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Synthesis of Fluorine-Labeled Peptide Nucleic Acid Building Blocks as Sensors for the ^{19}F NMR Spectroscopic Detection of Different Hybridization Modes

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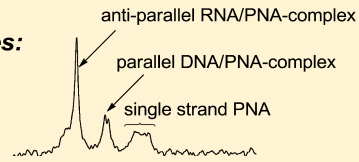
Supporting Information

ABSTRACT: Peptide nucleic acid (PNA) building blocks, bearing a fluorine sensor at C-5 of the uracil base [viz. trifluoromethyl and 3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl], were synthesized and incorporated to a PNA strand, and their applicability for the monitoring of different hybridization modes by ^{19}F NMR spectroscopy was studied. Both sensors gave unique ^{19}F resonance shifts in NMR when the PNA was targeted to a complementary antiparallel DNA, antiparallel RNA, parallel DNA, and parallel RNA. The 5-trifluoromethyl-derived sensor was additionally applied for the monitoring of interconversions from a parallel DNA/PNA complex to an antiparallel RNA/PNA complex and from a PNA/PNA complex to two DNA/PNA complexes (i.e., double-duplex invasion).



INTRODUCTION

^{19}F is a NMR-sensitive nucleus (83% of the ^{1}H) with a wide chemical shift dispersion and the shift responds readily to changes of local van der Waals interactions and electrostatic fields.[1,2] These characteristics make ^{19}F NMR spectroscopy useful for the monitoring of biologically important events, since even subtle conformational changes on biomacromolecules can be detected by a simple one-dimensional NMR technique without background interference.[3,4] ^{19}F NMR spectroscopy has successfully been applied in protein chemistry, for example, to the monitoring of folding/unfolding equilibrium,[5,6] protein/protein,[7] protein/ligand,[8,9] peptide/lipid bilayer,[10−12] and peptide/cell membrane[13] interactions ,and the same practice has been adopted for DNA/RNA chemistry for the detection of related processes.[14−29] Among these studies, ^{19}F NMR spectrocopy has proven to be useful especially for the detection of complex and dynamic equilibria, in which double helices are interconverted or converted to higher order hybridized structures. For example, hairpin/hairpin and duplex/hairpin equilibrium of self-complementary RNAs,[16,20] invasion of 2′-*O*-methyl oligoribonucleotides to a hairpin-type RNA-model,[22,25] and DNA-duplex/triplex equilibrium[24] have been studied.

Peptide nucleic acid (PNA)[30] is another interesting target to be studied by ^{19}F NMR spectroscopy, since its action is frequently involved in complex equilibria of hybridization. For example, PNA sequences rich in pyrimidines may bind to a complementary DNA oligonucleotide forming a $(PNA)_2$DNA triplex and/or by strand invasion resulting a P-loop complex.[9,31−33] Due to the achirality of unorganized PNA, competitive binding between complementary parallel and antiparallel DNA sequences may also take place in case these two optional regions are available. Among the alternative invasion mechanisms, the so-called double-duplex invasion is an additional binding mode in which a PNA/PNA duplex invades to a DNA/DNA duplex.[34] Identification of these processes are complex, but crucial for understanding the mechanism of PNA-based drug candidates or artificial restriction cutters, for which ^{19}F NMR may offer a real time insight. The present study describes the preparation of two ^{19}F-labeled PNA-building blocks (**1** and **2**), which are compatible with the solid-phase peptide synthesis using the Fmoc chemistry. Once incorporated into a PNA sequence, behavior of these sensors in different binding modes has been studied.

RESULTS AND DISCUSSION

Fluorine labeled PNA building blocks **1** and **2** were synthesized according to Scheme 1. Alkylation of 5-(trifluoromethyl)uracil (**3**) with *tert*-butyl bromoacetate gave the desired (uracil-N^1-yl)acetate **4**. The *tert*-butyl group of **4** was removed by TFA and the exposed carboxylate **5** was coupled to Fmoc/*t*-Bu-protected aminoethylglycinate using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The *tert*-butyl group of **6** was removed by TFA, which gave the CF_3 labeled PNA-building block **1** in 47% overall yield from **3**. 5-Iodoracil (**7**) was alkylated with *tert*-butyl bromoacetate as described for **3** above and the remained imide N^3 was subsequently protected by a PMB group (**8**).[35] Sonogashira coupling[36] between **8** and 3,3-bis(trifluoromethyl)-4,4,4-trifluoro-1-butyne[21] was found to be slow (10 days) and required gradual addition of the Pd^0 catalyst but finally gave **9** in an acceptable 59% yield. Progress of the reaction was

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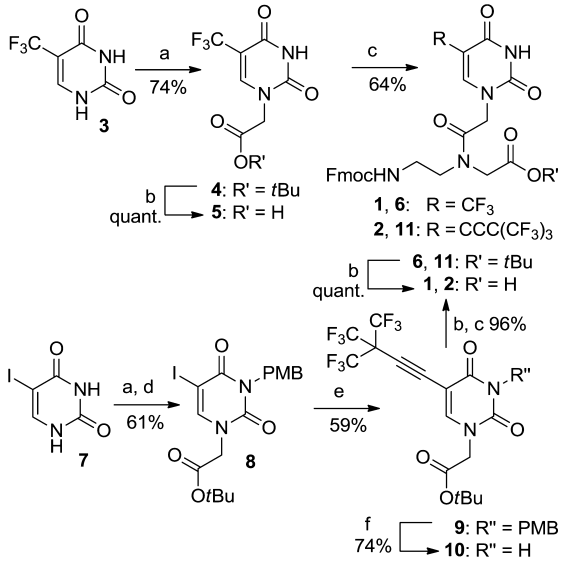
Scheme 1[a]



[a]Conditions: (a) $BrCH_2COO$-*t*-Bu, K_2CO_3, DMF; (b) TFA, CH_2Cl_2; (c) *N*-(FmocNHCH$_2$CH$_2$)GlyO-*t*-Bu, HBTU, DIEA, DMF; (d) PMBCl, NaH, DMF; (e) $HCCC(CF_3)_3$, CuI, $(Ph_3)_4Pd^0$, DIEA, DMF; (f) CAN, aq DMF.

followed by RP HPLC. The reaction time may be reduced by using an excess of the alkyne,[21] but it should be noted that use of only 1 equiv of expensive bis(trifluoromethyl)-4,4,4-trifluoro-1-butyne is appropriate in this case. The slow reaction rate (side reactions additionally observed without PMB protection) was related to the *tert*-butyl acetate at N^1 of uracil, since the corresponding reaction to 5-iodoridine has been reported to be nearly quantitative.[21] The PMB protection of **9** was removed by ceric ammonium nitrate, and the synthesis for **2** from **10** was then carried out as described for **1** from **4** above. The 3,3-bis(trifluoromethyl)-4,4,4-trifluoro-1-but-1-ynyl-labeled PNA building block **2** was obtained in 26% overall yield from **7**.

The building blocks (**1** and **2**) were incorporated into a model PNA sequence [H-Lys-GCACCXGTCC-Lys-NH_2,[37] X = residue of **1** (F_3-PNA) or **2** (F_9-PNA), Table 1] using automated solid-supported synthesis following the Fmoc chemistry on a 10 μmol scale. For each coupling, 5.0 equiv of building block (0.25 mol L^{-1} predissolved in NMP), 5.0 equiv of HBTU, and 10 equiv of DIEA were used (30 min in at rt) followed by a capping step with an acetic anhydride treatment (Ac_2O/pyridine/NMP, 1:25:25, v/v/v, 1 min at rt). After couplings of **1** and **2**, aliquots of the solid-supported PNA sequences were released and analyzed by RP HPLC. With this routine Fmoc cycle, coupling efficiencies for **1** and **2** were ca. 90%. The chain elongation was continued without further optimization. In order to minimize the risk for contamination by deleted sequences, the PNAs were released and purified as Fmoc-protected form. The Fmoc group was removed in solution, followed by a rough RP HPLC purification, to give the desired and homogenized ^{19}F labeled PNAs. It should be noted that 2′-deoxy-5-(trifluoromethyl)uridine residue in oligonucleotides has been reported to be prone to ammonolysis to form a 5-cyanouridine residue.[38] Both PNA building blocks **1** and **2** proved stable in the repeated piperidine tretaments. Authenticity and homogeneity of the products was verified by MS(ESI) spectroscopy (Figure 1).

Table 1. UV−Melting Temperatures (°C) for the Complexes. PNA_{Std}/F_3−PNA/F_9−PNA = H-Lys-GCACCXGTCC-Lys-NH_2 (X = T/Residue of 1/Residue of 2), Anti-Parallel DNA/RNA = 5′-GGACAGGT/UGC-3′, Parallel DNA/RNA = 5′-CGT/UGGACAGG-3′

	antiparallel DNA	antiparallel RNA	parallel DNA	parallel RNA
PNA_{Std}	54.5	65.4	43.1	50.3
F_3−PNA	53.4 (−1.1)	64.0 (−1.4)	42.4 (−0.7)	51.0 (−1.4)
F_9−PNA	56.5 (+2.0)	67.0 (+1.6)	41.7 (−1.4)	52.2 (+1.9)

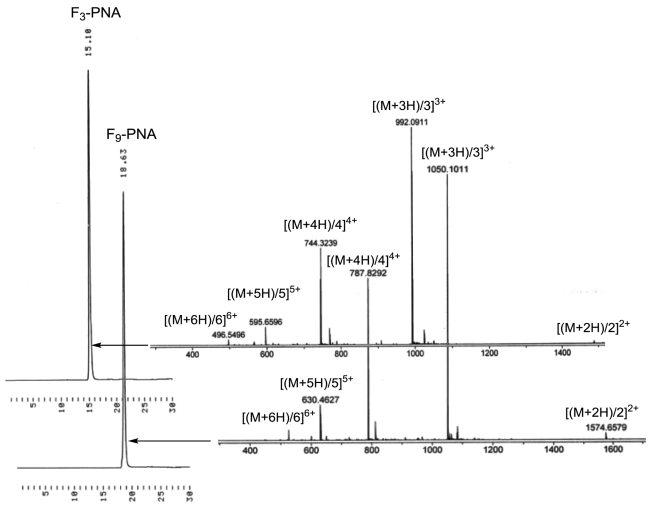


Figure 1. RP HPLC chromatograms of homogenized fluorine-labeled PNAs (F_3- and F_9-PNA) and their MS(ESI) spectra. RP HPLC conditions: see the Experimental Section.

Prior to ^{19}F NMR measurements, the effect of both ^{19}F sensors (**1** and **2**) on duplex stability was evaluated by UV and CD spectroscopy. Each combination of duplexes (i.e., both ^{19}F-labeled PNAs with antiparallel RNA and DNA and with parallel DNA and RNA) was compared to that with unmodified PNA (PNA_{Std}, Table 1). As seen, thermal denaturations were only slightly affected by the sensors. F_3 sensor (**1**) decreased (from −0.7 to −1.4 °C) the melting temperature in each case, whereas F_9 sensor (**2**) increased that with antiparallel RNA and DNA (+1.6 °C and +2.0 °C) and with parallel RNA (+1.9 °C) but decreased that with parallel DNA (−1.4 °C). In CD spectroscopy, F_3 sensor (**1**) disturbed formation of the parallel DNA complex, which could be seen by the gentling of CD curves at increasing temperature. This was probably related to a less ordered parallel oligonucleotide/PNA complex in which CF_3 modification close to stacked base moieties caused a broader denaturation range but did not remarkably decrease the inflection point. This may also be seen in UV spectroscopy in which slightly biphasic melting curves instead of ideal *S*-curves for parallel RNA/F_3−PNA and parallel DNA/F_3−PNA complexes were observed. These results are consistent with previous studies in which the effects of corresponding ^{19}F-nucleoside sensors [i.e., 2′-deoxy-5-(trifluoromethyl)uridine[39] and 2′-deoxy-5-[3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl]uridine[21]] on DNA duplexes were evaluated. The trifluoromethyl modification at C-5 of uracil base decreases duplex stability more than the 3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl modification.

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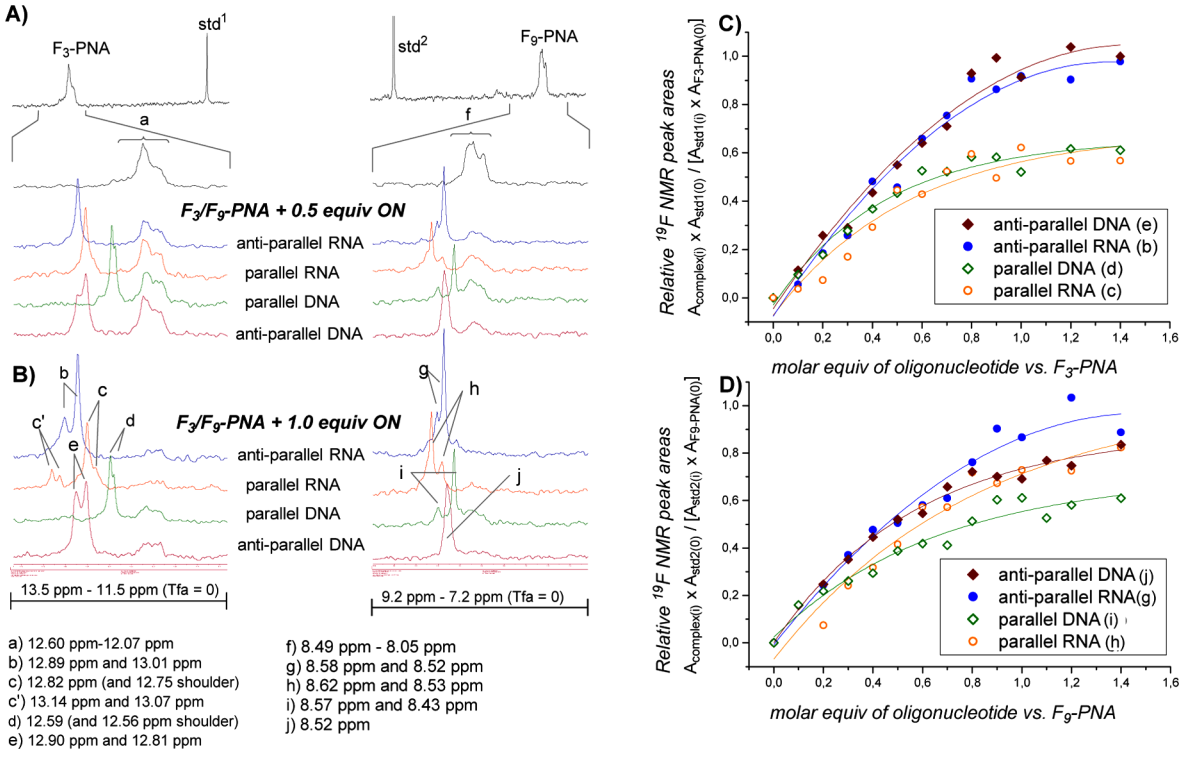

Figure 2. ^{19}F NMR spectra of F_3- and F_9-PNA and their complexes (A, B) and the corresponding titration curves (C, D) according to relative peak areas of ^{19}F resonance signals.

^{19}F NMR measurements were carried out using 60 μmol L^{-1} solutions of F_3-PNA and 20 μmol L^{-1} solutions of F_9-PNA in NaH_2PO_4 (10 mmol L^{-1}, 100 mmol L^{-1} NaCl, pH = 7.0) -buffered D_2O–H_2O (1:9, *v*/*v*) at 37 °C. Internal standards [2′-deoxy-5-[3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl]-uridine[21] = Std1, [5-(trifluoromethyl)uracil-N^1-yl]acetate (**5**) = Std2] were used to facilitate integration of relative peak areas. It may be emphasized that according to UV and CD spectroscopy of the oligonucleotide/PNA complexes at 37 °C (the temperature used for the ^{19}F NMR measurements), only the antiparallel RNA/PNA complexes were fully hybridized and the other complexes were partially unwound. Both ^{19}F-labeled PNAs were gradually titrated with antiparallel DNA, antiparallel RNA, parallel DNA and parallel RNA. Examples of ^{19}F NMR spectra are shown in parts A and B of Figure 2. As seen, both ^{19}F-labeled PNAs alone gave broad and fragmented ^{19}F signals in the NMR (a and f) due to a number of slow rotameric equilibria of the amide bonds. Relatively sharp signals (b–e and g–j) to downfield appeared when the PNAs were mixed with their complementary oligonucleotides. Hybridization gives a more defined structure, as indicated by the ^{19}F NMR signals. Two main signals were observed (more or less in each case), which most likely are related to cis/trans-amide rotamerism of the sensors in hybridized PNAs. A unique ^{19}F NMR shift for each complex was obtained. The shift differences (h–j: 0.09 ppm) with the F_9-sensor (**2**) fell to a narrower range than those (b–d: 0.3 ppm) with the F_3-sensor (**1**), and the latter (**1**) may, hence, be better for the experiments aimed at monitoring the competition between PNA complexes. Relative peak areas $[A_{complex(i)} \times A_{std(0)}/(A_{std(i)} \times A_{PNA(0)})]$ versus molar equivalents of the added oligonucleotides are shown in parts C and D of Figure 2. As seen in Figure 2C, formation of the signals (c and d) of parallel DNA/F_3-PNA and parallel RNA/F_3-PNA complexes are saturated in ca. 60% of the required stoichiometric 1:1 complex formation. With the parallel RNA this may be seen in NMR spectra as increasing fragmentation (*c*′) of the signals upon titration, while with the parallel DNA part of the starting material (F_3-PNA) was not completely consumed even with a higher (1.4 equiv) excess of the DNA. The moderate saturation curve of the parallel-DNA/F_3-PNA complex may be explained by a low thermal melting temperature (42.4 °C), but the increased signal fragmentation refers also to formation of less specific lowest energy secondary structures. PNA duplexes, especially with parallel oligonucleotides, are not purely defined structures. Aggregation of PNA or even formation of higher ordered structures may play a role since the concentration was relatively high (60 μmol L^{-1}), and a palindromic region GGACAGG/CCXGTCC is included in these model sequences. A similar trend was observed also with F_9-PNA: ^{19}F resonance signals (h and i) of the structurally less defined parallel oligonucleotide/PNA complexes were more fragmented than those (g and j) with the antiparallel oligonucleotide/PNA complexes. Integration of these signals (g–j) was unfortunately disturbed (Figure 2D) by partial overlapping with the resonances of the single strand F_9-PNA.

The shift difference between the signals (b and d) of parallel DNA/F_3-PNA- and antiparallel RNA/F_3-PNA complexes was large enough (0.3 ppm) to be applied in the monitoring of a competitive hybridization. This was demonstrated by adding antiparallel RNA gradually to a mixture of a parallel DNA/F_3-PNA complex, and the conversion was followed by ^{19}F NMR. As seen in Figure 3A, the signals (b and d) of these duplexes were well separated, and the conversion in the relative peak areas could be followed (Figure 3C). An interesting application would additionally be the monitoring of double duplex invasion, which was demonstrated by a simplified experiment: F_3-PNA was hybridized with a complementary antiparallel PNA (H-Lys-GGACAGGTGC-Lys-NH_2), and the resulting PNA/

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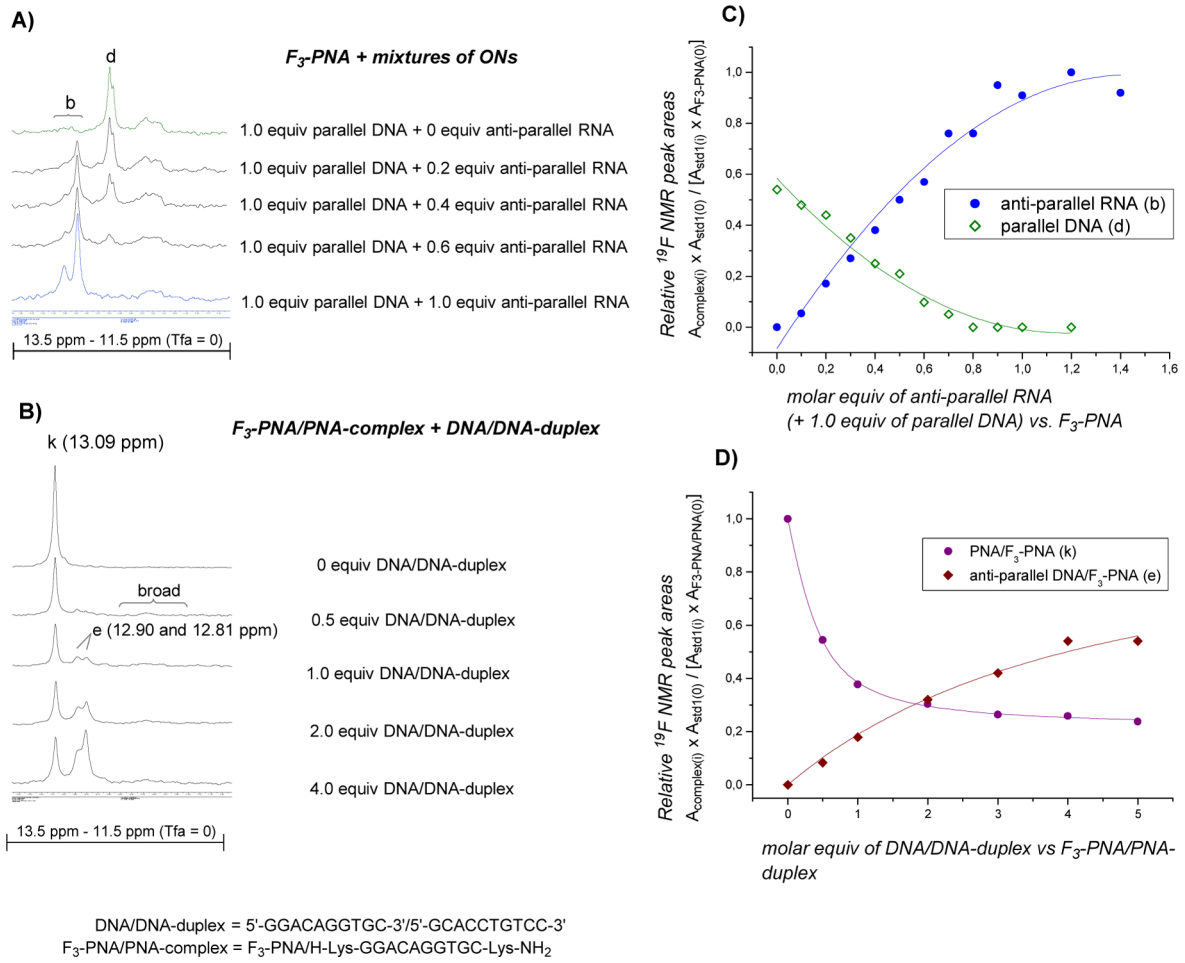

Figure 3. ^{19}F NMR spectra of interconverted F_3-PNA complexes (A, B) and the corresponding titration curves (C and D) according to relative peak areas of ^{19}F resonance signals.

PNA complex (T_m = 78.0 °C) was titrated with a DNA/DNA duplex (5′-GGACAGGTGC-3′/5′-GCACCTGTCC-3′, T_m = 48.0 °C). In contrast to oligonucleotide/PNA complexes, the PNA/PNA complex gave a sharp singlet at 13.09 ppm (Figure 3B). Upon titration with the DNA/DNA duplex, the signals (*e* = 12.90 and 12.81 ppm) of the antiparallel DNA/F_3-PNA complex (T_m = 53.4 °C) were present as expected (Figure 3B). Conversion between these two PNA complexes (now from a PNA/PNA complex to two PNA/DNA complexes) could be followed. As seen in Figure 3D, the starting material (F_3-PNA/PNA) did not follow stoichiometric complex formation, which indicates that in addition to the formation of the expected products, minor secondary structures are also formed. It is worth mentioning that all species were fully hybridized during this dynamic process, which complicates the monitoring by conventional detection methods (see the CD spectra of all species in the Supporting Information).

CONCLUSION

Two fluorine-labeled PNA building blocks (**1** and **2**) were prepared and incorporated into a PNA strand, and the applicability of these sensors for the monitoring of PNA complex equilibrium was evaluated. The ^{19}F resonance expectedly depended on the complementary oligonucleotide (antiparallel DNA/RNA and parallel DNA/RNA), resulting in a unique shift for each of the complexes studied. With 5-[3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl]uracil-derived sensor (**2**) the resulted signals shifted in a relatively narrow field compared to those with 5-(trifluoromethyl)uracil-derived sensor (**1**). In this regard, the latter (**1**) showed more potential, and its applicability was additionally demonstrated for the monitoring of competition between the antiparallel RNA/PNA and parallel DNA/PNA complex. In these relatively simple demonstrations, the results of ^{19}F NMR spectroscopy may be verified by more conventional and convenient detection methods (like CD spectroscopy and fluorescense techniques, of which sensitivity is superior compared to NMR-related detection methods), but the behavior of the sensors proved promising for application for the monitoring of more complex and dynamic equilibria of PNA complexes. Applicability of the 5-(trifluoromethyl)uracil-derived sensor (**1**) for the monitoring of double-duplex invasion (competition between PNA/PNA complex and two PNA/DNA duplexes) was then evaluated. This dynamic process (detection of which is hardly achievable by other detection methods) could successively be followed by ^{19}F NMR sepctroscopy. In contrast to the corresponding ^{19}F NMR applications related to DNA and RNA, the slow rotameric equilibria of the amide bonds disturb detection of uncomplexed PNAs. The ^{19}F NMR spectroscopic detection of PNA should hence be useful to studies in which interconversion of duplexes or their conversion to triple helices takes place.

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EXPERIMENTAL SECTION

General Remarks. ^{1}H and ^{13}C NMR spectra of **1**, **2**, **4**, **6**, and **8**−**11** were recorded at a frequency of 500 and 125 MHz, respectively. The chemical shifts are given in ppm from internal TMS. ^{19}F NMR spectra of **1**, **2**, **4**, **6**, and **9**−**11** were recorded at a frequency of 376.1 MHz. ^{19}F resonances were referenced relative to external CCl_3F. Rotameric mixtures could be seen in ^{13}C NMR spectra of **1**,**2**, **6**, and **11** (more visible in the ^{1}H NMR), but in ^{19}F NMR these are observed only in the spectrum of **6**. For the ^{19}F NMR measurements of F_3-PNA and F_9-PNA and their RNA/DNA complexes, a frequency of 470.6 MHz was used and ^{19}F resonances were referenced to internal Tfa (0 ppm). Typical experimental parameters were chosen as follows: ^{19}F excitation pulse 4.0 μs, acquisition time 1.17 s, prescan delay 6.0 s, relaxation delay 0.8 s; the usual number of scans was 2048. The mass spectra were recorded using an ESI ionization method. RP HPLC analysis and purification of PNAs were performed using an analytical Phenomenex peptide C-18 column (3.6 μm, 250 × 4.6 mm). A gradient elution from 0.1% aq Tfa to 0.1% Tfa in H_2O−acetonitrile (2:8, v/v) (0−30 min), flow rate 1.0 mL min^{-1} and λ = 260 nm were used. RNA and DNA sequences used in the study were synthesized by an automatic DNA/RNA synthesizer in a standard manner.

Sample preparation for ^{19}F NMR spectroscopy: lyophilized PNAs were dissolved in 10 mmol L^{-1} NaH_2PO_4 buffer (including 100 mmol L^{-1} NaCl, pH 6.5) in D_2O−H_2O (1:9 v/v). For the titration experiments, oligonucleotides were added as 1 and 5 mmol L^{-1} solutions to a 60 μmol L^{-1} solution of F_3-PNA and to a 20 μmol L^{-1} solution of F_9-PNA. Prior to each spectroscopic detection, the mixtures were heated to 90 °C for 1 min and then allowed to cool to room temperature.

*tert-Butyl [5-(Trifluoromethyl)uracil-N^1-yl]acetate (**4**).* *tert*-Butyl bromoacetate (0.45 mL, 3.0 mmol) was added to a mixture of 5-(trifluoromethyl)uracil (0.50 g, 2.8 mmol) and K_2CO_3 (0.38 g, 2.8 mmol) in dimethylformamide (3.0 mL). The mixture was stirred overnight at room temperature and poured into a two-phase mixture of 10% aqueous KH_2PO_4 and ethyl acetate. The organic layer was separated, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude product was crystallized in ethyl acetate to give 0.61 g (74%) of the product **4**. ^{1}H NMR (500 MHz, $CDCl_3$ + drops of CD_3OD) δ: 7.96 (s, 1H), 4.44 (s, 2H), 1.46 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$ + drops of CD_3OD) δ: 166.3, 159.8, 150.2, 146.2 (q, J = 5.6 Hz), 121.8 (q, J = 268 Hz), 104.5 (q, J = 32.9 Hz), 83.6, 49.5, 27.4. ^{19}F NMR (376 MHz, $CDCl_3$ + drops of CD_3OD) δ: −60.0. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{12}F_3N_2O_4$ 293.0749, found 293.0768.

*tert-Butyl N-[N-(9-Fluorenylmethoxycarbonyl)-2-aminoethyl]-N-[5-(trifluoromethyl)uracil-N^1-yl]acetylglycinate (**5**).* Compound **4** (0.30 g, 1.0 mmol) was dissolved in a mixture of trifluoroacetic acid and dichloromethane (1:3, v/v, 8.0 mL), stirred for 2 h at room temperature, and evaporated to dryness. The residue was coevaporated twice with dichloromethane, dried under vacuum over phosphorus pentoxide, and dissolved in a mixture of *tert*-butyl *N*-[*N*-(9-fluorenylmethoxycarbonyl)-2-aminoethyl]glycinate (0.44 g, 1.0 mmol) and HBTU (0.43 g, 1.1 mmol) in *N*,*N*-dimethylformamide (4.0 mL), and then DIEA (356 μL, 2.0 mmol) was added. The mixture was stirred at room temperature for 2 h and poured into a two-phase mixture of 10% aqueous KH_2PO_4 and ethyl acetate. The organic phase was separated, washed with saturated $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (a gradient elution from 50% EtOAc in petroleum ether to 100% EtOAc) to yield 0.40 g (64%) of the product **5** as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$ + drops of CD_3OD) δ: 7.77 (b, 1H), 7.71 (b, 2H), 7.56 (b, 2H), 7.35 (b, 2H), 7.65 (b, 2H), 6.28, 5.98, 5.84, and 5.55 (each b, 2H), 4.67, 4.39, and 4.32 (each b, 2H), 4.60 and 4.49 (both b, 2H), 4.16 (b, 1H), 4.02, 3.88, and 3.78 (each b, 2H), 3.65, 3.43, and 2.85 (each b, 2H), 3.33, 3.27, and 2.85 (each b, 2H), 1.46 and 1.41 (both b, 9H). ^{13}C NMR (125 MHz, $CDCl_3$ + drops of CD_3OD) δ: (168.8, 168.6), (167.6, 167.0), 159.7, 157.1, 150.5, (146.8, 146.6), (143.8, 143.7), 141.2, 127.7, 127.0, (124.6, 124.5), 121.9 (q, J = 270 Hz), 119.9, 104.9 (m), (84.0, 83.0), (66.75, 66.70), (50.9, 49.8), 48.6, (39.1, 39.0 and 38.4); ^{19}F NMR (376 MHz, $CDCl_3$ + drops of CD_3OD) δ: −63.6 and −63.7. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{30}H_{30}F_3N_4O_7$ 615.2067, found 615.2076.

*N-[N-(9-Fluorenylmethoxycarbonyl)-2-aminoethyl]-N-[5-(trifluoromethyl)uracil-N^1-yl]acetylglycine (**1**).* Compound **6** (0.38 g, 0.62 mmol) was dissolved in a mixture of trifluoroacetic acid and dichloromethane (1:3, v/v, 8.0 mL), and the reaction was monitored by analyzing aliquots of the mixture by RP HPLC. Once the reaction was completed (2 h), the mixture was evaporated to dryness, coevaporated twice with dichloromethane, and dried under vacuum over phosphorus pentoxide. Product **1** (0.35 g, quant) as a slightly pink powder was obtained. ^{1}H NMR (500 MHz, CD_3OD) δ: 8.11 and 8.08 (s and s, 1H), 7.77 (m, 2H), 7.65 (m, 2H), 7.38 (m, 2H), 7.31 (m, 2H), 4.82 and 4.66 (s and s, 2H), 4.42 and 4.36 (d and d, 2H, J = 6.5 Hz and J = 6.8 Hz), 4.26 and 4.12 (s and s, 2H), 4.22 (m, 1H), 3.51−3.38 (m, 2H), 3.40−3.31 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ: (171.1, 170.0), (168.2, 167.8), (160.1, 160.0), (157.6, 157.5), (150.5, 150.3), 147.5 (m), 143.9, 141.2, 127.4, 126.8, 124.8, 122.4 (q, J = 271 Hz), 119.5, 103.6 (m), 66.4, (49.1, 47.9), (48.52, 48.48), 47.8, 47.1, (38.5, 38.0). ^{19}F NMR (376 MHz, CD_3OD) δ: −62.1. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{26}H_{22}F_3N_4O_7$ 559.1441, found 559.1443.

*tert-Butyl [N^3-(4-Methoxybenzyl)-5-iodoracil-N^1-yl]acetate (**8**).* *tert*-Butyl bromoacetate (0.75 mL, 4.6 mmol) was added to a mixture of 5-iodoracil (**7**, 1.0 g, 4.2 mmol) in dimethylformamide (5.0 mL). The mixture was stirred overnight at room temperature and poured into a two-phase-mixture of 10% aq KH_2PO_4 and dichloromethane. The organic layer was separated, dried with Na_2SO_4, filtered, and evaporated to dryness. The residue was coevaporated with dry DMF and dissolved in the same solvent (10 mL), and then NaH (0.19 g, 60% dispersion in mineral oil, 4.6 mmol) and 4-methoxybenzyl-chloride (0.87 mL, 6.2 mmol) were added. The mixture was stirred overnight at room temperature and poured into saturated $NaHCO_3$, and the crude product **8** was extracted with diethyl ether. The organic layers were combined, dried with Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (20% EtOAc in petroleum ether) to yield 1.2 g (61%) of the product **8** as white foam. ^{1}H NMR (500 MHz, CD_3OD) δ: 7.56 (s, 1H), 7.45 (d, 2H, J = 8.6 Hz), 6.82 (d, 2H, J = 8.5 Hz), 5.11 (s, 2H), 4.36 (s, 2H), 3.78 (s, 3H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, CD_3OD) δ: 166.1, 160.0, 159.3, 151.1, 147.0, 130.9, 128.5, 113.8, 83.7, 68.1, 55.3, 50.6, 45.6, 28.0. HRMS (ESI-TOF) m/z: $[M+H]^+$ calcd for $C_{18}H_{22}IN_2O_5$ 473.0573, found 473.0562.

*tert-Butyl [N^3-(4-Methoxybenzyl)-5-(3,3-bis(trifluoromethyl)-4,4,4-trifluoro-1-butynyl)uracil-N^1-yl]acetate (**9**).* 3,3-Bis-(trifluoromethyl)-4,4,4-trifluoro-1-butyne (0.32 g, 1.3 mmol) was dissolved in DMF (1.0 mL) and added to a mixture of **2** (0.61 g, 1.3 mmol), CuI (50 mg, 0.26 mmol), and $(Ph_3)_4Pd^0$ (0.15 g, 0.13 mmol) in DMF (1.0 mL) under nitrogen atmosphere at 0 °C. The mixture was then allowed to warm to room temperature and stirred for 10 days. Upon reaction, an additional $(Ph_3)_4Pd^0$ (50 mg, 0.043 mmol) was added after 2, 4, and 6 days (n_{tot} 0.26 mmol). According to RP HPLC, the extra additions of $(Ph_3)_4Pd^0$ improved the reaction, but the conversion still remained at 76%. The mixture was evaporated to dryness, and the product was isolated by silica gel chromatography (20% EtOAc in petroleum ether) to give 0.45 g (59%) of **9** as white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.55 (s, 1H), 7.45 (m, 2H), 6.85 (m, 2H), 5.08 (s, 2H), 4.40 (s, 2H), 3.80 (s, 3H), 1.50 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 165.8, 160.2, 159.3, 150.2, 148.1, 130.7, 128.1, 120.1 (q, 290 Hz), 113.8, 96.4, 83.9, 83.2, 74.3, 57.6 (m, J = 30.8 Hz), 55.1, 51.0, 44.6, 27.9. ^{19}F NMR (376 MHz, $CDCl_3$) δ: −69.4. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{24}H_{20}F_9N_2O_5$ 587.1229, found 587.1209.

*tert-Butyl [5-(3,3-Bis(trifluoromethyl)-4,4,4-trifluoro-1-butynyl)-uracil-N^1-yl]acetate (**10**).* Ceric ammonium nitrate (CAN, 0.80 g, 1.5 mmol) was dissolved in H_2O (2.0 mL) and added to a mixture of **9** (0.43 g, 0.73 mmol) in DMF (10 mL). The mixture was stirred for 1 h at 60 °C once the orange color disappeared. An additional CAN (0.80 g, 1.5 mmol) was then added, and the mixture was stirred for 2 h. The virtually completed reaction (according to RP HPLC) was poured into

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water, and the crude product was extracted with ethyl acetate. The organic layers were combined, dried with Na_2SO_4, filtered, and evaporated to dryness. The residue was purified twice by silica gel chromatography (first 50% EtOAc in petroleum ether, then a gradient from 2 to 6% MeOH in DCM) to give 0.23 g (74%) of **10** as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.69 (s, 1H), 7.60 (s, 1H), 4.43 (s, 2H), 1.51 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 165.8, 160.5, 149.9, 149.7, 120.0 (q, J = 290 Hz), 97.2, 84.3, 82.3, 75.0, 57.5 (m, J = 30.8 Hz), 49.8, 27.9. ^{19}F NMR (376 MHz, $CDCl_3$) δ: −69.5. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{16}H_{12}F_9N_2O_4$ 467.0653, found 467.0653.

tert-Butyl N-[N-(9-Fluorenylmethoxycarbonyl)-2-aminoethyl]-N-[5-[3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl]uracil-N^1-yl]-acetylglycinate (11). Compound **11** was synthesized from **9** as described for **6** from **5** above. 0.21 g 0.44 mmol) of **10**, 0.19 g (0.44 mmol) of *tert*-butyl *N*-[*N*-(9-fluorenylmethoxycarbonyl)-2-aminoethyl]glycinate, 0.13 g (0.46 mmol) of HBTU, and 0.16 mL (0.92 mmol) of DIEA were used. After silica gel chromatography (a gradient elution from 50% to 30% petroleum ether in EtOAc) 0.34 g (96%) of the product **11** as white foam was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.75 (d, J = 7.6 Hz, 2H), 7.58 (m, 2H), 7.47 (s, 1H), 7.39 (dd, J = 7.4 and 7.5 Hz), 7.30 (dd, 2H, J = 7.4 and 7.6 Hz), 6.00 and 5.59 (both b, 1H), 4.48 and 4.36 (both d, 2H, J = 7.2 Hz), 4.46 (s, 2H), 4.20 (m, 1H), 4.07 and 3.96 (both s, 2H), 3.8−3.2 (m, 2H), 3.4−2.8 (m, 2H), 1.51 and 1.47 (both s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: (168.6, 168.4), (167.3, 166.6), (160.9, 160.8), (156.84, 156,78), 150.8, (150.6, 149.7), (143.9, 143.8), 141.3, 141.2, (127.75, 127.69), (127.11, 127.06), (125.2, 124.9), 120.1 (q, J = 290 Hz), (119.95, 119.92), (96.8, 96.6), 83.8, (82.8, 82.7), 74.6, (66.9, 66.7), 57.5 (m, J = 30.6 Hz), (50.8, 49.6), (48.79, 48.75), (48.3, 48.2), (47.2, 47.1), (39.1, 38.7), 27.9. ^{19}F NMR (376 MHz, $CDCl_3$) δ: −69.4. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{35}H_{30}F_9N_4O_7$ 789.1971, found 789.1942.

N-[N-(9-Fluorenylmethoxycarbonyl)-2-aminoethyl]-N-[5-[3,3-bis(trifluoromethyl)-4,4,4-trifluorobut-1-ynyl]uracil-N^1-yl]acetyl Glycinate 2. Compound **10** (0.30 g, 0.38 mmol) was treated with trifluoroacetic acid as described for the preparation of **1** from **5** above. The desired compound **2** was quantitatively obtained as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$ + drops of CD_3OD) δ: 7.71 (m, 2H), 7.71 and 7.66 (both s, 1H), 7.55 (m, 2H), 7.35 (m, 2H), 7.26 (m, 2H), 6.33 and 6.02 (both b, 1H), 4.71 and 4.53 (both s, 2H), 4.44 and 4.33 (both b, 2H), 4.15 (m, 1H), 4.09 and 3.99 (both s, 2H), 3.73−3.14 (m, 2H), 3.42−2.84 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$ + drops of CD_3OD) δ: (171.3, 170.9), (167.5, 167.1), (161.6, 161.5), 157.1, 127.7, 127.0, (125.0, 124.9), 120.1 (q, J = 290 Hz), 119.9, (96.5, 96.3), (82.9, 82.8), 74.4, (66.76, 66.56), 57.5 (m, J = 31.0 Hz), (49.8, 48.7), (48.7, 48.6), (48.5, 48.3), (47.13, 47.08), (38.9, 38.4). ^{19}F NMR (376 MHz, $CDCl_3$) δ: −67.2. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{31}H_{22}F_9N_4O_7$ 733.1345, found 733.1319.

Synthesis of F_3- and F_9-PNA. PNA synthesis was carried out on a Rink amide derived Chem Matrix resin on a 10 μmol scale using commercially available Fmoc/Boc-protected PNA-building blocks **1**, **2** and Fmoc-Lys(Boc)-OH. An automatic peptide synthesizer was applied. For each coupling 5 equiv of amino acid (**1**, **2**, commercially available PNA building blocks, and lysine, 0.25 mol L^{-1} of each predissolved in NMP), 5 equiv of HBTU, and 10 equiv of DIEA and 30 min coupling time (at rt), followed by a capping step with an acetic anhydride treatment (Ac_2O, pyridine, NMP, 1:25:25, v/v/v, 1 min at rt) were used. Piperidine (20%) in NMP was used for the Fmoc deprotection (7 min at rt). The solid-supported PNAs were released as their Fmoc-protected form with a mixture of anisol and Tfa (1:10, v/v, for 2 h at rt), precipitated from cold diethyl ether, dissolved in 0.1% aqueous Tfa, and purified by RP HPLC. The product fractions were lyophilized to dryness and dissolved in 20% piperidine in DMF. After 5 min, incubation the mixtures were evaporated to dryness, the residues were dissolved in Tfa, precipitated from cold diethyl ether and repurified by RP HPLC. The product fractions were lyophilized to dryness to give the desired homogenized PNAs (RP HPLC chromatograms seen in Figure 1) as white powders. Authenticity of F_3- and F_9-PNA was verified by MS(ESI) spectroscopy (Figure 1).

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra for **1**, **2**, **4**, **6**, and **8**−**11**, crude RP HPLC profiles of PNA sequences bearing **1** and **2**, more detailed ^{19}F NMR titrations, and UV and CD spectra of F_3- and F_9-PNAs in the presence of complementary oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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