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Fluorinated Peptide Nucleic Acids with Fluoroacetyl Side Chain Bearing 5-(F/CF_3)-Uracil: Synthesis and Cell Uptake Studies

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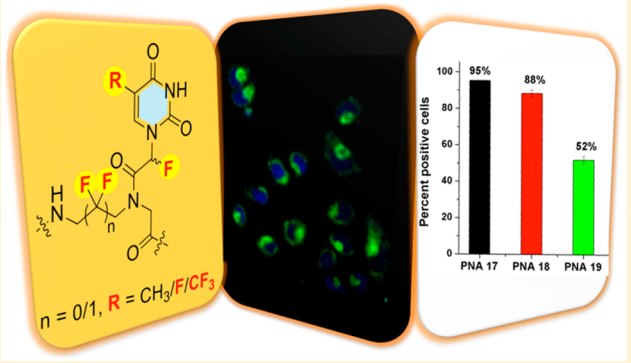
Supporting Information

ABSTRACT: Fluorine incorporation into organic molecules imparts favorable physicochemical properties such as lipophilicity, solubility and metabolic stability necessary for drug action. Toward such applications using peptide nucleic acids (PNA), we herein report the chemical synthesis of fluorinated PNA monomers and biophysical studies of derived PNA oligomers containing fluorine in in the acetyl side chain (−CHF−CO−) bearing nucleobase uracil (5-F/5-CF3-U). The crystal structures of fluorinated racemic PNA monomers reveal interesting base pairing of enantiomers and packing arrangements directed by the chiral F substituent. Reverse phase HPLC show higher hydrophobicity of fluorinated PNA oligomers, dependent on the number and site of the fluorine substitution: fluorine on carbon adjacent to the carbonyl group induces higher lipophilicity than fluorine on nucleobase or in the backbone. The PNA oligomers containing fluorinated bases form hybrids with cDNA/RNA with slightly lower stability compared to that of unmodified aeg PNA, perhaps due to electronic effects. The uptake of fluorinated homooligomeric PNAs by HeLa cells was as facile as that of nonfluorinated PNA. In conjunction with our previous work on PNAs fluorinated in backbone and at N-terminus, it is evident that the fluorinated PNAs have potential to emerge as a new class of PNA analogues for applications in functional inhibition of RNA.



INTRODUCTION

Peptide nucleic acids (**1**) are putative mimics of DNA/RNA.[1] With no known toxicity even at reasonably higher concentrations,[2] they are attractive as RNA functional blocking agents.[3,4] However, to be effective, their solubility and cell permeability need improvements. Toward these aims, a wide variety of PNA analogues with modifications in backbone or side chain, structural preorganization via cyclic monomers and PNAs possessing inherently cationic groups have been conceived.[5,6] Many chiral and cationic PNA analogues have shown improvements in desirable properties.[7−9] An interesting departure from such approaches would be to introduce fluorine in C−H bonds and maintain the inherent sterics of the PNA, but concurrently impart the physicochemical attributes needed for drug-like properties. The high electronegativity and a small atomic radius of fluorine endow it special properties such as low polarizability and polar hydrophobicity.[10] Introduction of a fluoro group in an organic molecule can substantially modulate their physicochemical properties, enhance metabolic stability and favor drug-like activity.[11] As fluorine is the smallest substituent that can be used as a replacement for the C−H bond, it adds only limited extra steric demand at receptor sites.[12] Fluorinated organic compounds have become highly important in the pharmaceutical and agrochemical industries with about 25% of current drugs, including many top-sellers contain at least one fluorine atom,[13,14] including the fluorinated oligonucleotides used for potential antisense activity.[15,16]

The first fluorinated peptide nucleic acid monomer conceived the replacement of central amide bond with fluoro vinyl group to obtain olefinic PNA[17] and its hybridization properties emphasized the important structural role of tertiary amide bond in PNA as an enabling element for recognition of complementary DNA. Subsequently, PNAs composed of fluorinated nucleobases demonstrated the merits of fluorine to probe PNA:DNA/RNA hybridization by ^{19}F NMR.[18] Most recently, we have described fluorinated PNA **2a** that incorporated additional $-CF_2-$ in the ethylenediamine fragment and shown that these have better cell permeability compared to nonfluorinated PNA **2b**, without compromising the DNA:RNA hybridizing ability.[19] We also found that conjugation of perfluoroalkyl chain to PNA (**3**) induces formation of nanoparticles in 150−200 nm size[20] that favors cell permeability. Building on these concepts, we now report synthesis of fluorinated PNA analogues having 5-F/5-CF_3 uracil (**4**, **5**) and incorporating F in the N-acetyl side chain (**6**-**8**) to assess their consequences on DNA/RNA hybridization properties and cell permeability. We also report reverse phase HPLC

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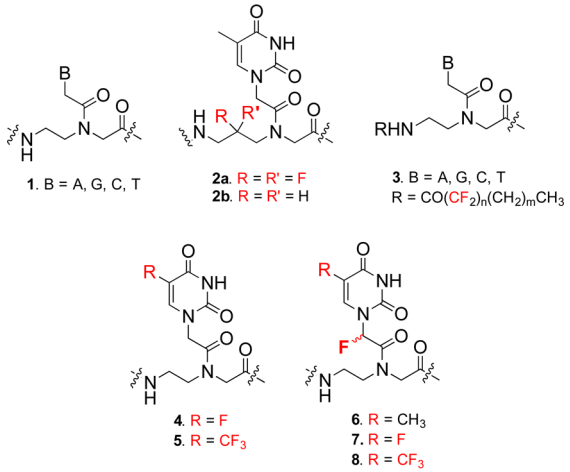
data of differently substituted fluoro PNAs, which indicate that the enhanced lipophilicity of derived PNAs that depends not only the number of fluorines but also on the site of incorporation. The fluorinated PNAs displayed thermal stability of duplexes with complementary DNA/RNA sequences that are slightly weaker, but significantly improve their cell permeability.



RESULTS AND DISCUSSION

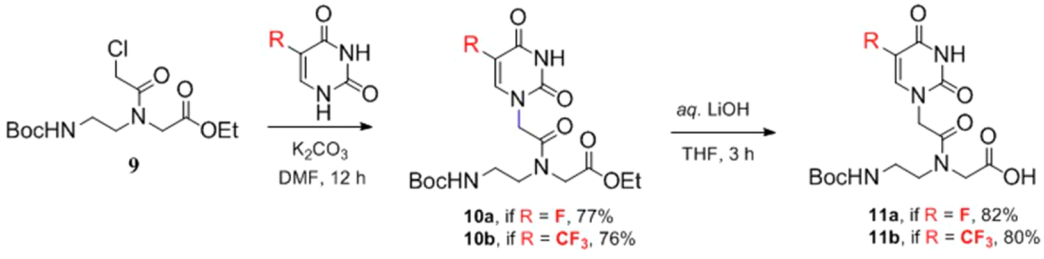
Synthesis of Fluorinated PNA Monomers and Oligomers. The nucleobase fluorinated PNA building blocks were synthesized from the known[21] chloro compound **9** (Scheme 1), which was coupled with the commercially available 5-fluoro/5-trifluoromethyl uracil (5-FU/5-CF_3U) using K_2CO_3 as base in DMF to obtain the esters **10a/b**. The esters were hydrolyzed to corresponding acid monomers **11a** and **11b** respectively.

PNA monomers (**16**) fluorinated in nucleobase and side chain were synthesized starting from 5-methyl/5-fluoro/5-trifluoromethyl uracil (**12**, R = T/5F−U/5-CF_3−U). These were individually treated with ethyl bromofluoroacetate in the presence of K_2CO_3 to obtain the corresponding N^1-fluoroalkylated products **13a/b/c** (Scheme 2). The esters were hydrolyzed to the corresponding acids **14a/b/c** using aq. KOH in THF. The acids were independently coupled with *N*-Boc-2-amino ethyl glycine ethyl ester in the presence of HBTU/DIPEA to obtain the PNA esters **15a/b/c** respectively as racemic mixtures. These were hydrolyzed to the respective acids to get the desired fluorinated PNA monomers **16a/b/c** and used without resolution for synthesis of PNA oligomers. The identities of all compounds were confirmed by their NMR (^{1}H, ^{13}C and ^{19}F) and mass spectral data. It should be noted that all monomers are enantiomeric pairs, with chirality on carbon carrying F substitution. Attempts to resolve the compound **13a** by different methods such as (i) crystallization or (ii) conversion to diastereomers through esterification with optically active (*S*)-2-phenyl ethanol (Scheme 3) or N-alkylation at N^3 with chiral (*S*)-2-phenyl ethanol (Scheme 3) failed.

The intermediates **13a/b/c** were crystallized from 50% ethyl acetate-petroleum ether, ethyl acetate and chloroform, respectively. The X-ray crystal diffraction data was collected at 200 K temperature using Mo Kα radiation (λ = 0.71073 Å). Crystal structures of compound **13a** and **13b** showed monoclinic symmetry with space group $P21/n$, whereas compound **13c** showed triclinic symmetry with space group $P1$, and compound **17a/b** showed monoclinic symmetry with space group $P2_1$. The crystal structures showed hydrogen bond pairing between the *R* and *S* enantiomers through N3H and either C(2) or C(4) carbonyls in varying patterns (Figure 1). Compound **17** crystallized with H-bonding between the two diastereomers (Figure 1). The presence of fluorine at δ-position induced the crystallization between the two enantiomers as well as diastereomers and the crystal structure analysis suggested interesting chiral recognition features.

Each of the monofluorinated 5-MeU **13a**(*R*/*S*) and the difluorinated **13b**(*R*/*S*) racemates formed base pairs within the enantiomeric pairs, with the N3H and C4 carbonyl of one enantiomer forming H-bond with the C4 carbonyl and N3H of the other enantiomer [**13a**(*R*) C(4)═O—H—N3 **13a**(*S*) and **13a**(*R*) N3—H—O═C(2) **13a**(*S*); **13b**(*R*) C(4)═O—H—N3 **13b**(*S*) and **13b**(*R*) N(3)—H—O═C(2) **13b**(*S*)]. In case of **13a/b**, the base pairs are slightly twisted and the ethyl group of both the enantiomers are tucked inside the adjacent base paired units as though their interaction also stabilized the assembly. The base paired *R* and *S* enantiomers of **13b**(*R*/*S*) are aligned in an antiparallel manner relative to each other with respect to the relative orientations of respective C(2)═O and C(5)−F bonds, and the ethyl groups are pointed away from the base stacks. Interestingly, the diastereomeric pair **17a/b**(*RS*/*SS*) derived from **13a**(*R*/*S*) showed base pairing comprising of N3−H and C2 carbonyl (and not C4 carbonyl unlike **13a/b**) [**17**(*S*) C(2)═O—H—N3 **17b**(*R*) and **17**(*S*) N3H—O═C(2) **17b**(*R*)]. The phenyl ring of *SS* diastereomer stacked with the T base of same residue, while the phenyl ring of *RS* diastereomer stacked with the T base of adjacent residue separated by 3.6 Å, suggesting stabilization of the assembly through additional π−π interactions. The bases in H-bonded pairs are antiparallel relative to each other. This structure is interesting since the presence of more than one diastereomer in a single crystal is less common. In case of tetrafluorinated **13c**(*R*/*S*) (5-CF_3 and δ-F), interbase pairing occurs between the *R* and *S* enantiomers through diagonally crossed single hydrogen bonds connecting C2 carbonyl N3H, possible because of the 90° relative twist of the bases in the pairs. In

Scheme 1. Synthesis of Nucleobase Modified (5FU-*aeg*, 5CF_3U-*aeg*) PNA Monomers (11a/b)



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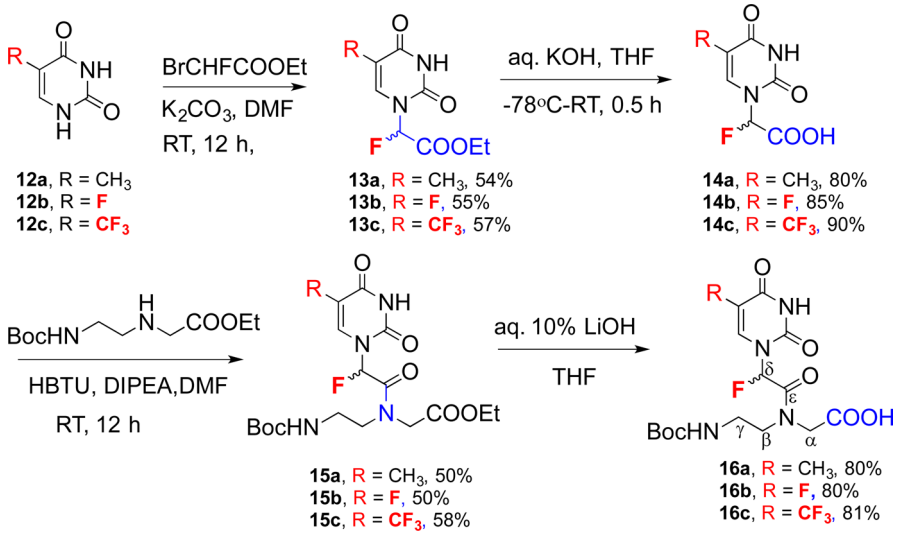
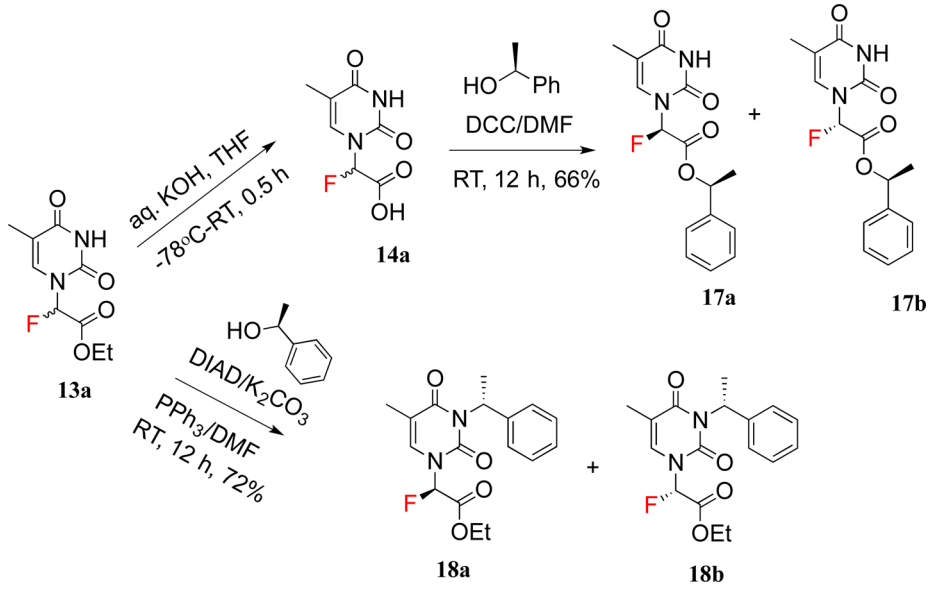
Scheme 2. Synthesis of Nucleobase and Side Chain Fluorinated (δ-F, *aeg*-T), (δ-F, 5FU-*aeg*), and (δ-F, 5CF$_3$U-*aeg*) PNA Monomers (16a/b/c)



Scheme 3. Attempted Resolution of the Racemate of 13a



all crystals, the *R* and *S* enantiomers are in adjacent vertical planes with the *R*-enantiomers H-bonded with *S*-enantiomers in the horizontal plane. H-bonding is seen only between the *R*−*S* enantiomeric pairs, but not between *R*−*R* or *S*−*S* units in the same plane. These structures clearly reflect how the chiral δ-F bond controls the crystal packing, particularly in guiding the orientation of nearby substituted ethyl and the phenyl substituents of the enantio and diastereomers. In α-fluoroketones/esters, the C−F bond and carbonyl prefer to be in *anti* conformation[22,23] with O−C−C−F dihedral angle of 180°; however, in the present structures, this angle showed considerable departure, with (±) 19° for **13a**, (±) 150° for **13b**, (±) 24° for **13c**, 16° for **17***S* and −22° for **17***R*, clearly reflecting stability due to H-bonded base pairs and the packing of ethyl/phenyl substituents. Another effect of chiral center would be to influence the rotamer population around the tertiary amide bond in solution. Side chain modified fluoro derivatives δ-F-*aeg*-T, δ-F-5FU-*aeg* and δ-F-5CF3U-*aeg* showed ∼69%, ∼68% and ∼70% major rotameric populations respectively (as determined from ^{1}H NMR), whereas unmodified *aeg*-T showed 62% major rotameric population. Thus, introduction of fluorine in side chain of the *aeg* PNA backbone has only marginal effect on rotameric population, although the present results cannot characterize the structure of major rotamer.

As attempts toward resolution of the enantiomers failed, the synthesis of PNA oligomers incorporating various fluorinated units were done using the racemic monomers **9**−**11**. The different fluorinated monomers were incorporated into PNA sequences corresponding to the homo-oligomeric H-TTTTTTTT-Lys and the mixed sequence H-TTACCT-CAGT-Lys. All PNA sequences were synthesized on solid support (MBHA resin) using standard Boc-chemistry. The solid support was first derivatized with L-lysine and the loading value of the resin was adjusted to 0.35 mmol/g. The various PNA sequences were assembled using the unmodified and fluorinated PNA monomers, coupled in sequence at appropriate sites using HBTU/HOBt as coupling reagent in DMF.

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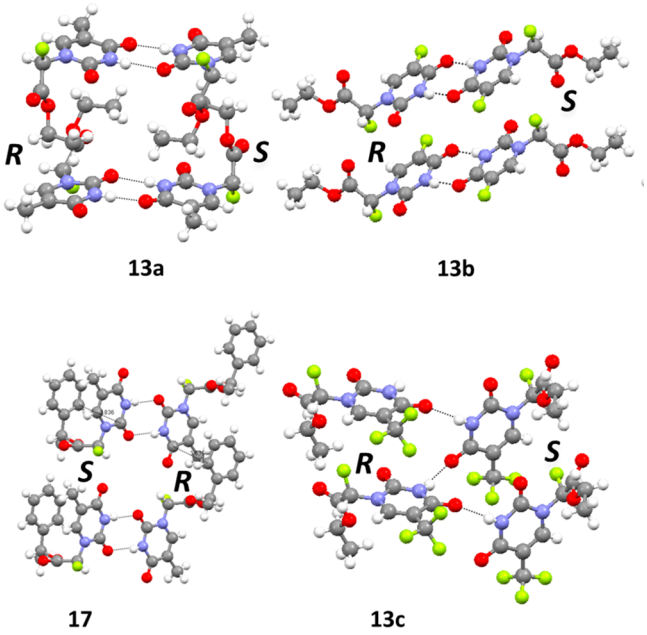

Figure 1. Hydrogen bonding between enantiomers of compounds **13a**, **13b**, **13c** and diastereomers of compound **17**.

After the assembly, the PNAs were cleaved from the resin by reaction with TFMSA/TFA, purified on reverse phase HPLC and characterized using MALDI-TOF mass spectrometry.

The reverse phase HPLC t_R values of fluorinated PNAs (PNA **2**–**5** and PNA **7**) irrespective of the site of fluorination, were always found to be higher than the analogous non-fluorinated PNAs (PNA **1** and PNA **6**), suggesting that fluorination generally enhanced the hydrophobicity of PNAs. The HPLC profiles for various fluorinated and nonfluorinated PNAs run under identical solvent elution conditions on analytical C18 RP column are shown Figure 2 and the t_R values are listed in Table 1. While the homooligomer 5CF_3U-*aeg* (PNA **2**, U^{CF3} with 24×F atoms) had higher t_R (17.7 min) compared to that of nonfluorinated *aeg*-T (**T**, PNA **1**, 10.1 min) with Δt_R of 7.6 min, PNA **3** (T_F) having a total of 8xδ-F substituents, showed Δt_R of 4.1 min compared to nonfluorinated PNA **1**. A Δt_R of 4.3 was seen between fluorinated PNA **5** (U^F_F, 5FU+δ-F, 16×F) compared to PNA **4** (U^F, 5FU, 8×F). These results suggest that t_R or hydrophobicity is also a function of the site of F substitution and C–F α to an electronegative function seems to be more effective in inducing

Table 1. HPLC t_R Values of PNAs 1–7[a]

PNA	H-PNA-Lys	HPLC t_R (min)
PNA 1	TTTTTTTTT	10.1
PNA 2	$U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}$	17.7
PNA 3	$T_FT_FT_FT_FT_FT_FT_FT_FT_F$	14.2
PNA 4	$U^FU^FU^FU^FU^FU^FU^FU^FU^F$	10.0
PNA 5	$U^F_FU^F_FU^F_FU^F_FU^F_FU^F_FU^F_FU^F_FU^F_F$	14.3
PNA 6	$T_{CH2}T_{CH2}T_{CH2}T_{CH2}T_{CH2}T_{CH2}T_{CH2}T_{CH2}T_{CH2}$	11.0
PNA 7	$T_{CF2}T_{CF2}T_{CF2}T_{CF2}T_{CF2}T_{CF2}T_{CF2}T_{CF2}T_{CF2}$	13.4

[a]HPLC profile and MALDI-TOF spectra are given in Supporting Information. U^{CF3}, T_F, U^F and U^F_F are derived from the monomers **11b**, **16a**, **16a** (R = H) and **16b**. T_{CH2} and T_{CF2} correspond to aminopropyl glycyl PNA and its fluorinated analogue,[18] respectively.

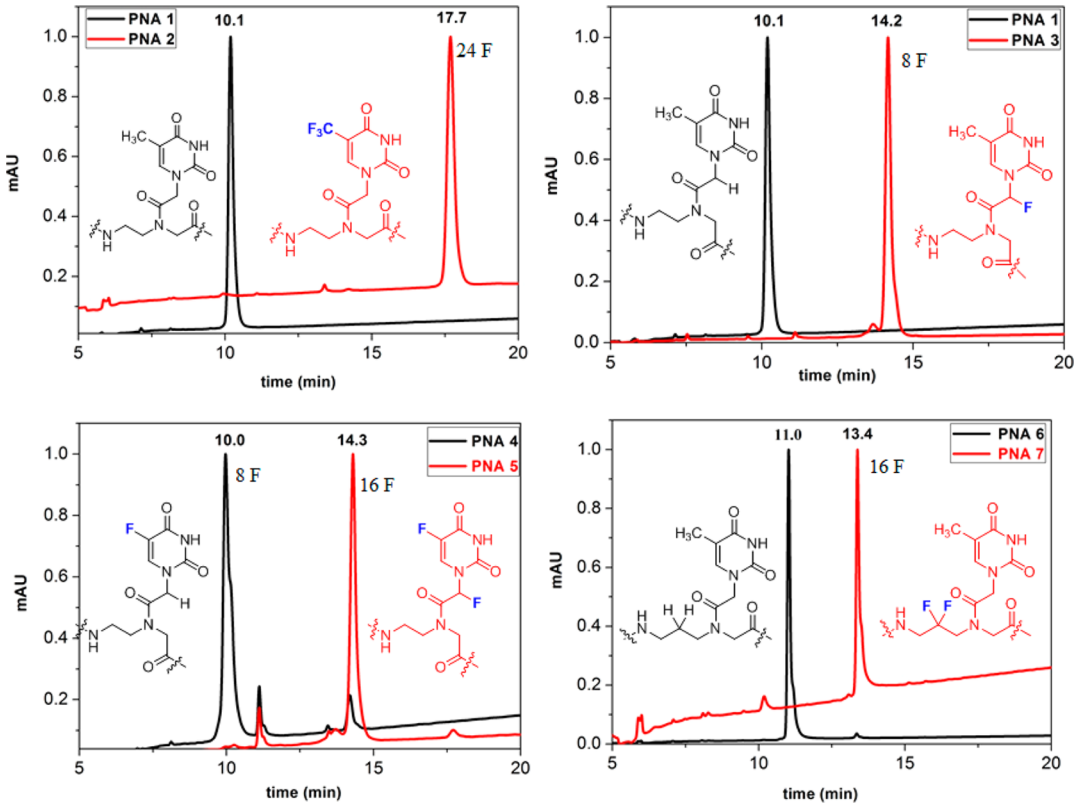

Figure 2. Comparison of HPLC retention times (t_R) of *aeg*-T (T) and δ-F, *aeg*-T (T^F) PNA octamers; *aeg*-T (T) and 5CF_3U-*aeg* (U^{CF3}) PNA octamers; 5FU-*aeg* (U^F) and δ-F, 5FU-*aeg* (U^F_F) PNA octamers and *apg* (T) and γ-CF_2-*apg* (T_{CF2}) PNA octamers.

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net hydrophobicity compared to that of neighboring aliphatic moiety (CO > C=C > CH_2). It may be noted that PNA **1** (C5−CH_3) and PNA **4** (C5−F) have almost similar t_R values (10.1 and 10.0 respectively), indicating that both methyl and fluorine are equally hydrophobic. Since RP HPLC t_R values are a recognized method for hydrophobicity determination[23] the present results relating the number and site of fluorination to HPLC t_R may guide the design of PNAs for better cell permeability.

UV-T_m Data of 5FU-*aeg* Modified PNAs 9−12. The effect of fluorinated side chain and nucleobase on the ability of PNAs to form duplexes with complementary DNA and RNA was studied by temperature dependent UV spectral studies.

5FU-aeg PNA. Incorporation of single 5FU-*aeg* PNA unit at N-terminus (PNA **9**, Table 2) did not affect the stability of duplexes from either cDNA (ΔT_m = 1.1°) or cRNA over that of unmodified control *aeg* PNA **8**. When the substitution was in the middle (PNA **10**, Table 2), slight destabilization was observed for duplexes with both cDNA **1** (ΔT_m of −2.7°) for duplex and cRNA **1** (ΔT_m of −4.2°) compared to that of unmodified *aeg* PNA **8**. Increasing the number of modifications to 2 and 3 as in PNA **11** and PNA **12** (Table 2), showed further decrease in stability of hybrids with ΔT_ms of −4.3° and −3.6 ° for duplexes with cDNA **1** and ΔT_m of −4.8° and −6.6 °C for duplex from cRNA **1** compared to analogous duplexes of unmodified *aeg* PNA **8**. The 5FU-*aeg* PNAs though destabilized the derived duplexes with cDNA **1** and cRNA **1**, the destabilization of duplexes were marginally higher for cRNA **1** than that for cDNA **1**.

Table 2. UV-T_m Values of Fluorinated and Unmodified Mixmer PNAs[a]

		DNA		RNA	
PNA	PNA sequence	DNA **1**	ΔT_m	RNA **1**	ΔT_m
PNA **8**	H-TTACCTCAGT-Lys	48.6	–	60.6	–
PNA **9**	H-T*U*^{F}ACCTCAGT-Lys	49.7	+1.1	60.2	−0.4
PNA **10**	H-TTACC*U*^{F}CAGT-Lys	45.9	−2.7	56.4	−4.2
PNA **11**	H-T*U*^{F}ACC*U*^{F}CAGT-Lys	44.3	−4.3	55.8	−4.8
PNA **12**	H-T*U*^{F}ACC*U*^{F}CAG*U*^{F}-Lys	45.0	−3.6	54.0	−6.6
PNA **13**	H-TU^{CF3}ACCTCAGT-Lys	43.1	−5.5	58.1	−2.5
PNA **14**	H-TTACCU^{CF3}CAGT-Lys	41.2	−7.4	50.2	−10.4
PNA **15**	H-TU^{CF3}ACCU^{CF3}CAGT-Lys	45.9	−2.7	55.9	−4.7
PNA **16**	H-TU^{CF3}ACCU^{CF3}CAGU^{CF3}-Lys	49.4	+0.8	60.0	−0.6

[a]See the Supporting Information for mass spectra. ΔT_m indicates the difference in T_m of duplexes of modified PNA:DNA **1**/RNA **1** and unmodified *aeg* PNA:DNA **1**/RNA **1**, respectively. The values are accurate to ±0.5 °C. DNA **1** = 3′AATGGAGTCA5′, RNA **1** = 3′AAUGGAGUCA5′.

5CF$_3$U-aeg PNA. The PNA **13** (Table 2) with modification at N-terminus destabilized the duplexes with cDNA **1**/cRNA **1** by 5.5 °C/2.5 °C, while PNA **14** with middle modification destabilized the corresponding hybrids with cDNA **1**/cRNA **1** by 7.4/10.4 °C. The PNA **15** (Table 2) with double modifications at N-terminus and in middle destabilized the duplex with cDNA**1**/cRNA **1** by 2.7/4.7 °C and the PNA **16** (Table 2) with triple modifications stabilized the cDNA **1**/cRNA **1** hybrid as compared to that of control unmodified PNA **8**. Overall, the 5CF$_3$U-*aeg* PNAs (**13**-**16**) show binding with their complementary DNA/RNA sequences with slight reduction in T_m compared to unmodified *aeg* PNA **8** and higher T_m for duplexes with cRNA compared to that with cDNA. The destabilization order in case of cDNA is in the order of M < N < Bi < Tri whereas in case of cRNA it is in the order of M < Bi < N < Tri. This is perhaps a consequence of electronic effects of the fluoro groups (C5−F/CF3) present on nucleobase.

The sequence specificity of nucleobase fluorinated PNAs (PNA **9**−**16**) toward their cDNA/RNA sequences were examined by hybridization with single base mismatch sequences (Supporting Information S42). 5FU-*aeg* PNAs (PNA **9**−**12**) showed higher sequence discrimination with mismatch DNA **2**/RNA **2** than that of unmodified *aeg* PNA **8**. 5CF$_3$U-*aeg* PNAs (PNA **13**−**16**) showed higher sequence discrimination for mismatch RNA **2** than that with mismatch DNA **2**.

Cell Uptake Studies of PNAs 17−19. The fluorinated homo-oligomer PNAs (PNAs **2**−**3**) were examined for their cell-uptake ability in HeLa cell line as compared to the control PNA **1**. The correspondingly labeled fluorescent PNAs (PNA **17**, PNA **18** and PNA **19**) were synthesized on solid support by coupling with 5/6-carboxyfluoresecin at the N-terminus of the PNAs, through a lysine unit (Table 3).

Cell permeability and uptake quantification of these PNAs in HeLa cells were done by confocal microscopy and FACS analysis. The cells were incubated with the fluorescently labeled PNAs (PNA **17**-PNA **19**; 2 μM) for 24 h followed by treatment with nuclear stain (Hoechst 33342, blue). The live cell confocal images for the PNA **17**, PNA **18** and PNA **19** in HeLa cells (Figure 3) indicate that all the three PNAs (PNAs **17**−**19**, Table 3) were taken by the HeLa cells and localized in cytoplasm. The presence of PNAs in cytoplasm was seen from the superimposed images of nuclear stain blue dye and PNA green fluorescent images that are shown in Figure 3.

The quantitative estimation of the cell-penetrating abilities of the PNA homooligomers (**17**−**19**) was done by fluorescence activated cell sorter (FACS) analysis (Figure 4). The mean fluorescence intensities were found to be high for both the nonfluorinated PNA **17** (95%) and fluorinated PNA **18** (5F−U, 88%) and more than that of 5-CF$_3$ PNA **19** (52%). The molecular origin of the lower cell uptake of PNA **19** could not be ascertained from the present work with data limited for representative nonchiral homooligomers. More data on mixed sequences consisting of pure enantiomers and toxicity studies are needed for any generalization.

Table 3. PNAs Used for Cell Uptake Studies[a]

PNA	PNA sequence	mol. formula	calcd mass	obsvd mass	HPLC t_R (min)
PNA 17	TTTTTTTTT-Lys-CF	$C_{121}H_{149}N_{37}NaO_{40}$	2783.0660	2783.8934	12.2
PNA 18	$T_FT_FT_FT_FT_FT_FT_FT_F$-Lys-CF	$C_{121}H_{142}F_8N_{37}O_{40}$	2905.0087	2905.0827	14.1
PNA 19	U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}U^{CF3}-Lys-CF	$C_{121}H_{126}F_{24}N_{37}O_{40}$	3192.8580	3192.9503	16.2

[a]CF = Carboxyfluorescein.

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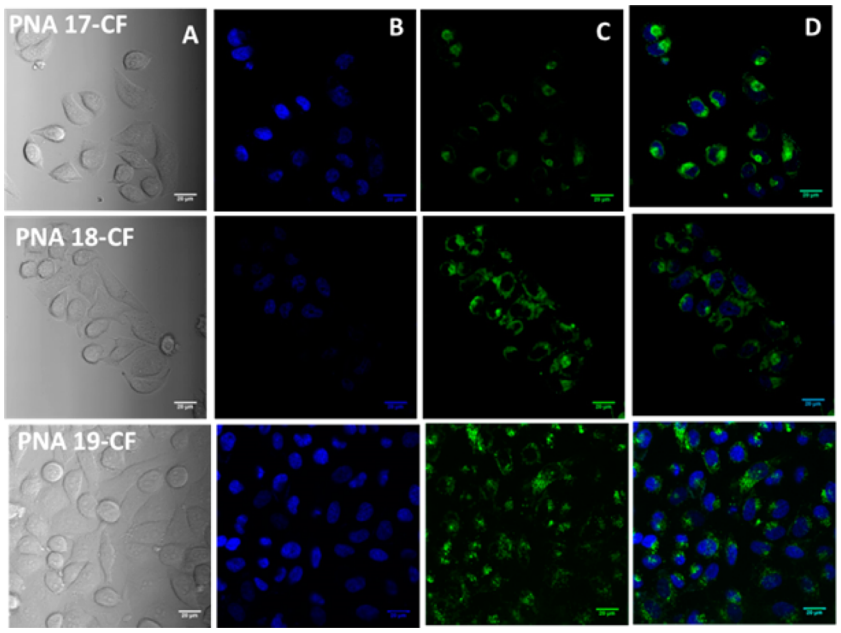

Figure 3. Confocal images of fluorescent PNAs **17**, PNA **18** and PNA **19** in HeLa cells: (A) Bright field image, (B) Nuclear stain from Hoechst 33442, (C) Green fluorescence image from PNA, and (D) Superimposed image of B and C.

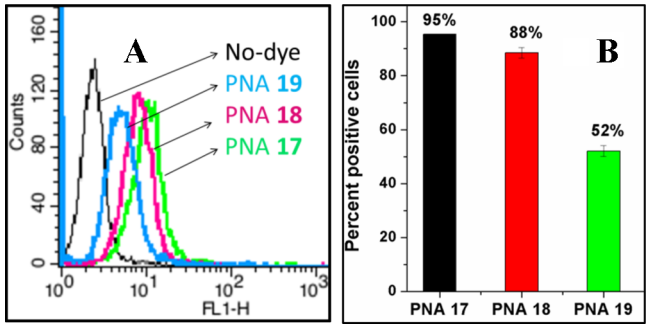

Figure 4. (A) Overlay histogram of mean fluorescence of HeLa cells: (Black) control cells without dye; (Green) PNA **17** and (Red) PNA **18** and (Light blue) PNA **19**. (B) Percent of positive cells in HeLa cells.

CONCLUSION

In continuation of our approaches to access newer PNA analogues, fluorinated in backbone or at the N-terminus, this report describes chemical synthesis of PNA oligomers fluorinated in the side chain. The synthesis of relevant monomers used the racemic fluorinating reagent bromofluoroethyl acetate for N-alkylation of nucleobases that eventually lead to racemic PNA monomers, which could not be resolved. The crystal structures of the racemic monomers indicated specific homo base pairing through H-bonding between the *R* and *S* enantiomers in **13** and continuous single hydrogen bonds in diastereomers **17**, highlighting the role of chiral F in influencing the crystal structure. The reverse phase HPLC of the derived PNA oligomers fluorinated at C5 of nucleobase uracil and side chain indicated that in homooligomeric series the hydrophobic/lipophilic character increased with the number of fluorines. The extent of hydrophobicity also depended on the site of fluorine substitution in the PNA. The nucleobase modified PNAs bind with their cDNA/RNA sequences with sequence specificity, but with slight destabilization of corresponding hybrids. However, 5F−U and 5CF_3-U PNAs exhibited sequence discrimination of mismatched DNA/RNA sequences, with slightly better discrimination of RNA. The fluorinated PNAs exhibited cell uptake as good as nonfluorinated PNAs and this work in conjunction with our previous results[18,19] implies that the fluorination can be a promising strategy to explore PNA analogues as potential new inhibitors of RNA function. Attempts to obtain stereochemically pure fluorinated PNA oligomers employing chirally pure monomers acquired either from the enantiomerically pure reagent or through chromatographic resolution are in progress to access fluorinated PNAs with desirable biochemical and cell penetrating properties.

EXPERIMENTAL PROCEDURES

All reactions were carried out in oven-dried glassware under argon atmosphere. Low temperature experiments were conducted using dry ice acetone combination. All commercial solvents were distilled prior to use and analytical thin layer chromatography (TLC) was performed on precoated 250 um layer thickness silica gel GF254 sheets (Merck 5554) plates. Visualization of compounds on silica plate was performed by UV light and/or staining with ninhydrin solution. ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR were recorded using 400 MHz NMR spectrometer at 400, 100, and 376.6 MHz respectively and chemical shifts were expressed in parts per million (ppm). Hexafluorobenzene was used as internal reference for ^{19}F NMR. High resolution mass spectra for all reaction intermediates were recorded using high definition mass spectrometry. Mass spectra for PNA oligomers were obtained by MALDI-TOF mass spectrometry using DHB as matrix. PNA oligomers were purified by HPLC system using semipreparative C18 (10 × 250 mm) column.

Ethyl 2-(*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-chloroacetamido)acetate (9). To a solution of *N*-Boc-2-amino ethyl glycine ethyl ester (5.0 g, 20.0 mmol) in acetonitrile (10.0 mL), triethylamine (2.8 mL, 20.0 mmol) and chloroacetyl chloride (1.6 mL, 20.0 mmol) were added at 0 °C dropwise, and stirring was continued for 5 h, monitoring the completion of reaction by TLC, after completion of reaction, water was added and extracted with ethyl acetate (3 × 15 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and product was purified by column chromatography, eluting with petroleum ether/EtOAc

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(1:1) to obtain the compound **9** as a sticky oil (Yield 5.79 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (t, 3H), 1.36 (s, 9H), 3.13−3.21 (m, 2H), 3.46 (t, 2H), 3.96 (s, 2H), 4.10−4.15 (m, 4H), 5.51 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.8, 28.1, 38.1, 38.4, 40.5, 40.9, 48.0, 49.3, 50.3, 61.3, 61.8, 79.0, 79.5, 155.8, 167.3, 168.8, 169.3.

Ethyl 2-(*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetate (10a). To a solution of the previously synthesized compound **9** (2.0 g, 6.2 mmol) in DMF (5.0 mL), 5-fluorouracil (0.8 g, 6.2 mmol) and K_2CO_3 (0.9 g, 6.2 mmol) were added at RT and stirring was continued at same temperature for 12 h, monitoring the completion of reaction by TLC, after completion of reaction, water was added and extracted with ethyl acetate (3 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (1:1) to obtain the compound **10a** as a transparent sticky solid (Yield 1.99 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26−1.34 (m, 3H), 1.44 (s, 9H), 3.2−3.33 (m, 2H), 3.52 (t, J = 8 Hz, 2H), 4.06 (s, 2H), 4.18−4.27 (m, 2H), 4.46 (min) 4.60 (maj) (s, 2H), 5.66 (br s, 1H), 7.29−7.34 (m, 1H), 9.69 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −165.85 (s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 28.4, 38.7, 48.3, 48.8, 61.9, 80.2, 129.55 (d, $J_{C-F(allylic)}$ = 36 Hz), 140.4 (d, $J_{C-F(gem)}$ = 237 Hz), 149.8, 156.1, 157.3, 157.5, 167.0, 169.3, 169.7; HRMS (ESI-TOF) m/z Calcd for $C_{17}H_{25}FN_4NaO_7$ $[M + Na]^+$ 439.1605, found 439.1606.

2-(*N*-2((*tert*-Butoxycarbonyl)amino)ethyl)-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (11a). White amorphous solid. To the ester compound **10a** (2.1 g, 5.1 mmol) in THF (5.0 mL), 10% LiOH was added at 0 °C and stirred for 3 h. After completion of reaction, the mixture was acidified with sat. aq.$KHSO_4$ solution. The product was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain the desired monomer **11a** in good yields as a white solid (1.62 g, 82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.35 (maj) 1.37 (min) (s, 9H), 2.99−3.13 (m, 2H), 3.27−3.32 (m, 2H), 3.65 (s, 2H), 4.43 (maj) 4.57 (min) (s, 2H), 7.01 (maj) 7.18 (min) (t, 1H), 7.76−7.85 (m, 1H), 8.42 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −170.72 (s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.7, 38.1, 47.9, 48.5, 52.9, 78.0, 131.4 (d, $J_{C-F(allylic)}$ = 31 Hz), 139.3 (d, J_{C-F} = 227 Hz), 150.6, 156.1, 158.0, 166.7, 167.8, 171.4; HRMS (ESI-TOF) m/z Calcd for $C_{15}H_{21}FN_4NaO_7$ $[M + Na]^+$ 411.1292, found 411.1285.

Synthesis of Ethyl-2-(*N*-(2-((*tert*-butoxycarbonyl)amino)-ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetate (10b). To a solution of the compound **9** (1.0 g, 3.1 mmol) in DMF (5.0 mL), K_2CO_3 (430 mg, 3.10 mmol) and 5-trifluoromethyluracil (560 mg, 3.1 mmol) were added at RT and stirring was continued at same temperature for 12 h, monitoring the completion of reaction by TLC, after completion of reaction, water and aq. $KHSO_4$ were added and extracted with ethyl acetate (3 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and product was purified by column chromatography, eluting with petroleum ether/EtOAc (1:1) to obtain the compound **10b** as a white sticky solid (Yield 1.10 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25−1.33 (m, 3H), 1.43 (s, 9H), 3.25−3.34 (m, 2H), 3.52 (m, 2H), 4.07 (s, 2H), 4.17−4.27 (m, 2H), 4.56 (min) 4.71 (maj)(s, 2H), 5.11(min) 5.61 (maj) (br d, 1H), 7.68−7.73 (m, 1H), 9.78 (maj) 9.85 (min) (br s,1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −64.60 (s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 28.4, 29.7, 38.7, 48.8, 61.9, 62.5, 80.2, 105.13 (q, $J_{C-F(allylic)}$ = 32 Hz) 120.5, 123.2, 146.3, 150.1, 156.3, 159.2, 162.9, 166.8, 167.2, 169.5; HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{25}F_3N_4NaO_7$ $[M + Na]^+$ 489.1573, found 489.1571.

Synthesis of 2-(*N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)-acetamido)acetic acid (11b). White amorphous solid. The ester compound **10b** (1.0 g, 2.1 mmol) in THF was saponified with 10% LiOH at 0 °C for 3 h. After completion of reaction, the mixture was acidified with sat. aq. $KHSO_4$ solution and then extracted with ethyl acetate, dried over Na_2SO_4 and evaporated under reduced pressure to obtain the desired monomer **11b** in good yields as a white solid (Yield 0.75 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.31(min) 1.33 (maj) (s, 9H), 2.96−3.15 (m, 2H), 3.25−3.35 (m, 2H), 3.93 (maj) 4.04 (min) (s, 2H), 4.58 (min) 4.76 (maj) (s, 2H), 6.73 (min) 6.89 (maj) (br, 1H), 8.18 (min) 8.24 (maj) (s, 1H), 11.81 (br s,1H); ^{19}F NMR (376.6 MHz, DMSO-d_6, C_6F_6 as internal reference) δ −61.95 (s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.6, 38.5, 47.3, 48.3, 48.8, 78.2, 78.5, 99.9, 121.5, 121.8, 124.5, 148.5, 150.6, 156.1, 156.2, 159.8, 167.1, 167.6, 170.9; HRMS (ESI-TOF) m/z Calcd for $C_{16}H_{21}F_3N_4NaO_7$ $[M + Na]^+$ 461.1260, found 461.1264.

Ethyl 2-fluoro-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetate (13a). To a solution of thymine **12a** (2.0 g, 15.90 mmol) and K_2CO_3 (2.2 g, 15.9 mmol) in anhydrous DMF (10.0 mL), ethyl bromofluoroacetate (1.9 mL, 15.9 mmol) was added at RT and the stirring was continued for 12 h at the same temperature. Yellow colored precipitate was observed, after completion of reaction, (monitored by TLC) water was added to the reaction mixture and extracted with ethyl acetate (3 × 30 mL). The organic layer was washed with brine solution and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the product was purified by column chromatography, eluting with EtOAc/petroleum ether (30:70) to afford the compound **13a** as sticky oil (Yield 1.97 g, 54%). IR (neat) 3025, 3005, 1731, 1453, 1371, 1279, 1225, 1067, 775 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (t, J = 8 Hz, 3H), 1.96 (s, 3H), 4.36 (q, J = 8 Hz, 2H), 6.64 (d, J = 48 Hz, 1H), 7.11 (s, 1H), 9.23 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −157.61 (d, J = 48.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.4, 14.0, 63.5, 87.3 (d, $J_{C-F(gem)}$ = 215 Hz), 113.2, 135.3, 150.2, 163.8, 163.9, 64.3; HRMS (ESI-TOF) m/z Calcd for $C_9H_{12}FN_2O_4$ $[M + H]^+$ 231.0781, found 231.0780.

2-Fluoro-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetic acid (14a). To a solution of the compound **13a** (2.0 g, 8.7 mmol) in THF (10.0 mL), 2.5 M KOH (1.0 mL, 7.4 mmol) was added at −78 °C. After 5−10 min stirring, reaction mixture was warmed to room temperature and monitored by TLC. After completion of reaction, proton exchange resin Dowex-H+ was added in order to acidify the reaction mixture. Then the acid compound was filtered, and the solvent was removed under a vacuum to obtain the compound **14a** as a white solid (Yield 1.40 g, 80%). IR (neat) 3019, 2969, 1738, 1663, 1435, 1369, 1221, 1098 cm^{-1}; ^{1}H NMR (400 MHz, D_2O) δ 1.57 (s, 3H), 6.13 (d, J = 52 Hz, 1H), 7.15 (s, 1H), 11.04 (br s, 1H); ^{19}F NMR (376.6 MHz, D_2O, C_6F_6 as internal reference) δ −156.3 (d, J = 60.2 Hz); ^{13}C NMR (100 MHz, D_2O) δ 11.3, 90.5 (d, $J_{C-F(gem)}$ = 217 Hz), 112.2, 139.4, 151.5, 166.5, 169.2; HRMS (ESI-TOF) m/z Calcd for $C_7H_8FN_2O_4$ 203.0467 $[M + H]^+$, found 203.0468.

Ethyl 2-(*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-fluoro-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-acetamido)acetate (15a). To the acid compound **14a** (1.4 g, 6.9 mmol) in anhydrous DMF (4.0 mL), aminoethylglycine ethylester (1.4 g, 6.9 mmol) was added and cooled to 0 °C. After 5 min stirring, HBTU (2.9 g, 7.6 mmol) and DIPEA (3.6 mL, 20.8 mmol) were added to the reaction mixture and stirred for 12 h at RT. After completion of reaction, the reaction mixture was neutralized with sat. aq. $KHSO_4$, and extracted with ethyl acetate (3 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (50:50) to afford the compound **15a** as sticky solid (Yield 1.48 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25−1.31 (m, 3H), 1.42 (s, 9H), 1.94 (maj) 1.95 (min) (s, 3H), 3.29−3.34 (comp, 2H), 3.42−3.67 (comp, 2H), 4.10−4.25 (comp, 4H), 4.94 (min.) 5.29 (maj) (br s, 1H), 6.97 (min) 7.17 (maj) (d, 1H), 7.32 (min) 7.37 (maj) (s, 1H), 9.16 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ (−155.59)- (−152.67) (m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.5, 14.1, 28.3, 29.7, 38.5, 48.9, 61.9, 80.1 (d, $J_{C-F(gem)}$ = 209 Hz), 112.7, 135.5,150.1, 156.1, 163.2, 164.3, 168.6, 168.8; HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{27}FN_4NaO_7$ $[M + Na]^+$ 453.1761, found 453.1766.

2-(*N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)-2-fluoro-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)-

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acetic acid (16a). White amorphous solid. To the ester compound **15a** (1.5 g, 3.5 mmol) in THF (5.0 mL), 10% LiOH was added at 0 °C and stirred for 3 h. After completion of reaction, the mixture was acidified with sat. aq.$KHSO_4$ solution. The product was extracted with ethyl acetate and dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure to obtain the desired monomer **16a** in good yields as white solid (Yield 1.15 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (s, 9H), 1.83 (s, 3H), 2.62−2.78 (m, 2H), 2.85−2.96 (m, 2H), 3.51−3.64 (m, 2H), 4.93 (min) 5.05 (maj) (br s, 1H), 6.31 (min) 6.46 (maj) (d, 1H, J = 44 Hz), 6.71 (min) 6.77 (maj) (s, 1H), 9.21 (br s, 1H); ^{13}C NMR (100 MHz, ACN-d_3) δ 12.2, 28.3, 31.4, 38.4, 49.1, 79.7, 81.3, 86.6, (d, $J_{C-F(gem)}$ = 129 Hz), 113.4, 135.8, 151.2, 156.9, 164.5, 170.7; HRMS (ESI-TOF) m/z Calcd for $C_{16}H_{23}FN_4NaO_7$ $[M + Na]^+$ 425.1448, found 425.1436.

Ethyl 2-fluoro-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetate (13b). To a solution of 5-fluorouracil **12b** (2.0 g, 15.4 mmol) in anhydrous DMF (10.0 mL), K_2CO_3 (2.2 g, 15.4 mmol) and ethyl bromofluoroacetate (1.9 mL, 15.4 mmol) were added at RT and stirring was continued at RT for 12 h, pink colored precipitate was observed. After completion of reaction, water and aq. $KHSO_4$ (10 mL) were added and extracted with ethyl acetate (3 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (70:30) to obtain the compound **13b** as sticky oil (Yield 1.98 g, 55%). IR (neat) 3083, 3020, 1735, 1462, 1370, 1223, 1162, 1070 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (t, J = 8 Hz, 3H), 4.36−4.42 (m, 2H), 6.67 (d, J = 48 Hz, 1H), 7.425 (d, J = 4 Hz, 1H), 9.50 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −158.63 (d, J = 45.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.0, 63.9, 87.34 (d, $J_{C-F(gem)}$ = 217 Hz), 123.9 (d, $J_{C-F(allylic)}$ = 34 Hz), 141.4 (d, $J_{C-F(gem)}$ = 242 Hz), 148.6, 156.2, 156.5, 163.4, 163.7; HRMS (ESI-TOF) m/z Calcd for $C_8H_9F_2N_2O_4$ $[M + H]^+$ 235.0530, found 235.0527.

2-Fluoro-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetic acid (14b). To a solution of compound **13b** (1.9 g, 8.4 mmol) in THF (10.0 mL,) 2.5 M KOH (1.0 mL, 16.9 mmol) was added at −78 °C. After 5−10 min stirring, reaction was brought to room temperature slowly and monitored by TLC. After completion of reaction, proton exchange resin Dowex-H+ was added in order to acidify the reaction mixture. The acid product was filtered and solvent was removed under a vacuum to obtain the compound **14b** as a white solid (Yield 1.47 g, 85%). IR (neat) 3020, 2970, 1737, 1434, 1370, 1222 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.40 (d, J = 44 Hz, 1H), 8.25 (d, J = 4 Hz, 1H), 12.20 (br s, 1H); ^{19}F NMR (DMSO-d_6, C_6F_6 as internal reference 376.6 MHz) δ −154.57 (d, J = 12 Hz), −167.11; ^{13}C NMR (100 MHz, DMSO-d_6) δ 90.2 (d, $J_{C-F(gem)}$ = 215 Hz), 127.9 ((d, $J_{C-F\ (allylic)}$ = 35 Hz),140.2 (d, $J_{C-F(gem)}$ = 232 Hz), 148.87, 157.2, 157.4, 164.9, 165.2; HRMS (ESI-TOF) m/z Calcd $C_6H_5F_2N_2O_4$ $[M + H]^+$ 207.0217, found 207.0212.

Ethyl-2-(*N*-(2-((*tert*-butoxycarbonyl)amino)ethyl)-2-fluoro-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)-acetate (15b). To the acid compound **14b** (1.5 g, 7.0 mmol) in anhydrous DMF (4.0 mL), aminoethylglycine ethylester (1.7 g, 7.0 mmol) was added and cooled to 0 °C. After 5 min stirring, HBTU (2.7 g, 7.0 mmol) and DIPEA (3.0 mL, 17.6 mmol) were added to the reaction mixture and stirred for 12 h at RT. After completion of reaction, the reaction mixture was neutralized with sat. aq. $KHSO_4$ and extracted with ethyl acetate (3 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (50:50) to obtain the compound **15b** as a transparent sticky solid (Yield 1.52 g, 50%). IR (neat) 3007, 2970, 1739, 1444, 1369, 1221 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23−1.28 (m, 3H), 1.39 (s, 9H), 3.29−3.65 (comp, 4H), 4.05−4.24 (comp, 4H), 5.15 (min) 5.46 (maj) (br s, 1H), 7.01 (min) 7.16 (maj) (d, 1H), 7.62 (min) 7.64 (maj) (d, J = 8 Hz, 1H), 10.29 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −163.33 (min) −163.45 (maj) (s), −155.68 (d, J = 45.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 28.2, 38.4, 48.9, 62.0, 80.2, 85.0 (maj) 86.0 (min) (d, $J_{C-F(gem)}$ = 210 Hz), 124.4 (d, $J_{C-F(allylic)}$ = 35 Hz), 140.9 (d, $J_{C-F(gem)}$ = 239 Hz), 148.9, 156.3, 156.8, 157.1, 163.6, 163.9, 168.7; HRMS (ESI-TOF) m/z Calcd for $C_{17}H_{24}F_2N_4NaO_7$ $[M + Na]^+$ 457.1511, found 457.1506.

2-(*N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)-2-fluoro-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamido)acetic acid (16b). White amorphous solid. To the ester compound **15b** (1.5 g, 3.5 mmol) in THF (5.0 mL), 10% LiOH was added at 0 °C and stirred for 3 h. After completion of reaction, the mixture was acidified with sat. aq. $KHSO_4$. The product was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain the desired monomer **16b** as a white solid (Yield 1.14 g, 80%). IR (neat) 3742, 3676, 3647, 3617, 2360, 2325, 1716, 1515, 1459, 1422, 1370, 1256, 1223, 1170 cm^{-1}; ^{1}H NMR (400 MHz, ACN-d_3) δ 1.38 (s, 9H), 3.21−3.47 (m, 4H), 3.94−4.17 (m, 2H), 5.39 (min) 5.53 (maj) (br s, 1H), 6.93 (min) 7.09 (maj) (d, J = 48 Hz, 1H), 7.63 (br s, 1H), 9.72 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −150.33 (maj) −151.37 (min) (d, J = 45.2 Hz), −161.36 (min) 161.70 (maj) (s); HRMS (ESI-TOF) m/z Cacld for $C_{15}H_{20}F_2N_4NaO_7$ $[M + Na]^+$ 429.1197, found 429.1198.

Synthesis of Ethyl 2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)-2-fluoroacetate (13c). To the solution of 5-trifluoromethyluracil **12c** (500 mg, 2.8 mmol) in anhydrous DMF (2.0 mL), K_2CO_3 (380 mg, 2.8 mmol) and ethyl bromofluoroacetate (0.3 mL, 2.8 mmol) were added at RT and stirring was continued at RT for 12 h, pink colored precipitate was observed. After completion of reaction, water and aq. $KHSO_4$ (10 mL) were added and extracted with ethyl acetate (3 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (70:30) to obtain the compound **13c** as a sticky oil (Yield 0. 47 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (t, J = 8 Hz, 3H), 4.41 (q, J = 4 Hz, 2H), 6.65 (d, J = 44 Hz, 1H), 7.84 (s, 1H), 9.17 (br s, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −158.26 (d, J = 4.5 Hz), −65.12 (s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.9, 64.1, 87.6 (d, $J_{C-F(gem)}$ = 220 Hz), 108.8 (q, $J_{C-F(allylic)}$ = 34 Hz), 120.99 (q, $J_{C-F(gem)}$ = 269 Hz), 141.1 148.4, 157.5, 163.0, 163.3; HRMS (ESI-TOF) m/z Cacld for $C_9H_8F_4N_2NaO_4$ $[M + Na]^+$ 307.0318, found 307.0320.

Synthesis of 2-(2,4-Dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)-2-fluoroacetic acid (14c). To a solution of compound **13c** (470 mg, 1.6 mmol) in THF (2.0 mL), 2.5 M KOH (1.0 mL, 10.0 mmol) was added at −78 °C. After 5−10 min stirring, reaction was brought to room temperature slowly and monitored by TLC. After completion of reaction, proton exchange resin Dowex-H+ was added in order to acidify the reaction mixture. The acid product was filtered and solvent was removed under a vacuum to obtain the compound **14c** as a white solid (Yield 0. 38 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.42 (d, J = 48 Hz, 1H), 8.54 (s, 1H), 12.24 (s, 1H); ^{19}F NMR (376.6 MHz, DMSO-d_6, C_6F_6 as internal reference) δ −155.0 (d, J = 45.2), −62.72(s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 91.6(d, $J_{C-F(gem)}$ = 218 Hz), 104.9, 121.4, 147.0, 149.4, 159.5, 165.2, 165.5; HRMS (ESI-TOF) m/z Cacld for $C_7H_5F_4N_2O_4$ $[M + H]^+$ 257.0185, found 257.0204.

Synthesis of ethyl-2-(*N*-(2-((*tert*-Butoxycarbonyl)amino)-ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)-2-fluoroacetamido)acetate (15c). To the acid compound **14c** (300 mg, 1.17 mmol) in anhydrous DMF (2.5 mL), aminoethylglycine ethylester (280 mg, 1.17 mmol) was added and cooled to 0 °C. After 5 min stirring, HBTU (440 mg, 1.17 mmol) and DIPEA (0.60 mL, 3.5 mmol) were added to the reaction mixture and stirred for 12 h at RT. After completion of reaction, reaction mixture was neutralized with sat. aq. $KHSO_4$ and extracted with ethyl acetate (3 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (1:1) to obtain the compound **15c** as a transparent sticky solid (Yield 0.32 g, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (t, 3H), 1.40 (s, 9H), 3.26−3.57 (m, 4H), 4.04 (min) 4.11(maj) (s, 2H), 4.18−4.28 (m, 2H), 5.13 (min) 5.48 (maj) (br s, 1H), 7.05 (min) 7.23 (maj) (d, J = 44 Hz, 1H), 7.37 (q, J = 4 Hz, 1H), 8.03 (br s, 1H); ^{19}F NMR

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(376.6 MHz, $CDCl_3$, C_6F_6 as internal reference) δ −155.6 (d, J = 45.2), −62.4 (s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.0, 28.3, 38.7, 49.1, 62.1, 62.5, 80.3, 85.1 (maj) 86.3 (min) (d, $J_{C-F(gem)}$ = 211 Hz), 106.9 (q, $J_{C-F(allylic)}$ = 34 Hz), 141.7, 149.1, 156.4, 158.3, 163.4, 163.7, 168.7; HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{24}F_4N_4NaO_7$ $[M + Na]^+$ 507.1478, found 507.1479.

Synthesis of 2-(*N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)-2-(2,4-dioxo-5-(trifluoromethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)-2-fluoroacetamido)acetic acid (16c). To the ester compound **15c** (300 mg, 0.62 mmol) in THF (5.0 mL), 10% LiOH was added at 0 °C and stirred for 3 h. After completion of reaction, the mixture was acidified with sat. aq. $KHSO_4$ solution and product was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain the desired monomer **16c** as a white solid (Yield 0.23 g, 81%). ^{1}H NMR (400 MHz, MeOH-d_4) δ 1.40 (s, 9H), 3.24−3.27 (m, 2H), 3.45−3.60 (m, 2H), 4.03 (min.) 4.07 (br s, 1H), 4.22−4.35 (m, 2H), 7.15 (min) 7.27 (maj) (d, J = 44 Hz, 1H) 8.15 (s, 1H); ^{19}F NMR (376.6 MHz, DMSO-d_6, C_6F_6 as internal reference) δ −155.46 (d, J = 45.2 Hz), −62.44 (s); ^{13}C NMR (100 MHz, MeOH-d_4) δ 27.1, 29.8, 37.3, 37.9, 79.3, 85.9 (d, $J_{C-F(gem)}$ = 110 Hz), 106.2 (d, $J_{C-F(allylic)}$ = 33 Hz), 123.2, 141.9 (maj) 142.0 (min), 149.2, 157.0, 157.1, 158.9, 164.1,170.5; HRMS (ESI-TOF) m/z Calcd for $C_{16}H_{20}F_4N_4NaO_7$ $[M + Na]^+$ 479.1166, found 479.1173.

(*S*)-1-Phenylethyl 2-fluoro-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetate (17). To a solution of acid compound **14a** (300 mg, 1.5 mmol) and (S)-1-phenylethanol (0.4 mL, 3.0 mmol) in anhydrous DMF (10.0 mL), DCC predissolved in DMF (369 mg, 1.8 mmol) was added at RT and stirring was continued at RT for 12 h. After completion of reaction, DCU was filtered and then water was added to the filtrate and extracted with ethyl acetate (3 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with petroleum ether/EtOAc (1:9) to obtain the diastereomeric mixture compound **17** as a sticky solid (Yield 0.25 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.63 (t, J = 8 Hz, 3H), 1.80−1.90 (m, 3H), 6.01−6.08 (m, J = 8 Hz, 1H), 6.61−6.77(m, J = 16 Hz, 1H), 6.69 (dd, 1H), 7.31−7.40 (m, 5H); 9.11−9.15 (bd, 1H); ^{19}F NMR (376.6 MHz, $CDCl_3$) δ −155.99 (d, J = 49 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.3, 12.4, 21.6, 21.7, 76.2, 86.67 (d, $J_{C-F(gem)}$ = 217 Hz), 87.07 (d, $J_{C-F(gem)}$ = 217 Hz), 113.0, 113.1, 126.3, 128.7, 134.9, 135.0, 139.7, 149.9, 163.1, 163.5; HRMS (ESI-TOF) m/z Calcd for $C_{15}H_{15}FN_2NaO_4$ $[M + Na]^+$ 329.0914, found 329.0921.

Ethyl 2-fluoro-2-(5-methyl-2,4-dioxo-3-((*R*)-1-phenylethyl)-3,4-dihydropyrimidin-1(2*H*)-yl)acetate (18). To a solution of *S*-phenyl alcohol (0.2 mL, 1.7 mmol) in DMF, DIAD (0.3 mL, 1.7 mmol) and PPh_3 (456 mg, 1.7 mmol) were added at ice-cold condition and stirred for 15 min, after the formation of active intermediate, compound **13a** (200 mg, 0.9 mmol) and K_2CO_3 (120 mg, 0.9 mmol) were added at the same temperature, and stirring was continued at RT for 12 h. After completion of reaction (monitored by TLC), water was added to the reaction mixture and the product was extracted with ethyl acetate (3 × 10 mL). The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and product was purified by column chromatography, eluting with EtOAc/petroleum ether (15:85) to obtain the diastereomeric mixture compound **18** as a sticky solid (Yield 0.13 g, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30−1.34 (m, 3H) 1.82 (dd, J = 8 Hz, 2H), 1.94 (d, J = 4 Hz, 1H), 4.26−4.36 (m, 2H), 6.25−6.29 (m, 1H), 6.49 (dd, 1H), 7.07 (s, 1H), 7.23−7.43 (m, 5H); ^{19}F NMR (376.6 MHz, $CDCl_3$) δ −155.69 (dd, J = 45.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.3, 13.9, 14.0, 51.3, 51.4, 63.3, 87.2, 89.4, 89.6, 112.4, 112.5, 127.3, 127.4, 128.1, 128.7, 133.7, 133.9, 139.7, 163.0, 163.9, 164.3; HRMS (ESI-TOF) m/z Calcd for $C_{17}H_{19}FN_2NaO_4$ $[M + Na]^+$ 357.1227, found 357.1229.

UV−T_m Measurements. The samples for T_m measurements were prepared by mixing PNA and cDNA/RNA (4 μM each, 1:1) in phosphate buffer (10 mM, pH 7.2) containing NaCl (10 mM), annealed at 90 °C for 3 min and slow cooling to RT. The UV absorbance at 260 nm was recorded with a heating rate of 1.0 °C/min temperature increment from 16 to 85 °C. The normalized absorbance at 260 nm was plotted as a function of the temperature and the T_m was determined from the first derivative plots with respect to temperature and is accurate to ±0.5 °C. The concentration of DNA, RNA and PNA were calculated with the help of extinction coefficients of nucleobases (A = 15.4, T = 8.8, C = 7.3 and G = 11.7).

Circular Dichroism. CD spectra of the PNA:DNA and PNA:RNA duplexes (4 μM) and the relevant single strands were recorded in sodium phosphate buffer (10 mM, pH 7.2) with NaCl (10 mM) at 20 °C. The CD spectra were recorded from 300 to 190 nm by addition of 5 scans, with a resolution of 0.1 nm, bandwidth of 1.0 nm, sensitivity of 2 mdeg, response of 2 s and a scan speed of 50 nm/min.

Confocal Microscopy. NIH 3T3 or HeLa cells were plated in 8-well chambered cover glass in 200 μL Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) at the concentration of 1.5 × 10^4 to 2 × 10^4 cells per well. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 for 12 h. The 5(6)-carboxy fluorescein tagged PNAs (2 μM) were added to the corresponding and the cells were incubated for 24 h at 37 °C in a atmosphere of 5% CO_2. They were washed thrice with ice-cold PBS and replenished with DMEM (200 μL) medium containing Hoechst 33342 and ER-red (1 μM each) and incubated at 37 °C for 30 min. The excess nuclear stain Hoechst and ER-red were washed with cold PBS, fresh OPTIMEM medium was added and the cells were immediately visualized on 60× objective of Zeiss LSM 710 laser scanning confocal microscope using 405 and 488 nm filters for Hoechst and carboxy fluorescein, respectively.

FACS Sample Preparation. NIH 3T3 or HeLa cells were plated in 9 × 60 mm dishes in DMEM medium (2.0 mL) at a concentration of 2 × 10^6 cells per dish. The 5(6)-carboxyfluorescein tagged PNA stock solutions were added to the corresponding wells to achieve the final concentration of 1 μM. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was aspirated, cells were washed thrice with ice-cold PBS and collected by trypsinization using 0.5 mL of 0.05% trypsin-EDTA for each dish. DMEM medium (2 mL) was added and the cells were centrifuged for 5 min at 4 °C, and washed thrice with ice-cold PBS. To the cell suspension 200 μL of 1% PFA was added and incubated on ice for 10 min for fixation of the cells. The cell suspension was washed with ice cold PBS for 5 min at 4 °C, filtered through 70 μm cell strainers and analyzed on FACS Calibur flow cytometer. The data obtained from FACS were processed using CellQuest Pro software. The cell permeation of PNA oligomers has been confirmed by histograms obtained from the experiment. The percent positive cells for each individual PNA were plotted on Microcal Origin 8.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01009.

Crystal data (ZIP)

^{1}H, ^{13}C and ^{19}F NMR and mass spectra of compounds **9**−**20**; crystal structures of compounds **13a**−**c** and **17**; HPLC and MALDI-TOF of PNA **1**−**19**; CD spectra and UV−melting profiles of modified PNA:DNA/RNA duplexes (PDF)

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Notes

The authors declare no competing financial interest.

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