtered through celite pad. Celite pad was washed with hot dioxane (2 x 10 mL). Dioxane was evaporated and the residue was purified by silica gel column chromatography to afford the product **b** as a white solid (0.56 g, 42%). $R_f$ = 0.63 (petroleum ether:EtOAc = 10:90); $^1$H-NMR (400 MHz, $d_6$-DMSO): δ = 11.85 (br, 1H), 8.21 (ma.) and 8.15 (mi.) (s, 1H), 7.64 (d, *J* = 7.6 Hz, 1H), 7.49 (d, *J* = 7.6 Hz, 1H), 7.38 (s, 1H), 7.30-7.21 (m, 2H), 6.99 (ma.) and 6.77 (mi.) (t, *J* = 5.2 Hz, 1H), 4.93 (ma.) and 4.75 (mi.) (s, 2H), 4.33-4.07 (m, 4H), 3.46-3.37 (m, 2H), 3.23-3.04 (m, 2H), 1.40 (ma.) and 1.36 (mi.) (s, 9H), 1.27 (mi.) and 1.18 (ma.) (t, *J* = 7.0 Hz, 3H) ppm; $^{13}$C-NMR (100 MHz, $d_6$-DMSO): δ = 169.0, 167.2, 160.7, 155.8, 152.9, 149.9, 148.9, 142.6, 131.5, 128.8, 124.5, 123.2, 121.2, 110.4, 103.9, 78.1, 60.6, 48.3, 47.9, 47.0, 38.1, 28.2, 14.0 ppm; HRMS: (*m/z*): Calc. for $C_{25}H_{30}N_4O_8Na$: 537.1961 $[M+Na]^+$; found: 537.1960.

**2-(2-(5-(benzofuran-2-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)-*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)acetamido)acetic acid 2**:

To a solution of compound **b** (0.17 g, 0.33 mmol, 1.0 equiv.) in methanol (10 mL) was added lithium hydroxide (3% solution in $H_2O$, 1.5 mL ) and the reaction mixture was stirred for 3 h at RT. Reaction mixture was evaporated to dryness, and residue was dissolved in water (15 mL) and washed with diethyl ether (10 ml). To the aqueous extract was added saturated $KHSO_4$ solution (~2 mL) to adjust the pH to ~4. The product was then extracted in EtOAc (2 × 40 mL) and solvent was evaporated to afford the product **2** as a white solid (0.15 g, 93%). $R_f$ = 0.26 ($CH_2Cl_2$:MeOH = 70:30); $^1$H-NMR

(400 MHz, $d_6$-DMSO): δ = 11.83 (ma.) and 11.81 (mi.) (br, 1H), 8.22 (ma.) and 8.14 (mi.) (s, 1 H), 7.64 (d, *J* = 6.0 Hz, 1H), 7.51-7.49 (m, 1H), 7.37 (s, 1H), 7.30-7.22 (m, 2H), 6.97 (ma.) and 6.76 (mi.), 4.91 (ma.) and 4.73 (mi.) (s, 2H), 4.20 (mi.) and 4.00 (ma.) (s, 2H), 3.42 ($_{app}$t, *J* = 5.0 Hz, 2H), 3.22-3.17 (ma.) and 3.06-3.03 (mi.) (m, 2H), 1.40 (ma.) and 1.36 (mi.) (s, 9H) ppm; $^{13}$C-NMR (100 MHz, $d_6$-DMSO): δ = 170.8 (ma.) and 170.5 (mi.), 167.4 (mi.) and 167.0 (ma.), 160.7, 155.8 (ma.) and 155.6 (mi.), 152.9, 149.9, 148.9, 142.7, 128.9, 124.5, 123.2, 121.2, 110.4, 103.9, 78.1, 48.3, 47.7, 46.9, 38.0, 28.2 ppm; HRMS: (*m/z*): Calc. for $C_{23}H_{26}N_4O_8Na$: 509.1648 $[M+Na]^+$; found: 509.1648.

**5. Solid phase synthesis fluorescently modified) PNA oligomers**

*General procedure:* All PNA oligomers contained two lysine residues at the C terminus for improved solubility. PNA oligomers were synthesized on MBHA resin using Boc protected *aeg*-PNA monomers and fluorescent PNA monomer **2**. Typically, MBHA resin (25 mg, 0.35 mmol/g) was allowed to swell in $CH_2Cl_2$ (2 mL) for 2 h. The solvent was removed and the resin was treated with 50% DIPEA in $CH_2Cl_2$ (3 mL) for 10 min. This step was repeated two more times. The resin was then washed sequentially with $CH_2Cl_2$ (3 x 3 mL), DMF (3 x 3 mL) and $CH_2Cl_2$ (3 x 3 mL). The resin was dried under nitrogen flow for few minutes. The coupling reactions were performed in DMF (0.6 mL) with appropriate Boc-protected PNA monomers (3.0 equiv.) in the presence of HOBt (3.0 equiv.), HBTU (3.0 equiv.) and DIPEA (3.0 equiv.) using microwave PNA synthesizer. The conditions for microwave used were 20 W power and 65° C temperature. The coupling was performed for total 10 minutes in two intervals of 5 minutes. The resin was washed with $CH_2Cl_2$ (3 x 3 mL), DMF (3 x 3 mL) and $CH_2Cl_2$ (3 x 3 mL) and dried under nitrogen flow for few minutes. The Boc group was deprotected using 50% TFA in $CH_2Cl_2$ (3 mL) for 15 min. This step was repeated two more times, and the resin was then washed with $CH_2Cl_2$ (3 x 3 mL), DMF (3 x 3 mL) and $CH_2Cl_2$ (3 x 3 mL) and dried under nitrogen flow for few minutes. The resin was further treated with 10% DIPEA in $CH_2Cl_2$ (3 mL) for 10 min three times. The resin was washed again and the coupling, Boc deprotection, neutralization and washing steps were performed as mentioned above to synthesize the desired PNA sequence. After the final coupling, the dried resin

was transferred to a glass vial and treated with thioanisole (30 µL) and ethandithiol (12 µL) in an ice bath for 10 min. TFA (300 µL) was then added and stirred for another 10 min. Next, TFMSA (24 µL) was added to the above mixture and was stirred for 2 h at room temperature. The resin was filtered, and the filtrate was concentrated and precipitated as a white solid by adding cold diethyl ether (1 mL). The solvent was decanted and the crude product was dissolved in autoclaved water and purified by RP-HPLC. The integrity of PNA oligomers was confirmed by MALDI-TOF mass analysis and quantified by UV-Vis spectroscopy using the following extinction coefficients at 260 nm ($\varepsilon_A$ = 13700, $\varepsilon_T$ = 8600, $\varepsilon_G$ = 11700, $\varepsilon_C$ = 6600 $M^{-1}cm^{-1}$, $\varepsilon_{\mathbf{2}}$ = 12210 $M^{-1}cm^{-1}$).[31] See Table 1 for details.

**6. MALDI-TOF mass measurements**

Molecular weight of PNA oligomers were determined using Applied Biosystems 4800 Plus MALDI-TOF/TOF analyser. 1 µL of a ~150 µM stock solution of PNA oligomer was combined with 2,5-dihydroxybenzoic acid (DHB) or α-Cyano-4-hydroxycinnamic acid (CHCA) matrix. The samples were spotted on a plate and air dried before mass analysis. For MALDI-TOF mass analysis of PNA oligomers (Table 1).

**7. Photophysical characterization of fluorescently modified PNA oligomers**

For photophysical characterization of PNA **3**–**6** in different nucleobases environment, PNA **3**–**6** (10 µM) was annealed to custom DNA and RNA oligonucleotides (**3a**–**6c** and **3d**–**5f) (**10 µM) by heating a 1:1 mixture of PNA and DNA/RNA in 10 mM phosphate buffer (pH 7.1, 100 mM NaCl, 0.1 mM EDTA) at 90°C for 3 min. Samples were then cooled slowly to RT, and were placed in crushed ice for ~60 min. Samples were diluted to give a final concentration of 1 µM in phosphate buffer. Fluorescently modified duplexes were excited at 330 nm with an excitation slit width of 2 nm and emission slit width of 4 nm. Fluorescence experiments were performed in duplicate in a microfluorescence cuvette (Hellma, path length 1.0 cm) on a Horiba scientific Fluoromax- 4 instrument.

For photophysical characterization of multichromophoric PNA **10**–**14,** they were annealed to custom DNA oligonucleotides (**3a**–**6c** and **3d**–**5f) (**5 µM) by heating a 1:1

mixture of PNA and DNA in 10 mM phosphate buffer (pH 7.1, 100 mM NaCl, 0.1 mM EDTA) at 90°C for 3 min. Samples were then cooled slowly to RT, and were placed in crushed ice for ~60 min. Samples were diluted to give a final concentration of 0.5 μM in phosphate buffer. Fluorescently modified duplexes were excited at 330 nm with an excitation slit width of 3 nm and emission slit width of 5 nm. Fluorescence experiments were performed in duplicate in a microfluorescence cuvette (Hellma, path length 1.0 cm) on a Horiba Jobin Yvon, Fluorolog-3 instrument. For studying the effect of varying concentration of complementary DNA on fluorescence intensity PNA **3** (100 nm) and DNA **7** were annealed to custom DNA oligonucleotides (**3d, 8** and **3a)** by heating different concentration ratio mixture of PNA and DNA in 10 mM phosphate buffer (pH 7.1, 100 mM NaCl, 0.1 mM EDTA) at 90°C for 3 min. Fluorescently modified duplexes were excited at 330 nm with an excitation slit width of 2 nm and emission slit width of 4 nm. Fluorescence experiments were performed in duplicate in a microfluorescence cuvette (Hellma, path length 1.0 cm) on a Horiba Jobin Yvon, Fluorolog-3 instrument.

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## Appendix (selected spectra)

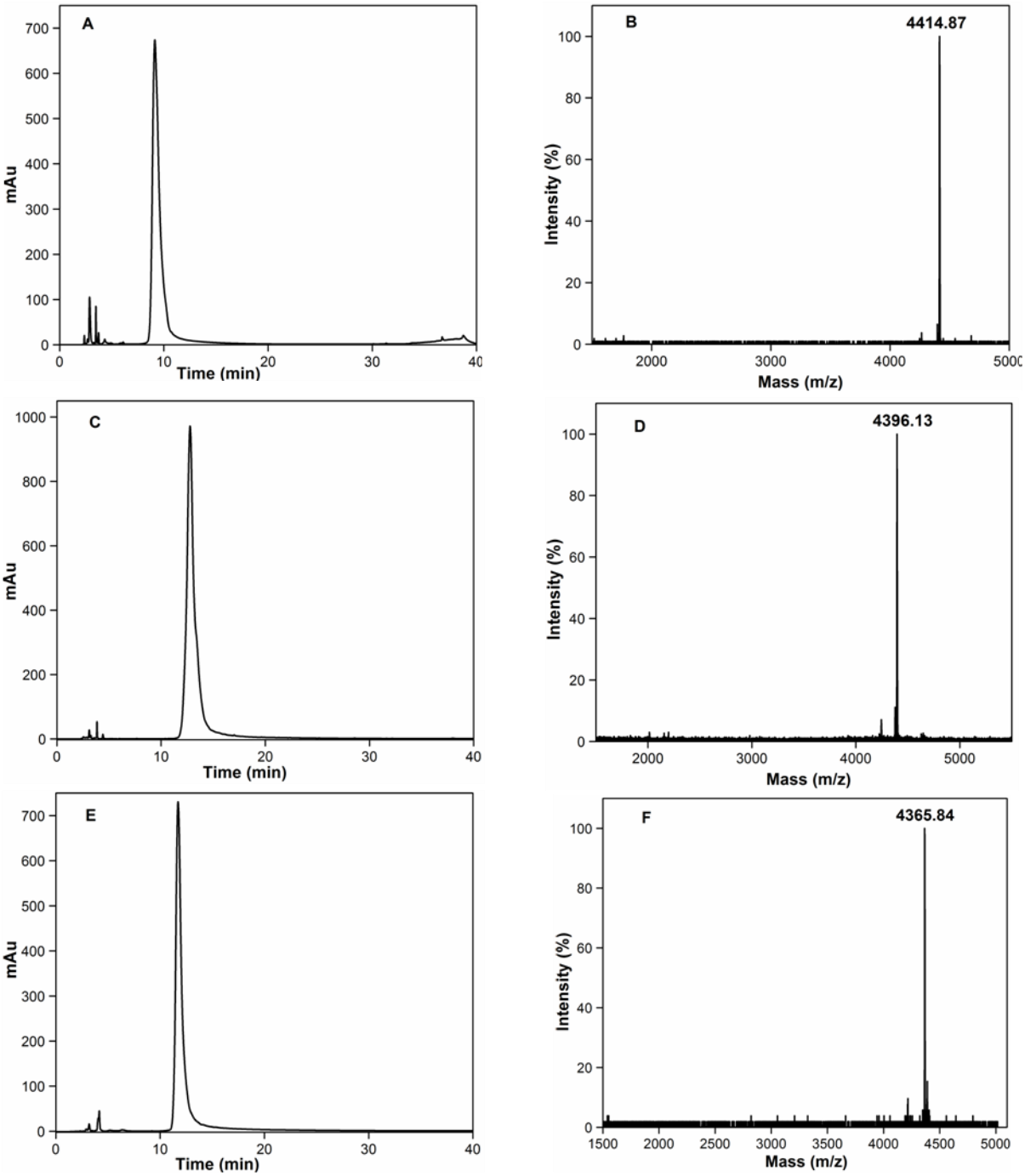


**Figure 14: (A)** RP-HPLC chromatogram of PNA oligomer **3 (B)** MALDI-TOF mass spectrum of PNA oligomer **3**. **(C)** RP-HPLC chromatogram of PNA oligomer **4 (D)** MALDI-TOF mass spectrum of PNA oligomer **4 (E)** RP-HPLC chromatogram of PNA oligomer **5. (F)** MALDI-TOF mass spectrum of PNA oligomer **5.** Mobile phase A: 5% acetonitrile in $H_2O$ containing 0.1% TFA, mobile phase B: 50% acetonitrile in $H_2O$ containing 0.1% TFA. Flow rate: 1 mL/min. Gradient: 0–50% B in 25 min and 50–100% B in 15 min. (See Table 1 for mass data

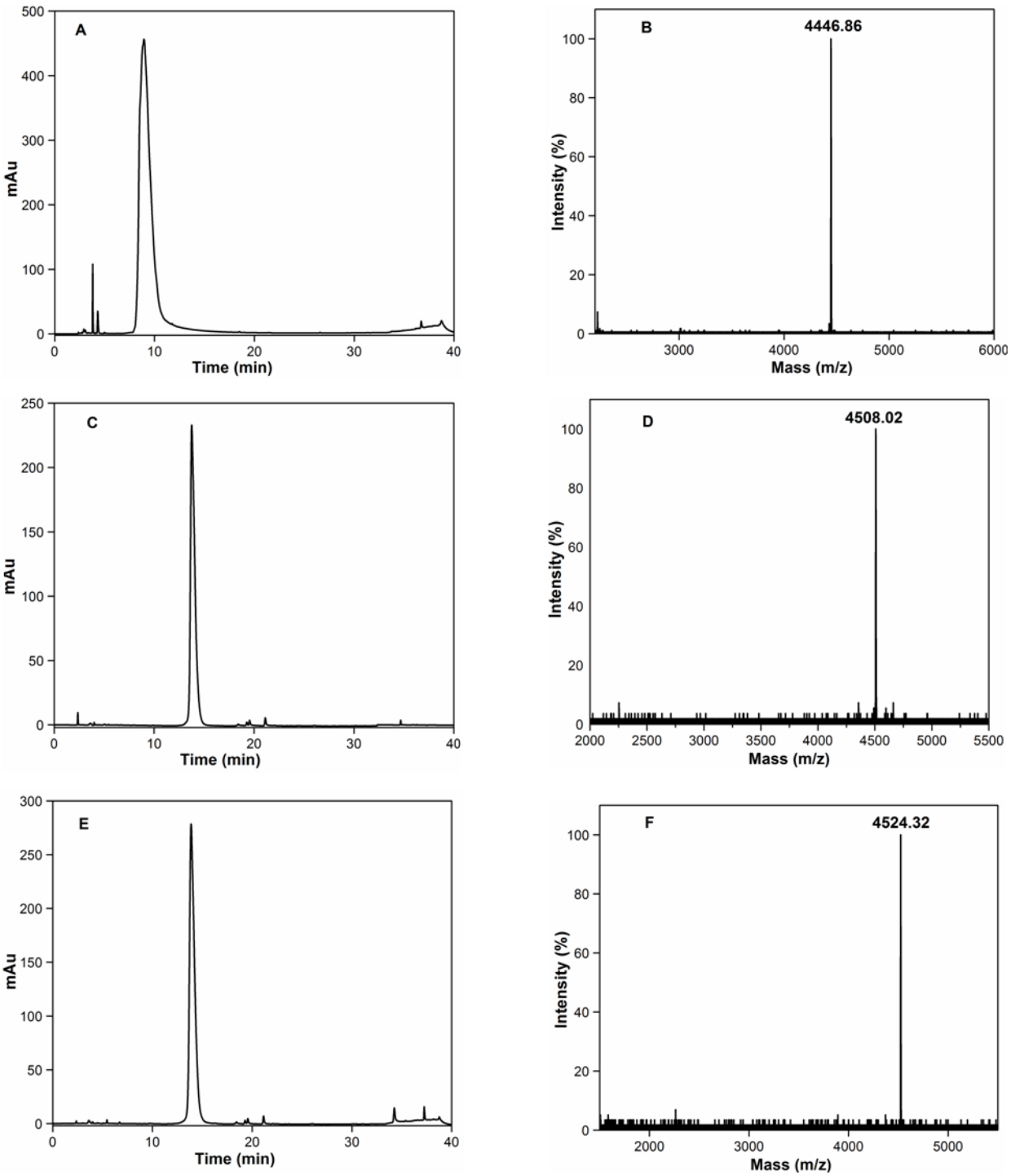


**Figure 15: (A)** RP-HPLC chromatogram of PNA oligomer **6 (B)** MALDI-TOF mass spectrum of PNA oligomer **6**. **(C)** RP-HPLC chromatogram of PNA oligomer **10 (D)** MALDI-TOF mass spectrum of PNA oligomer **10 (E)** RP-HPLC chromatogram of PNA oligomer **11 (F)** MALDI-TOF mass spectrum of PNA oligomer **11**. Mobile phase A: 5% acetonitrile in $H_2O$ containing 0.1% TFA, mobile phase B: 50% acetonitrile in $H_2O$ containing 0.1% TFA. Flow rate: 1 mL/min. Gradient: 0–50% B in 25 min and 50–100% B in 15 min. (See Table 1 for mass data).

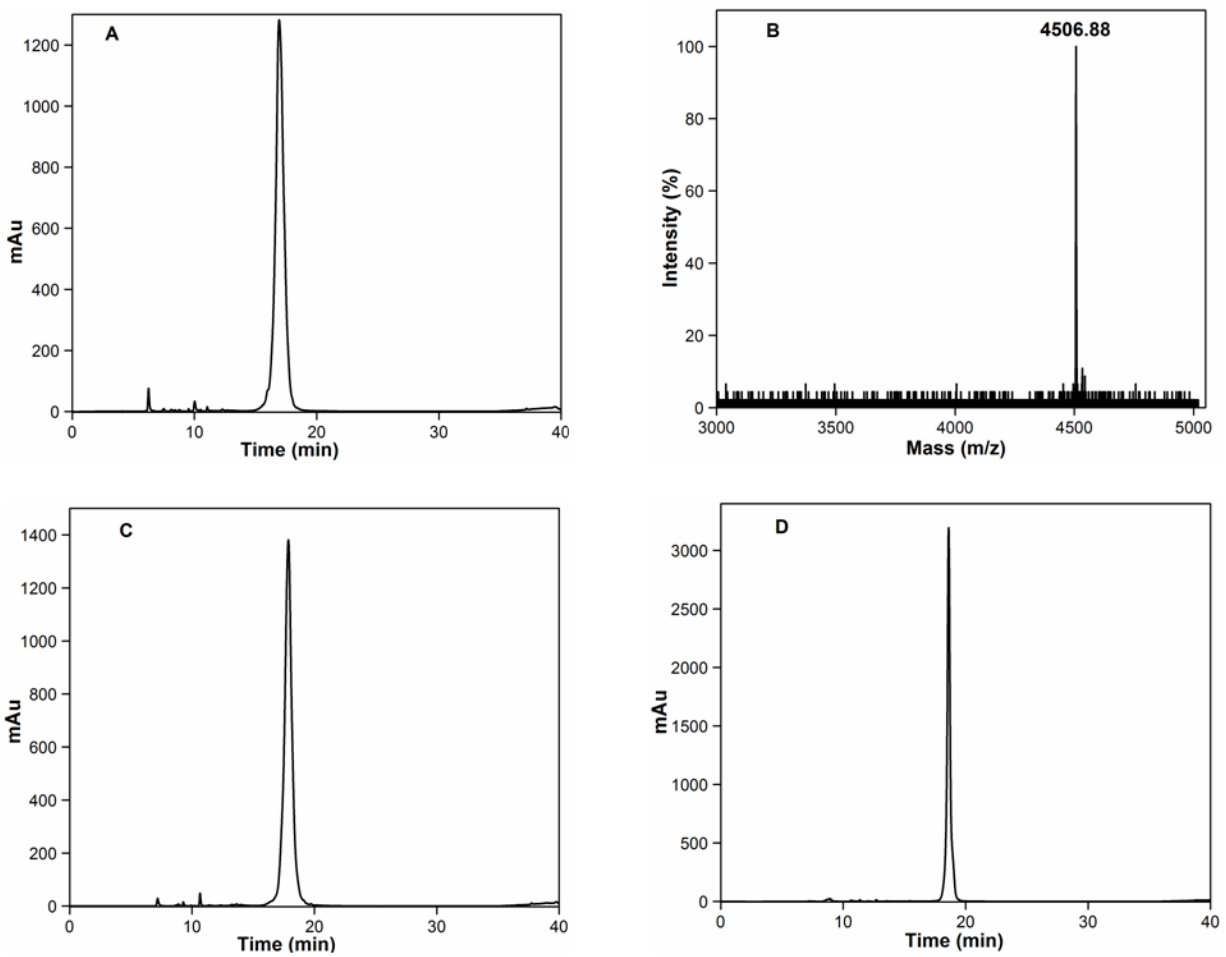


**Figure 16: (A)** RP-HPLC chromatogram of PNA oligomer **12 (B)** MALDI-TOF mass spectrum of PNA oligomer **12 (C)** RP-HPLC chromatogram of PNA oligomer **13 (D)** RP-HPLC chromatogram of PNA oligomer **14**. Mobile phase A: 5% acetonitrile in $H_2O$ containing 0.1% TFA, mobile phase B: 50% acetonitrile in $H_2O$ containing 0.1% TFA. Flow rate: 1 mL/min. Gradient: 0–50% B in 25 min and 50–100% B in 15 min. (See Table 1 for mass data).

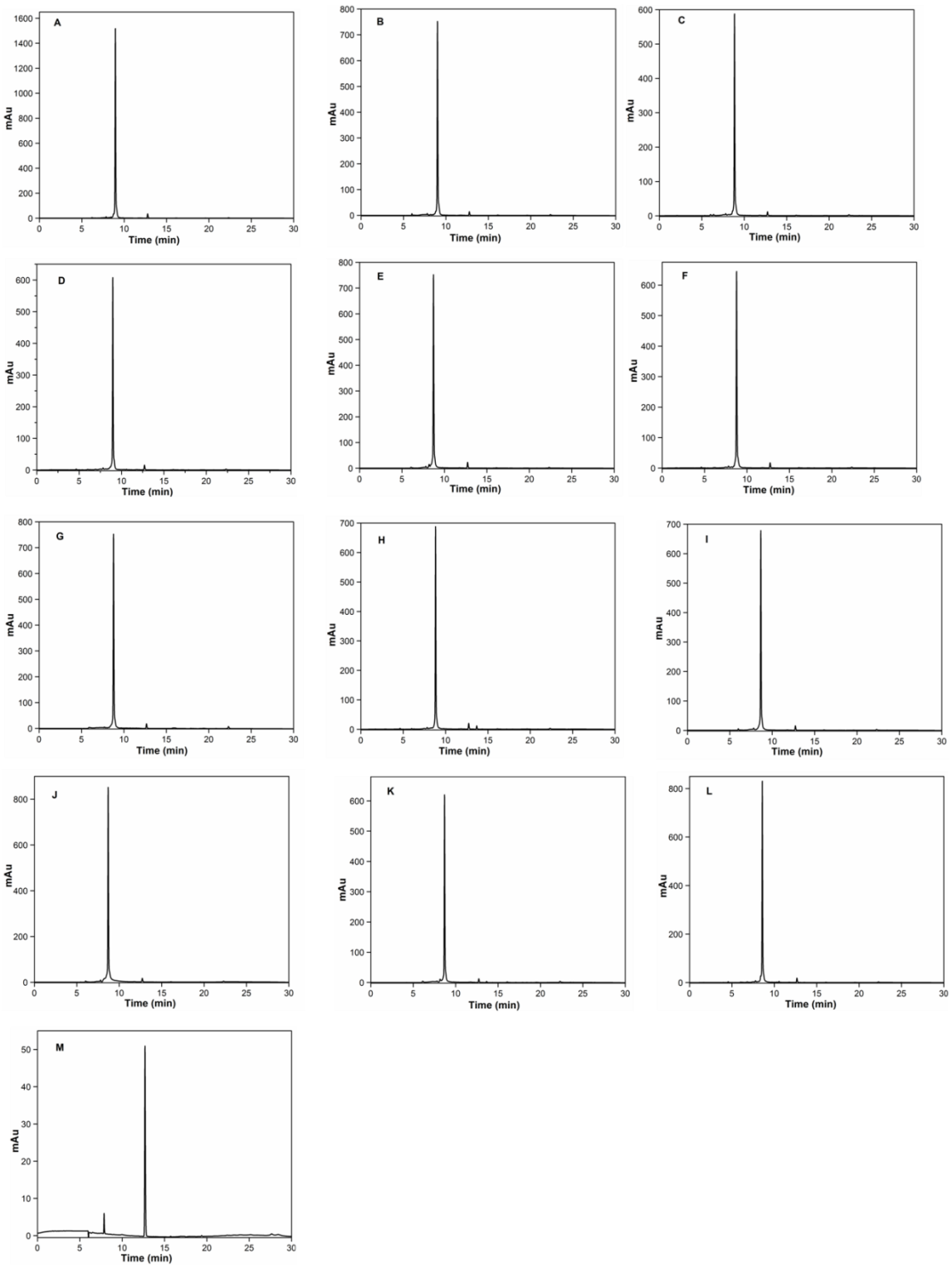


**Figure 17:** (**A-M**) RP-HPLC chromatogram of DNA ONs **3d**, **3e**, **3f**, **3g**, **4d**, **4e**, **4f**, **4g**, **5d**, **5e**, **5f**, **5g** and **10a**, respectively. Mobile phase A: 25 mM triethylammonium acetate buffer (pH 7.5), mobile phase B: acetonitrile. Flow rate: 1 mL/min. Gradient: 0–50% B in 20 min and 50–100% B in 10 min.

$^{1}$H-NMR of compound **2** in $d_6$-DMSO

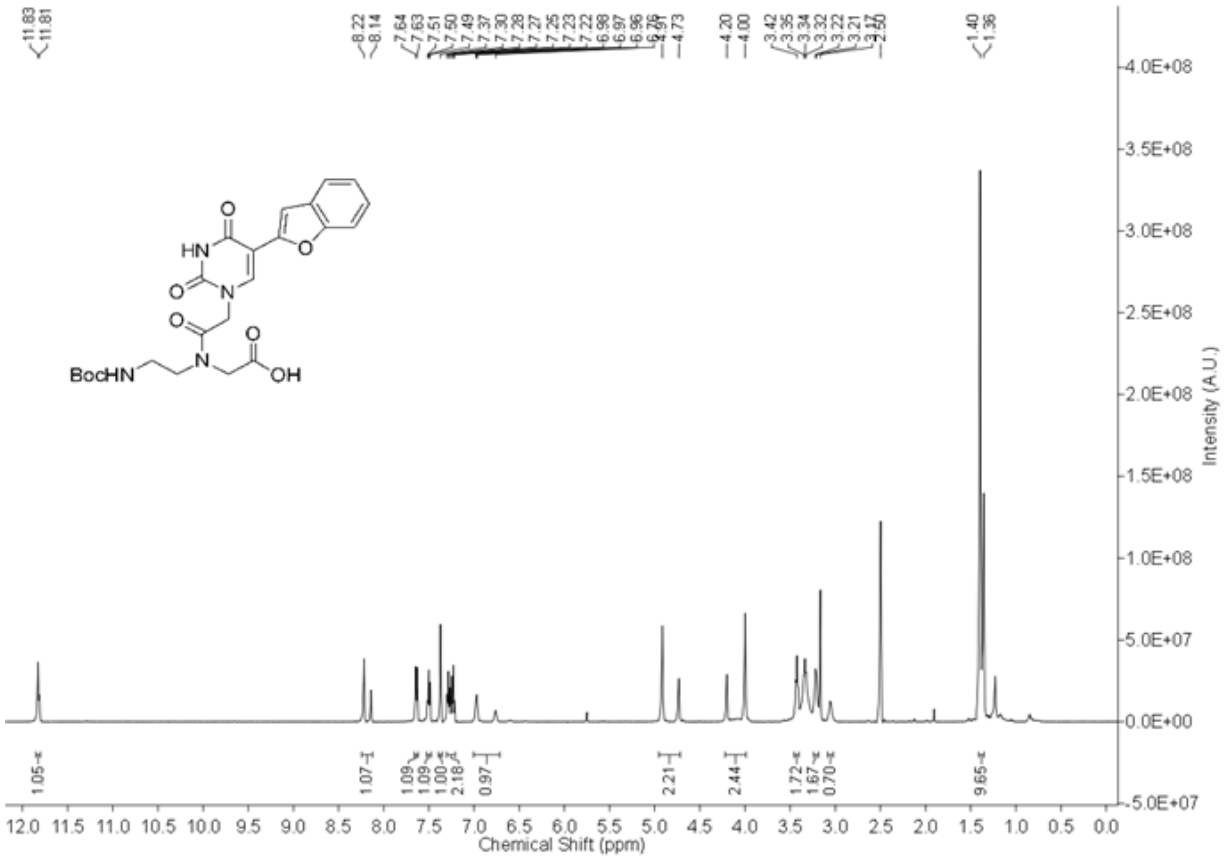


**Figure 18:** $^{1}$H-NMR of compound **2** in $d_6$-DMSO

$^{13}$C-NMR of compound **2** in $d_6$-DMSO

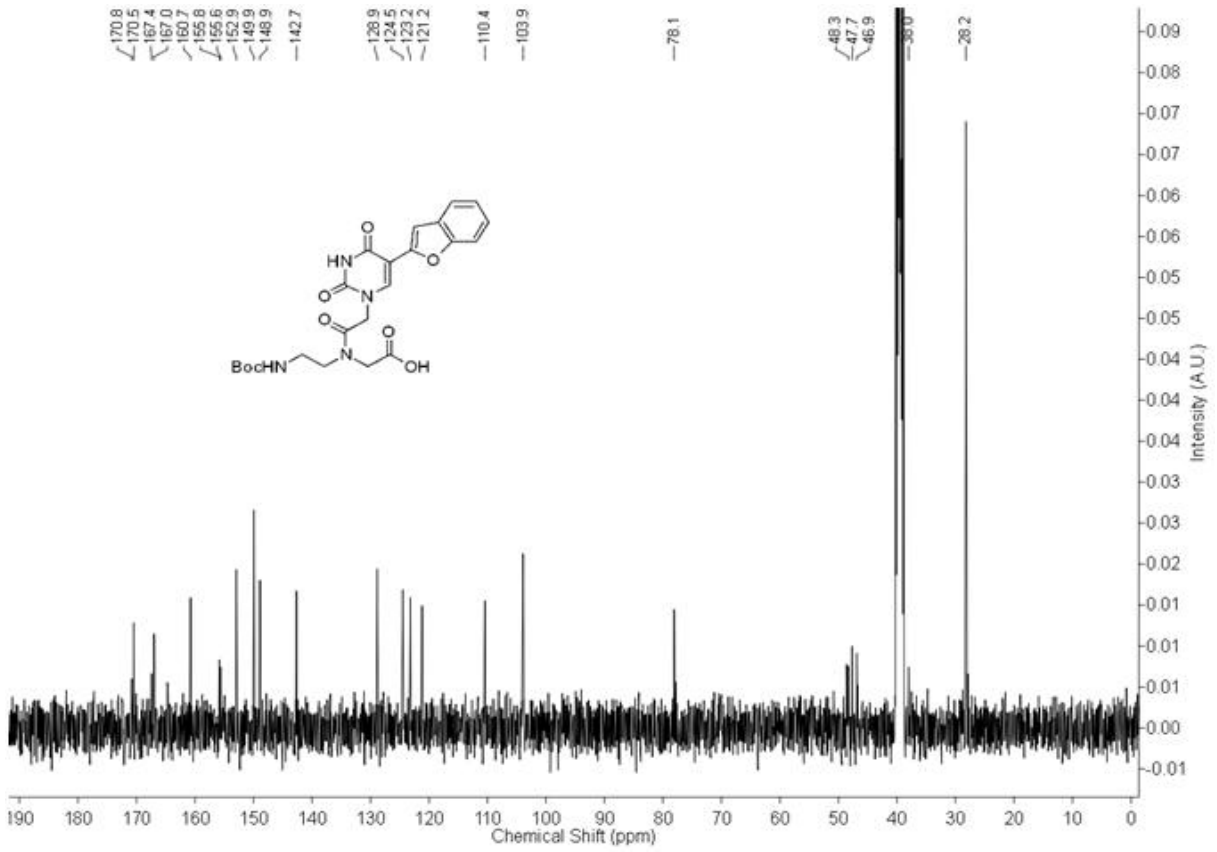


**Figure 19:** $^{13}$C-NMR of compound **2** in $d_6$-DMSO