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Design of 2′-phenylethynylpyrene excimer forming DNA/RNA probes for homogeneous SNP detection: The attachment manner matters

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ABSTRACT

1-Phenylethynylpyrene fluorophore (1-PEPy) has long-wavelength shifted emission and higher photostability compared to pyrene, retaining, however, pyrene's ability to form excimers. Here we report the synthesis of 2′-*O*-[3(and 4)-(pyren-1-ylethynyl)benzyl]-uridines and their tandem incorporation into *deoxyribo*- and 2′-*O*-Me-*ribo*-oligonucleotide probes. Excimer forming probes of type NN … NN**XX**NN … NN (**X** = 2′-*O*-[*meta*(or *para*)-(pyren-1-ylethynyl)-benzyl]uridine) containing two adjacent fluorescent nucleosides within an oligonucleotide are available in four types (*meta-meta*; *para-meta*; *meta-para*; *para-para*). Both DNA (N = deoxyribonucleotides) and 2′-*O*-Me-RNA (N = 2′-*O*-Me-ribo-nucleotides) probes were synthesized and their hybridization with complementary and singly mismatched DNA and RNA was studied. Several probes show a dramatic response of their excimer-to-monomer intensity ratio upon hybridization. Remarkably, most spectacular fluorescence changes were demonstrated for probes with *para-meta* and *meta-para* combination within 2′-*O*-Me-ribo-oligonucleotides. Using excimer forming probes, three natural SNP in *Helicobacter pylori* 23S RNA gene (A2144G, A2143G, A2143C) and the wild type gene can be distinguished.

1. Introduction

Fluorescently labeled oligonucleotides[1] are powerful tools in biotechnology, molecular biology and medical diagnostics. Numerous fluorochromes were conjugated to oligonucleotides – xanthenes,[2] cyanines,[2h,3] BODIPY,[4] coumarins,[5] quantum dots,[6] silver nanoclusters[7] etc. Recently a number of fluorescent environment-sensitive probes were developed for improved detection of DNA interactions.[8] Fluorescent polycyclic aromatic hydrocarbons, especially pyrene (Fig. 1), became widely applied labels for nucleic acids in the last two decades,[9] mainly due to its excimer forming ability.[9d,g] The broad band of a long wavelength pyrene excimer emission (typically around 450–500 nm) clearly differs from structured pyrene monomer emission (370–400 nm), and confirms that the pyrene residues are able to come into close proximity (≤4 Å). Excimer formation was used to study nucleic acids hybridization[10] and for detection of single point mutations.[11] However, pyrene and its simple (alkyl, carboxy) derivatives absorb at relatively short wavelengths, thus making them unsatisfactory for experiments in living cells due to excitation of cellular autofluorescence. This limitation of pyrene can be overcome by chemical functionalization affecting its spectroscopic and photophysical properties. We[12] and others[13] introduced the 1-(phenylethynyl) pyrene (1-PEPy; Fig. 1) fluorochrome as an improved dye for nucleic acid labeling. 1-PEPy is the subject of increasing interest due to its high fluorescence quantum yield,[13b,14] long-wave emission compared to pyrene, photostability, and propensity to form

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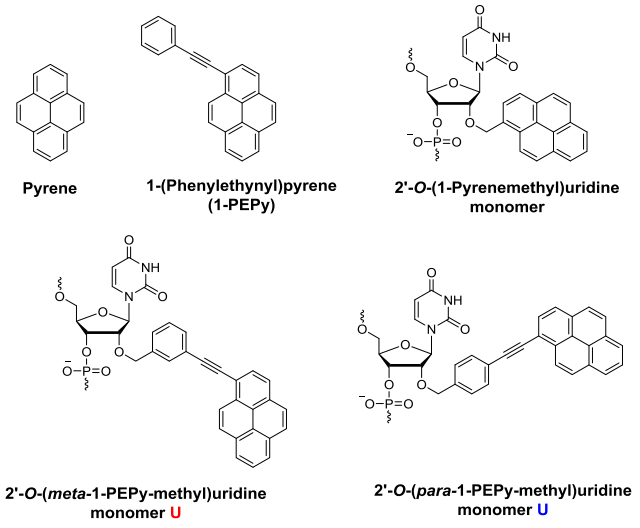


Fig. 1. Pyrene, 1-phenylethynylpyrene, and their nucleoside derivatives.

excimers. 1-PEPy attached to arabino-uridine via 2′-carbamate linker is able to form intra- and inter-strand excimers on DNA.[12f,j] Short excited lifetime of 1-PEPy (7.72 ns in EtOH) implies that its excimer confirms pre-association of two PEPy moieties, not only their mutual spacial attainability, making PEPy a specified probe of choice for structural studies of biomolecules.

Previously Yamana et al.[15] and others[16] synthesized oligonucleotides with 2′-*O*-pyren-1-ylmethyl nucleosides (uridine derivative is shown in Fig. 1) and demonstrated a variety of their useful applications. The fluorescence of singly 2′-*O*-(1-pyrenylmethyl)-labeled oligonucleotides is dramatically increased by hybridization with complementary RNA, but not DNA.[15i] This is caused by direction of the pyrene attached to the 2′-*O*-position of uridine via a methylene linker outside the RNA double helix, while the fluorochrome intercalates into the DNA duplexes. Next, bis(1-pyrenemethyl)-conjugated 2′-*O*-methyloligonucleotides were seen to be highly specific RNA-recognition probes.[15e] Karmakar and Hrdlicka used 2′-*O*-(1-pyrenylmethyl)uridine as a component in efficient SNP-discriminating RNA detection probes.[16f] Very recently, Imincan et al.[16j] used this nucleoside in improved light-up mutation detection in RNA tergets. Stimulated by the attractive features of 2′-*O*-(1-pyrenylmethyl) label,[16d,f,i] and taking into account improved spectral properties of 1-PEPy,[12f-i,13b] we have investigated *meta*- and *para*-1-PEPy dyes attached to uridine via 2′-*O*-methyl linkage.

In this paper, we describe the synthesis of 2′-*O*-(*meta*-1-PEPy-methyl)- and 2′-*O*-(*para*-1-PEPy-methyl)-uridine monomers (**U** and **U**, respectively, Fig. 1), their incorporation into DNA and 2′-*O*Me-RNA oligomers, and spectral properties of the conjugates containing double insertion of 1-PEPy moieties of the same type or a combination of *meta*- and *para*-1-PEPy monomers (Fig. 2).

2. Results and discussion

2.1. Synthesis of phosphoramidites and oligonucleotides

Modified phosphoramidites were synthesized as shown in Scheme 1. The starting Pom- and silyl-protected nucleoside **1**,[17] was reacted with 3- and 4-iodobenzylbromides in the presence of a strong phosphazene base to yield alkylation products **2a,b**. These were coupled with 1-ethynylpyrene[18] in common Sonogashira reaction conditions[12f,19] to afford the corresponding 1-PEPy-derivatives **3a,b**. Silyl protection was removed with triethylamine trihydrofluoride[20] to yield fluorescent nucleosides **4a,b**. These were converted into phosphoramidite reagents **6a,b** using standard procedures of 5′-*O*-dimethoxytritylation and 3′-*O*-phosphitylation.

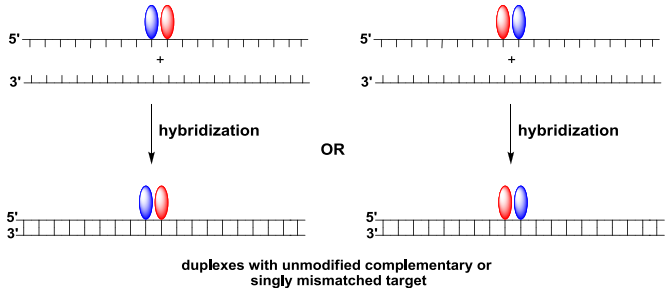
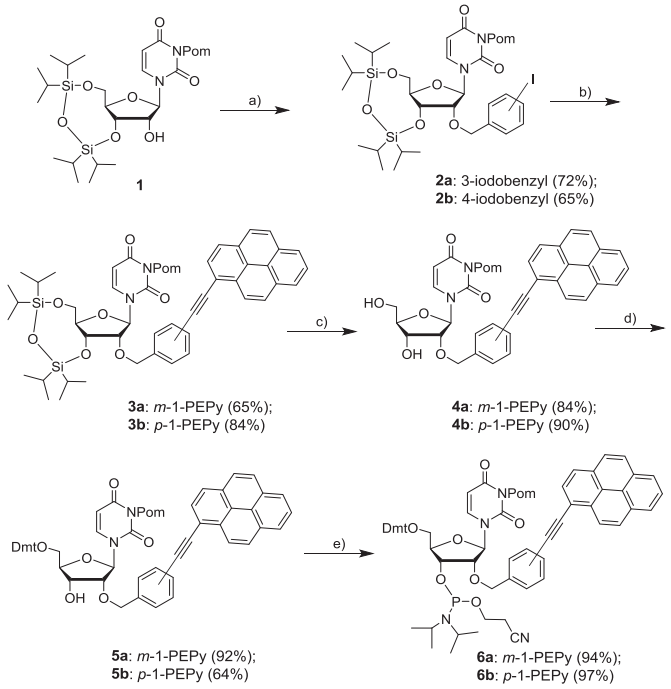


Fig. 2. Representative structures of the 2′-*O*-(1-PEPy) conjugates prepared in this study. Red and blue droplets indicate 2′-*O*-(meta-1-PEPy)- and 2′-*O*-(para-1-PEPy)-uridine monomers **U** and **U**, respectively (Fig. 1).



Scheme 1. Reagents and conditions: a) 3- or 4-iodobenzylbromide, BTPP, MeCN; b) 1-ethynylpyrene, $Pd(PPh_3)_4$, CuI, Et_3N, DMF; c) $Et_3N{\cdot}3HF$, THF; d) DmtCl, pyridine; e) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, diisopropylammonium tetrazolide, DCM. Pom = pivaloyloxymethyl, Dmt = 4,4′-dimethoxytrityl, BTPP = *tert*-butylimino-tri(-pyrrolidino)phosphorane, phosphazene base P_1-*t*-Bu-*tris*(tetramethylene).

Using modified phosphoramidites we prepared a series of fluorescent 20-mer deoxyribo- and 2′-*O*-methylribo-oligonucleotides (ONs) complemetary to the gene of 23S RNA *Helicobacter pylori*, an ulcerogenic bacterium (Table 1). 2′-*O*-Me RNA probes were reported to have some advantages *vs* DNA analogues, especially for RNA-directed probes.[21] Each probe contained a pair of PEPy-modified nucleosides, potentially able to form a PEPy excimer closely to the mutation site in the complementary strand. At the same time, we synthesized 20-mer DNA and RNA targets containing mismatches at positions 2143 and 2144 (see Supplementary data). The single nucleotide replacement mutations A2144G, A2143G and A2143C result in stability of the bacterium to clarithromycin,[22] thus their detection is important for clinical diagnostics. Since the probes are rather long, and designed for fluorescence measurements in ambient conditions in duplexes, the

Table 1
Oligonucleotides used in this study.[a]

	#	Sequence, 5′→3′
Model DNA targets	**DT-WT**	d(GCAAGACGG**AA**AGACCCCGT)
	DT-A43G	d(GCAAGACGG**GA**AGACCCCGT)
	DT-A43C	d(GCAAGACGG**CA**AGACCCCGT)
	DT-A44G	d(GCAAGACGG**AG**AGACCCCGT)
Model RNA targets	**RT-WT**	GCAAGACGG**AA**AGACCCCGU
	RT-A43G	GCAAGACGG**GA**AGACCCCGU
	RT-A43C	GCAAGACGG**CA**AGACCCCGU
	RT-A44G	GCAAGACGG**AG**AGACCCCGU
9,10-DNA probes	**DP-U^{9}U^{10}**	d(ACGGGGTC**UU**TCCGTCTTGC)
	DP-U^{9}U^{10}	d(ACGGGGTC**UU**TCCGTCTTGC)
	DP-U^{9}U^{10}	d(ACGGGGTC**UU**TCCGTCTTGC)
	DP-U^{9}U^{10}	d(ACGGGGTC**UU**TCCGTCTTGC)
10,11-DNA probes	**DP-U^{10}U^{11}**	d(ACGGGGTCT**UU**CCGTCTTGC)
	DP-U^{10}U^{11}	d(ACGGGGTCT**UU**CCGTCTTGC)
	DP-U^{10}U^{11}	d(ACGGGGTCT**UU**CCGTCTTGC)
	DP-U^{10}U^{11}	d(ACGGGGTCT**UU**CCGTCTTGC)
9,10-2′-OMe RNA probes	**MP-U^{9}U^{10}**	ACGGGGUC**UU**UCCGUCUUGC
	MP-U^{9}U^{10}	ACGGGGUC**UU**UCCGUCUUGC
	MP-U^{9}U^{10}	ACGGGGUC**UU**UCCGUCUUGC
	MP-U^{9}U^{10}	ACGGGGUC**UU**UCCGUCUUGC
10,11-2′-OMe RNA probes	**MP-U^{10}U^{11}**	ACGGGGUCU**UU**CCGUCUUGC
	MP-U^{10}U^{11}	ACGGGGUCU**UU**CCGUCUUGC
	MP-U^{10}U^{11}	ACGGGGUCU**UU**CCGUCUUGC
	MP-U^{10}U^{11}	ACGGGGUCU**UU**CCGUCUUGC

[a] DT = DNA target; RT = RNA target; DP = DNA probe; MP = 2′-OMe-RNA probe; **A** = nucleotide complementary to modification **U**/**U** or **U** of the target; **B** = mismatched nucleotide of the target (**B** = C, G); **U** = 2′-O-[3-(pyrene-1-ylethynyl)benzyl]uridine (*meta*-1-PEPy modification); **U** = 2′-O-[4-(pyrene-1-ylethynyl)benzyl]uridine (*para*-1-PEPy modification).

actual thermal stabilities of the probe–target duplexes were not determined.

2.2. Spectral properties of labeled oligonucleotides and duplexes carrying 2′-O-(1-PEPy-methyl)uridines

The steady-state fluorescence emission spectra of modified ONs and duplexes were obtained in a medium salt buffer (100 mM NaCl, 10 mM Na-phosphate, 0.1 mM EDTA, pH 7.0) using 0.2 μM concentration of complementary strands and an excitation wavelength of 360 nm. The emission wavelength range of 1-PEPy conjugates is 390–600 nm, fluorescence maxima of dye monomer and excimer are observed at ~405 nm and ~500 nm, respectively.

We used excimer-to-monomer fluorescence intensity ratio, I_{500}/I_{405}, as excimer formation criterion. DNA probes labeled at positions 9 and 10 exhibited ~2 times more intense fluorescence that 10,11-labeled analogues, and the spectra always contained both monomer and excimer emission bands. Upon hybridization of probes **DP** with complementary or singly mismatched DNA the ratio I_{500}/I_{405} is remarkably changed (Fig. 3). Thus, probes **DP-U^{9}U^{10}**, **DP-U^{9}U^{10}** and **DP-U^{10}U^{11}** display I_{500}/I_{405} of duplexes which allow detection of the A2144G replacement mutation in DNA target (**DT-A44G**), while **DP-U^{10}U^{11}** and **DP-U^{10}U^{11}** detect the A43C-mutant DNA. However, one can distinguish all the mutants from **DT-WT** (wild type) by consistent application of all the DNA probes followed by calculation of fluorescence ratio I_{500}/I_{405}. An excess of single stranded PCR-amplified DNA fragment containing the 2143–2144 region can be used as a target in particular assay procedure.[11e]

Next, 2′-OMe-RNA probes **MP** display remarkable excimer

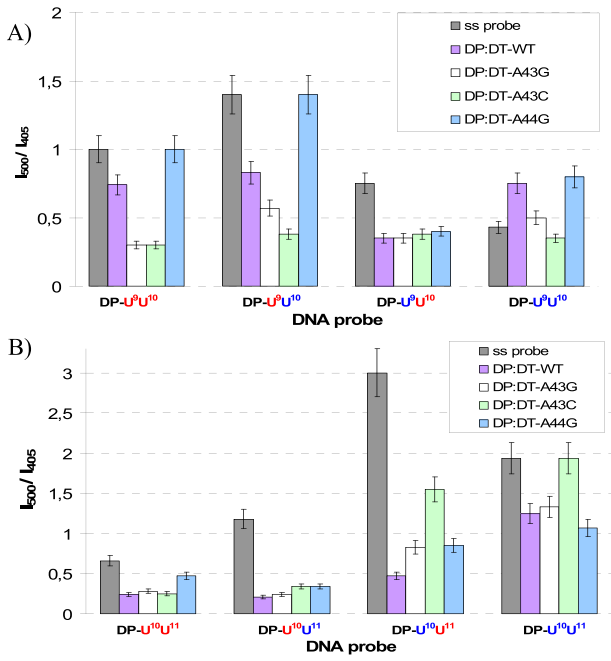


Fig. 3. Fluorescent properties of A) 9,10- and B) 10,11-labeled DNA probes and their duplexes with complementary or singly mismatched DNA targets. Spectra were obtained in a medium salt buffer (PBS) at 19 °C using an excitation wavelength of 360 nm and 0.2 μM concentration of ONs.

emission which is reduced upon hybridization with DNA (Fig. 4). This effect is more significant for *meta-meta*-1-PEPy-labeled probes than for *para-para*- and combined *para-meta*-1-PEPy analogues. As one can see, fluorescence of **MP-U^{9}U^{10}** and **MP-U^{10}U^{11}** is sensitive to the presence of mutation in DNA complement;

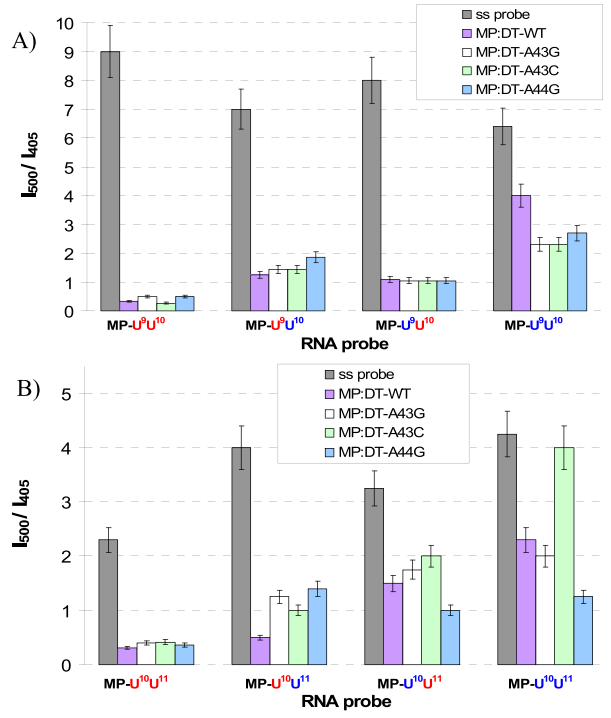


Fig. 4. Fluorescent properties of A) 9,10- and B) 10,11-labeled 2′-OMe-RNA probes and their duplexes with complementary or singly mismatched DNA. For conditions see Fig. 3.

MP-U^{10}U^{11} signals A44G-mutant DNA, while **MP-U^{10}U^{11}** allows detection of A44G and A43C SNPs. Again, consistent application of **MP** probes allows all the mutants from **DT-WT** sequence to be distinguished.

Fluorescence of the DNA probes (**DP**) is not significantly affected by hybridization with complementary or mutant RNA (Fig. 5A, Supplementary data). Among RNA probes, all the 9,10-labeled and 10,11-labeled probes containing two identical fluorochromes (**MP-U^{9}U^{10}**, **MP-U^{9}U^{10}**, **MP-U^{10}U^{11}** and **MP-U^{10}U^{11}**) are also not sensitive to RNA hybrid formation (see Supplementary data).

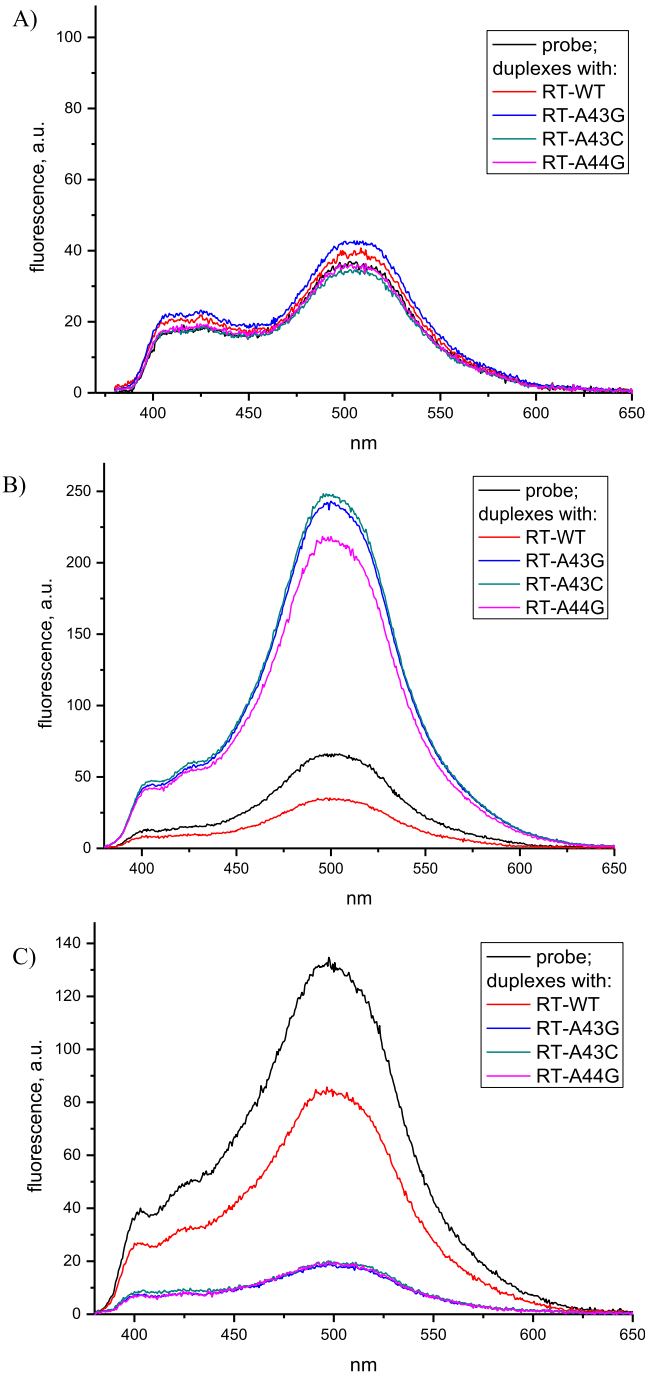


Fig. 5. Steady-state fluorescence emission spectra of probes A) **DP-U^{10}U^{11}**, B) **MP-U^{10}U^{11}**, C) **MP-U^{10}U^{11}** and their duplexes with complementary or mutant RNA. For conditions see Fig. 3.

Remarkable fluorescent properties were observed only for RNA duplexes formed by **MP-U^{10}U^{11}** and **MP-U^{10}U^{11}** containing combined *meta*- and *para*-1-PEPy fluorochromes (Fig. 4B and C). Thus, the fluorescence intensity of probe **MP-U^{10}U^{11}** at 500 nm is reduced ~2 times upon hybridization with **RT-WT**, while in the presence of mutations fluorescence intensity is enhanced ~4 times. Probe **MP-U^{10}U^{11}** containing the opposite location of isomeric modifications displays the opposite effect of mutations in the RNA target on its fluorescence properties. Hybridization of **MP-U^{10}U^{11}** with **RT-WT** results in ~40% decrease of fluorescence intensity, while hybridization with mutant RNA leads to ~6 times reduced fluorescence.

2.3. Molecular simulations

To understand such significant differences in excimer fluorescence of duplexes formed by modified oligonucleotides with the targets, we performed molecular dynamics simulation for each modified duplex in **MP-U^{10}U^{11}** series. Equilibration was achieved for all duplex conformations during at least 150 ns. The simulation has been restarted several times to get better sampling, and then distances between two PEPy moieties in the duplexes and for each PEPy residue with the RNA backbone were determined. As excimer can be formed only in case of overlapped PEPy residues we defined overlapping as a contact between PEPy atoms at the distance about 3.6 Å. Overlapping was found to be in a good agreement with the experimental data on duplex fluorescence. Higher overlapping corresponds to higher excimer fluorescence. Normalized data for the **MP-U^{10}U^{11}** series is presented in Fig. 6.

Another feature that can influence the excimer fluorescence is PEPy interactions with the RNA that could lead to the quenching of fluorescence. To evaluate the impact of excimer fluorescence quenching by the RNA backbone we measured the distances between PEPy moieties and RNA. In most cases such interactions were limited or totally absent. However the fluctuations of the distances differed significantly from one duplex to another (see Supplementary data). An example of PEPy-backbone distance fluctuation in duplexes of **MP-U^{10}U^{11}**, **MP-U^{10}U^{11}**, **MP-U^{10}U^{11}** with **RT-WT** target is presented in Fig. 7. In the first and last cases, distances are rather stable while in the case of **MP-U^{10}U^{11}** distance varies a lot, although dye residues are always located outside of the duplex (Fig. 8). As a result, observed excimer fluorescence for **MP-U^{10}U^{11}/RT-WT** duplex is rather low and insensitive to SNP (Fig. 5B). We want to emphasize that intra PEPy interactions do not drastically change A-U base pair geometry not only for perfect but also for most mismatched duplexes (see Supplementary data). Thus, maximal overlapping of PEPy residues in the duplex and stability of the distances between PEPy residues and RNA backbone are the main driving forces that can increase excimer fluorescence.

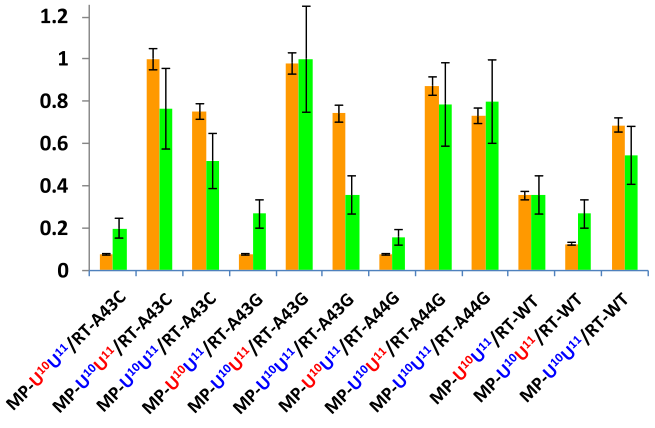


Fig. 6. Normalized steady-state fluorescence emission of probes (orange series) and normalized PEPy overlapping data from molecular simulations (green series) for duplexes of **MP-U^{10}U^{11}** series with wt and mutated RNA targets.

3. Conclusions

To conclude, we report the synthesis of 2′-*O*-[3(4)-(pyren-1-ylethynyl)benzyl]uridine monomers, their incorporation into deoxyribo- and 2′-OMe-ribo-oligonucleotides, fluorescent properties, and structural aspects for effective detection of nucleic acid hybridization. Double consequent insertion of these two regioisomeric labels into oligonucleotides led to excimer-forming probes. Some of them displayed remarkable changes in fluorescence spectra upon hybridization with complementary and singly mismatched DNA and RNA. This allowed all wt and mutant DNA and RNA targets in *H. pylori* sequences to be distinguished. Noteworthy, the most interesting fluorescent properties were observed for the doubly labeled conjugates containing combination of *meta*- and *para*-1-PEPy fluorochromes. In the presence of single base-pair mismatches in RNA targets, 2′-OMe-probe having *meta*- and *para*-1-PEPy displayed remarkably increased excimer fluorescence compared to fully matched duplexes. Taking into account stability of 2′-OMe-RNA to action of nucleases, as well as long wavelength emission of the pyrene excimer (λ_{max} ~500 nm), we propose employment of such probes for experiments in cell culture and *in vivo*. Thus, combining two isomeric 1-PEPy labels we received series of very promising probes for homogeneous fluorescence assays which might become useful for detection of nucleic acid hybridization, allowing accomplishment of experiments both *in vitro* and *in vivo*.

Hence, combined meta- and para-1-PEPy fluorochromes attached to 2′-OMe-RNA-oligonucleotides may become a useful tool for the detection of SNPs. Furthermore, 2′-OMe-RNA is stable to action of nucleases and 1-PEPy-labeled 2′-OMe-RNA probes may appear suitable for experiments in cell culture and *in vivo*.

4. Experimental section

4.1. General methods

Reagents obtained from commercial suppliers were used without further purification; 1-ethynylpyrene,[18] bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphane,[23] and diisopropylammonium tetrazolide[24] were prepared as described. Solvents were from Chimmed (Russia), mainly HPLC grade and used without further purification unless otherwise noted. DCM was always used freshly distilled over CaH_2. DMF was freshly distilled under reduced pressure. 500 MHz ^{1}H, 126 MHz ^{13}C, and 202 MHz ^{31}P NMR spectra were recorded on a Bruker DRX-500 spectrometer at 303 K and referenced to DMSO-d_6 (2.50 ppm for ^{1}H and 39.7 ppm for ^{13}C) and 85% aq. H_3PO_4, (0.00 ppm for ^{31}P); 600 MHz ^{1}H and 150 MHz ^{13}C NMR spectra were recorded on a Varian Unity NMR spectrometer at 303 K and referenced to $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C). Coupling constants are reported in Hz and refer to apparent multiplicities. The assignments of signals in ^{1}H and ^{13}C NMR spectra were performed using 2D ^{1}H–^{13}C HMBC and HSQC NMR spectra. High resolution mass spectra were recorded in positive ion mode using IonSpec FT ISR mass spectrometer (MALDI) or PE SCIEX QSTAR pulsar mass spectrometer (ESI). Analytical thin-layer chromatography was performed on Kieselgel 60 F_{254} precoated aluminium plates (Merck). Silica gel column chromatography was performed using Merck Kieselgel 60 0.040–0.063 mm.

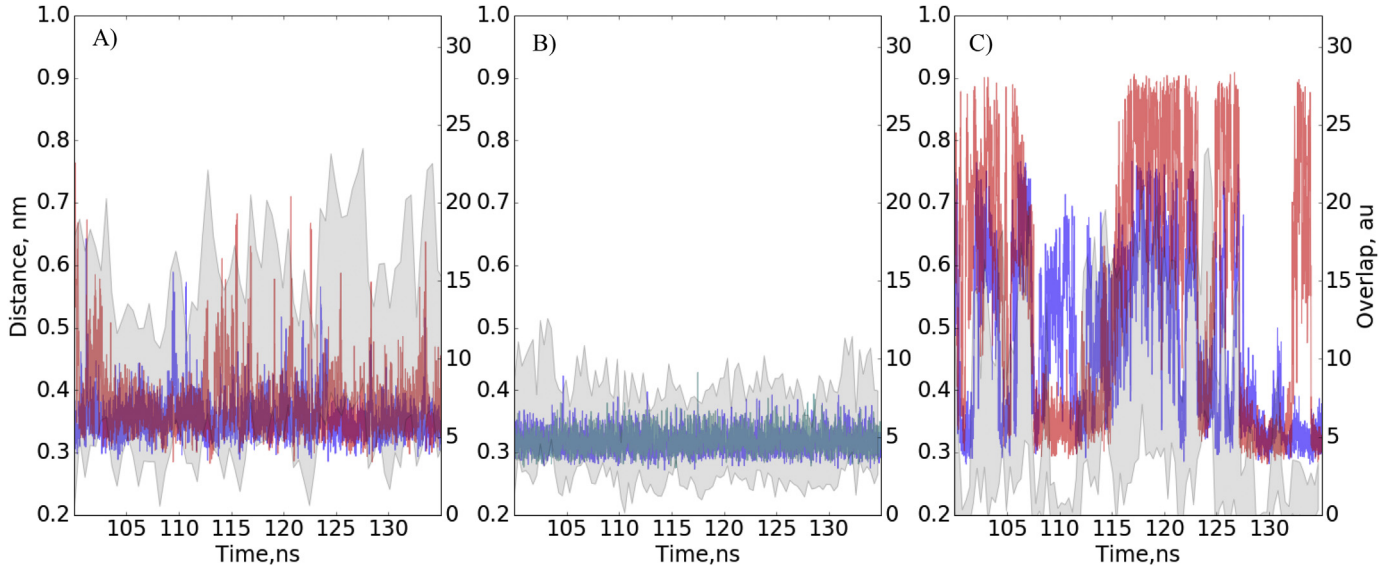


Fig. 7. MD simulated minimal distances between *meta*-PEPy residue (red) or *para*-PEPy residue (blue) and RNA backbone with gray overlap in arbitrary units is presented: A) **MP-U^{10}U^{11}/RT-WT**; B) **PP-U^{10}U^{11}/RT-WT**; C) **PM-U^{10}U^{11}/RT-WT**.

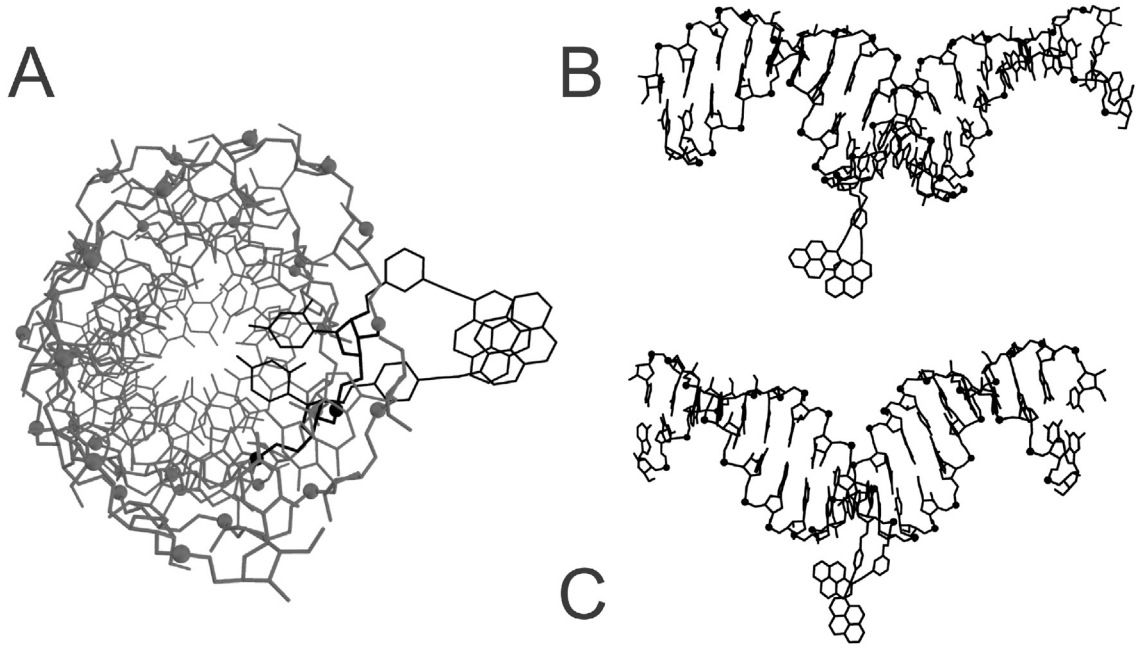


Fig. 8. Snapshots from the final part of MD simulation. A) **MP-U^{10}U^{11}/RT-WT** duplex; B) **PP-U^{10}U^{11}/RT-WT** duplex; C) **PM-U^{10}U^{11}/RT-WT** duplex.

4.2. Synthetic procedures

4.2.1. General procedure for the preparation of the compounds **2a,b**

N^3-Pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl) uridine[17] (1.65 g, 2.7 mmol) was dried by co-evaporation with dry MeCN (3 × 20 mL) and was then dissolved in dry MeCN–THF (50 mL, 1:1 v/v). Phosphazene base P1-*tert*-butyl-*tris*(tetramethylene) (1.29 mL, 4.05 mmol) followed immediately by 3- or 4-iodobenzylbromide (1.2 g, 4.05 mmol) were added to the stirred mixture at ambient temperature. TLC ($CHCl_3$–EtOH, 97:3 v/v) showed complete reaction after 3 h. The reaction mixture was evaporated to dryness in vacuo. The yellow oil was dissolved in $CHCl_3$ (100 mL) and washed with brine (1 × 100 mL) and water (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered, evaporated and co-evaporated with toluene (25 mL). The crude product was purified by column chromatography on silica gel (stepwise gradient of 0 → 4% EtOAc in toluene, v/v). The following compounds were obtained.

*4.2.1.1. 2′-O-(3-iodobenzyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl) uridine (**2a**).* Yield 1.61 g (72%), colorless oil, R_f 0.39 (5% EtOAc–toluene (v/v)). ^{1}H NMR (500 MHz; DMSO-d_6) δ 7.75 (m, 2H, H-6, H-2″), 7.64 (d, 1H, $J_{4'',5''}$ 7.7 Hz, H-4″), 7.38 (d, 1H, $J_{5'',6''}$ 7.7 Hz, H-6″), 7.13 (t, 1H, $J_{4'',5''} = J_{5'',6''}$ 7.7 Hz, H-5″), 5.83–5.72 (m, 4H, ^2J 11.2 Hz, H-5,1′, OCH_2N), 4.78 (m, 2H, ^2J 12.8 Hz, OCH_2Ar), 4.24 (m, 1H), 4.18 (m, 1H), 4.10 (m, 2H), 3.95 (m, 1H) (H-2′,3′,4′,5′), 1.10 (s, 9H, COCCH_3), 1.08–0.92 (m, 28H, SiCCHCH_3); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.59 (OCO), 161.26 (C4), 149.71 (C2), 140.86 (C6),

139.14 (C1″), 136.28 (C2″), 135.83 (C4″), 130.35 (C5″), 126.66 (C6″), 100.26 (C5), 94.83 (C3″), 89.40, 81.34 (C1′,2′), 80.37 (C4′), 70.32 (ArCH$_2$), 68.30 (NCH$_2$), 64.42 (C3′), 59.69 (C5′), 38.31 (*C*CH$_3$), 26.67 (3C, CCH$_3$), 17.37, 17.27, 17.20, 17.13, 17.03 (2C), 16.89, 16.84 (CH*C*H$_3$), 12.74, 12.35, 12.31, 12.13 (Si*C*). MALDI HRMS *m/z* 839.2206 [M+Na]$^+$ (calcd for $C_{34}H_{53}IN_2NaO_9Si_2^+$ 839.2226).

*4.2.1.2. 2′-O-(4-iodobenzyl)-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**2b**).* Yield 1.45 g (65%), white foam, R_f 0.42 (5% EtOAc–toluene (v/v)). ^{1}H NMR (500 MHz; DMSO-d_6) δ 7.75 (d, 1H, $J_{5,6}$ 8.2 Hz, H-6), 7.67 (d, 2H, *J* 8.2 Hz, H-3″,5″), 7.19 (d, 2H, *J* 8.2 Hz, H-2″,6″), 5.83–5.71 (m, 4H, H-5,1′, OC*H*$_2$N), 4.78 (s, 2H, OC*H*$_2$Ar), 4.22 (m, 1H), 4.17 (m, 1H), 4.09 (m, 2H), 3.94 (m, 1H) (H-2′,3′,4′,5′), 1.10 (s, 9H, COCC*H*$_3$), 1.07–0.89 (m, 28H, SiCC*HCH*$_3$); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.59 (O*C*O), 161.25 (C4), 149.70 (C2), 137.98 (C1″), 136.99 (2C, C3″,5″), 129.65 (2C, C2″,6″), 100.22 (C5), 93.44 (C4″), 89.40, 81.32 (C1′,2′), 80.17 (C4′), 70.60 (ArCH$_2$), 68.28 (NCH$_2$), 64.41 (C3′), 59.64 (C5′), 38.30 (*C*CH$_3$), 26.65 (3C, CCH$_3$), 17.36, 17.27, 17.19, 17.12, 16.98, 16.92, 16.81 (2C) (CH*C*H$_3$), 12.73, 12.35, 12.30, 12.06 (Si*C*). MALDI HRMS *m/z* 839.2227 [M+Na]$^+$ (calcd for $C_{34}H_{53}IN_2NaO_9Si_2^+$ 839.2226).

*4.2.2. General procedure for the preparation of the compounds **3a,b***

To a solution of corresponding starting nucleoside (1.304 g, 1.6 mmol) and 1-ethynylpyrene (433 mg, 1.9 mmol) in DMF (20 mL) under argon Pd(PPh$_3$)$_4$ (92 mg, 0.08 mmol), CuI (16 mg, 0.08 mmol) and triethylamine (327 μL, 3.2 mmol) were added, and the reaction mixture was stirred for 16 h at room temperature. The disappearance of the starting iodide was checked by TLC (10% EtOAc–toluene (v/v)). The mixture was then diluted with EtOAc (200 mL), washed with 0.1 M EDTA-(NH$_4$)$_2$ (2 × 200 mL) and water (5 × 200 mL), dried over Na$_2$SO$_4$, and evaporated to dryness. The residue was chromatographed on a silica gel column in step gradient 2 → 3 → 4% EtOAc in toluene. The following compounds were obtained.

*4.2.2.1. 2′-O-[3-(pyren-1-ylethynyl)benzyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**3a**).* Yield 947 mg (65%), yellow foam. R_f 0.42 (10% EtOAc–toluene (v/v)). ^{1}H NMR (600 MHz; CDCl$_3$) δ 8.65 (d, 1H, $J_{9''',10'''}$ 7.7 Hz, H-10‴), 8.26–8.17 (m, 4H, H-3‴,6‴,8‴,9‴), 8.13 (m, 1H, $J_{2''',3'''}$ 6.5 Hz, H-2‴), 8.10 (m, 1H, $J_{4''',5'''}$ 7.7 Hz, H-5‴), 8.05 (m, 1H, $J_{4''',5'''}$ 7.7 Hz, H-4‴), 8.03 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 6.1 Hz, H-7‴), 7.92 (d, 1H, $J_{5,6}$ 7.1 Hz, H-6), 7.79 (br.s, 1H, H-2″), 7.63 (d, 1H, $J_{4'',5''}$ 6.5 Hz, H-4″), 7.47 (d, 1H, $J_{4'',5''}$ 6.1 Hz, H-6″), 7.39 (m, 1H, H-5″), 5.98 (m, 2H, OC*H*$_2$N), 5.89 (br.s, 1H, H-1′), 5.72 (m, 1H, $J_{5,6}$ 7.1 Hz, H-5), 4.99 (m, 2H, OC*H*$_2$Ar), 4.27 (m, 2H), 4.20 (m, 1H), 4.00 (m, 1H), 3.89 (m, 1H) (H-2′,3′,4′,5′), 1.18 (s, 9H, CC*H*$_3$), 1.12–0.88 (m, 28H, SiCC*HCH*$_3$); ^{13}C NMR (150 MHz; CDCl$_3$) δ 177.46 (O*C*O), 161.70 (C4), 150.06 (C2), 138.30, 131.91, 131.27, 131.07, 130.91, 129.60, 128.48, 128.33, 128.16, 127.91, 127.25, 126.25, 125.57, 124.51, 124.35, 123.68, 117.79 (C1‴), 101.07 (C5), 95.03 (Cb), 89.30 (Ca), 88.67, 81.93, 81.39 (C1′,2′,4′), 71.56 (ArCH$_2$), 67.98 (NCH$_2$), 64.50 (C3′), 59.39 (C5′), 38.83 (*C*CH$_3$), 27.00 (3C, CCH$_3$), 17.43, 17.31, 17.04 (2C), 16.85 (2C), 16.64, 16.59 (CH*C*H$_3$), 13.38, 13.11, 12.84, 12.59 (Si*C*). MALDI HRMS *m/z* 937.3875 [M+Na]+ (calcd for $C_{52}H_{62}N_2NaO_9Si_2$+ 937.3886).

*4.2.2.2. 2′-O-[4-(pyren-1-ylethynyl)benzyl]-3′,5′-O-(tetraisopropyldisiloxan-1,3-diyl)uridine (**3b**).* Yield 1.23 g (84%), yellow foam. R_f 0.52 (10% EtOAc–toluene (v/v)). ^{1}H NMR (600 MHz; CDCl$_3$) δ 8.65 (d, 1H, $J_{9''',10'''}$ 7.7 Hz, H-10‴), 8.24–8.17 (m, 4H, H-3‴,6‴,8‴,9‴), 8.13 (m, 1H, $J_{2''',3'''}$ 6.6 Hz, H-2‴), 8.09 (m, 1H, $J_{4''',5'''}$ 7.1 Hz, H-5‴), 8.04 (m, 2H, H-4‴,7‴), 7.92 (d, 1H, $J_{5,6}$ 6.8 Hz, H-6), 7.68 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 6.6 Hz, H-3″,5″), 7.51 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 6.6 Hz, H-2″,6″), 5.98 (m, 2H, ^2J 8.2 Hz, OC*H*$_2$N), 5.88 (br.s, 1H, H-1′), 5.73 (m, 1H, $J_{5,6}$ 6.8 Hz, H-5), 4.99 (m, 2H, ^2J 11.0 Hz, OC*H*$_2$Ar), 4.27 (m, 2H), 4.20 (m, 1H), 4.01 (m, 1H), 3.88 (m, 1H) (H-2′,3′,4′,5′), 1.21 (s, 9H, CC*H*$_3$), 1.13–0.93 (m, 28H, SiCC*HCH*$_3$); ^{13}C NMR (150 MHz; CDCl$_3$) δ 177.47 (O*C*O), 161.68 (C4), 150.03 (C2), 138.25, 131.90, 131.66, 131.26, 131.08, 129.59, 128.33, 128.15, 127.77, 127.24, 126.24, 125.58, 124.51, 124.34, 122.87, 117.79 (C1‴), 101.07 (C5), 95.02 (Cb), 89.21 (Ca), 88.64, 81.91, 81.33 (C1′,2′,4′), 71.60 (ArCH$_2$), 68.06 (NCH$_2$), 64.49 (C3′), 59.39 (C5′), 38.84 (*C*CH$_3$), 27.01 (3C, CCH$_3$), 17.49, 17.42, 17.32, 17.04, 17.02, 16.85 (CH*C*H$_3$), 13.41, 13.14, 12.86, 12.61 (Si*C*). MALDI HRMS *m/z* 937.3841 [M+Na]$^+$ (calcd for $C_{52}H_{62}N_2NaO_9Si_2^+$ 937.3886).

*4.2.3. General procedure for the preparation of the nucleosides **4a,b***

To a solution of corresponding starting compound **3a,b** (915 mg, 1.0 mmol) in THF (3 mL) triethylamine thihydrofluoride (407 μL, 2.5 mmol) was added. The resulting solution was left at room temperature overnight. The completion of deprotection was checked by TLC (7% MeOH–CHCl$_3$ (v/v)). Reaction mixture was diluted with hexane (20 mL), the upper layer was discarded and the residue was washed with toluene-hexane (1:1 (v/v); 3 × 20 mL).

*4.2.3.1. 2′-O-[3-(pyren-1-ylethynyl)benzyl]uridine (**4a**).* Unprotected nucleoside was found to be soluble in CHCl$_3$, alcohols (MeOH, EtOH), and cannot be precipitated by trituration in any solvent. Crude product was chromatographed on a silica gel column with 3% MeOH–CHCl$_3$. Yield 564 mg (84%), yellow foam. R_f 0.42 (7% MeOH–CHCl$_3$ (v/v)). 1H NMR (500 MHz; DMSO-d_6) δ 8.63 (d, 1H, $J_{9''',10'''}$ 8.9 Hz, H-10‴), 8.38 (m, 3H, H-6‴,8‴,9‴), 8.33 (m, 1H, $J_{2''',3'''}$ 8.2 Hz, H-3‴), 8.27 (m, 2H, H-2‴,5‴), 8.23 (m, 1H, $J_{4''',5'''}$ 9.0 Hz, H-4‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.6 Hz, H-7‴), 8.04 (d, 1H, $J_{5,6}$ 8.2 Hz, H-6), 7.75 (br.s, 1H, H-2″), 7.69 (d, 1H, $J_{4'',5''}$ 6.9 Hz, H-4″), 7.44 (m, 2H, H-5″,6″), 6.03 (d, 1H, $J_{1',2'}$ = 5.0 Hz, H-1′), 5.80 (m, 3H, H-5, OC*H*$_2$N), 5.36 (d, 1H, *J* 5.8 Hz, 3′-O*H*), 5.21 (t, 1H, *J* 4.9 Hz, 5′-O*H*), 4.80 (m, 1H), 4.68 (m, 1H) (^2J 12.3 Hz, OC*H*$_2$Ar), 4.22 (m, 1H, H-3′), 4.08 (m, 1H, H-2′), 4.00 (m, 1H, H-4′), 3.75–3.57 (m, 2H, H-5′), 1.06 (s, 9H, CC*H*$_3$); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.60 (O*C*O), 161.11 (C4), 150.28 (C2), 139.98 (C6), 139.00 (C1″), 131.18 (C10a‴), 131.07 (C3a‴), 130.84 (C5a‴), 130.73, 130.56, 130.49, 129.69 (C2″,4″,2‴,8a‴), 128.93, 128.81 (C5″,9‴), 128.48 (C4‴), 127.97 (C6″), 127.30 (C5‴), 126.84 (C7‴), 126.09, 126.04 (C6‴,8‴), 125.00 (C3‴), 124.88 (C10‴), 123.73 (C10b‴), 123.47 (C10c‴), 122.44 (C3″), 116.73 (C1‴), 101.05 (C5), 95.17 (Cb), 88.31 (Ca), 87.29, 85.52, 80.97 (C1′,2′,4′), 70.61 (ArCH$_2$), 68.44 (NCH$_2$), 64.61 (C3′), 60.44 (C5′), 38.28 (*C*CH$_3$), 26.66 (3C, CCH$_3$). MALDI HRMS *m/z* 695.2369 [M+Na]$^+$ (calcd for $C_{40}H_{36}N_2NaO_8^+$ 695.2364).

*4.2.3.2. 2′-O-[4-(pyren-1-ylethynyl)benzyl]uridine (**4b**).* Crude material (brown oil) was triturated in 96% alcohol (5 mL), the crystalline nucleoside was filtered off, washed with EtOH (2 × 5 mL), diethyl ether (15 mL), and dried in vacuo. Yield 605 mg (90%), yellow solid. R_f 0.40 (7% MeOH–CHCl$_3$ (v/v)). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.62 (d, 1H, $J_{9''',10'''}$ 9.1 Hz, H-10‴), 8.38 (m, 3H, H-6‴,8‴,9‴), 8.32 (m, 1H, $J_{2''',3'''}$ 8.0 Hz, H-3‴), 8.26 (m, 2H, H-2‴,5‴), 8.22 (m, 1H, $J_{4''',5'''}$ 8.9 Hz, H-4‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.6 Hz, H-7‴), 8.02 (d, 1H, $J_{5,6}$ = 8.2 Hz, H-6), 7.72 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 8.0 Hz, H-3″,5″), 7.46 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 8.0 Hz, H-2″,6″), 6.02 (d, 1H, $J_{1',2'}$ 4.8 Hz, H-1′), 5.82 (m, 3H, H-5, OC*H*$_2$N), 5.35 (d, 1H, *J* 5.2 Hz, 3′-O*H*), 5.21 (br.s, 1H, 5′-O*H*), 4.80 (m, 1H), 4.67 (m, 1H) (^2J 12.8 Hz, OC*H*$_2$Ar), 4.22 (m, 1H, H-3′), 4.04 (m, 1H, H-2′), 4.00 (m, 1H, H-4′), 3.75–3.57 (m, 2H, H-5′), 1.13 (s, 9H, CC*H*$_3$); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.67 (O*C*O), 161.14 (C4), 150.29 (C2), 139.94 (C6), 139.23 (C1″), 131.45 (2C, C3″,5″), 131.15 (C10a‴), 131.04 (C3a‴), 130.84 (C5a‴), 130.56 (C8a‴), 129.65 (C2‴), 128.90 (C9‴), 128.46 (C4‴), 127.76 (2C, C2″,6″), 127.29 (C5‴), 126.83 (C7‴), 126.08, 126.04 (C6‴,8‴), 125.00 (C3‴), 124.89 (C10‴), 123.73 (C10b‴), 123.47 (C10c‴), 121.59 (C4″), 116.78 (C1‴), 101.07 (C5), 95.17 (Cb), 88.28 (Ca), 87.26, 85.54, 81.00 (C1′,2′,4′), 70.28 (ArCH$_2$), 68.43 (NCH$_2$),

64.71 (C3′), 60.44 (C5′), 38.35 (*C*CH$_3$), 26.72 (3C, C*C*H$_3$). MALDI HRMS *m/z* 695.2388 [M+Na]$^+$ (calcd for $C_{40}H_{36}N_2NaO_8^+$ 695.2364).

4.2.4. General procedure for the preparation of the compounds ***5a,b***

Corresponding unprotected nucleoside (**4a** or **4b**; 393 mg, 0.58 mmol) was co-evaporated with dry pyridine (3 × 15 mL) and then dissolved in pyridine (10 mL). The solution was cooled in an ice bath and DmtCl (204 mg, 0.6 mmol) was added in one portion. Reaction was kept at 0 °C for 4 h and then 16 h at room temperature. Reaction mixture was diluted with $CHCl_3$ (100 mL), washed with water (100 mL), 5% $NaHCO_3$ (100 mL), water (2 × 100 mL), then organic layer was dried over Na_2SO_4, evaporated, and the residue was chromatographed on a silica gel column with 3% acetone in toluene, containing 0.5% pyridine (v/v/v) giving the following compounds.

*4.2.4.1. 5′-O-(4,4′-dimethoxytrityl)-2′-O-[3-(pyren-1-ylethynyl)-benzyl]uridine (**5a**).* Yield 524 mg (92%), yellow foam. R_f 0.40 (10% acetone–toluene + 0.5% pyridine, v/v/v). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.62 (d, 1H, $J_{9''',10'''}$ 9.0 Hz, H-10‴), 8.39–8.31 (m, 4H, H-3‴,6‴,8‴,9‴), 8.27 (m, 2H, H-2‴,5‴), 8.23 (m, 1H, $J_{4''',5'''}$ 9.0 Hz, H-4‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.7 Hz, H-7‴), 7.83 (d, 1H, $J_{5,6}$ 8.2 Hz, H-6), 7.79 (br.s, 1H, H-2″), 7.69 (m, 1H, H-5″), 7.47 (m, 2H, H-4″,6″), 7.37 (m, 2H), 7.32 (m, 2H), 7.25 (m, 5H), 6.89 (d, 4H, *J* 8.7 Hz) (Dmt), 6.01 (d, 1H, $J_{1',2'}$ 3.6 Hz, H-1′), 5.79 (m, 2H, ^2J 9.4 Hz, OC*H*$_2$N), 5.45 (d, 1H, *J* 6.6 Hz, 3′-O*H*), 5.42 (d, 1H, *J* 8.2 Hz, H-5), 4.81 (m, 2H, ^2J 12.8 Hz, OC*H*$_2$Ar), 4.34 (m, 1H, H-3′), 4.13 (m, 2H, H-2′,4′), 3.72 (s, 6H, OC*H*$_3$), 3.40–3.26 (m, 2H, H-5′), 1.06 (s, 9H, CC*H*$_3$); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.61, 161.01, 158.21 (2C), 150.05, 144.59, 139.91, 138.98, 135.39, 135.09, 131.18, 131.06, 130.83, 130.75, 130.55, 130.42, 129.82 (4C), 129.67, 128.93, 128.90, 128.84, 128.47, 128.23, 127.98 (2C), 127.93, 127.77 (2C), 127.28, 126.08, 126.02, 124.98, 124.87, 123.72, 123.46, 122.50, 116.73, 113.33 (4C), 100.71, 95.19, 88.35, 88.21, 86.07, 83.12, 80.69, 70.71, 68.63, 64.62, 62.61, 55.08 (2C), 38.28, 26.65 (3C). MALDI HRMS *m/z* 997.3666 [M+Na]$^+$ (calcd for $C_{61}H_{54}N_2NaO_{10}^+$ 997.3671).

*4.2.4.2. 5′-O-(4,4′-dimethoxytrityl)-2′-O-[4-(pyren-1-ylethynyl)-benzyl]uridine (**5b**).* Yield 362 mg (64%), yellow foam. R_f 0.45 (10% acetone–toluene + 0.5% pyridine, v/v/v). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.62 (d, 1H, $J_{9''',10'''}$ 9.1 Hz, H-10‴), 8.38 (m, 3H, H-6‴,8‴,9‴), 8.32 (m, 1H, $J_{2''',3'''}$ 8.0 Hz, H-3‴), 8.27 (m, 2H, H-2‴,5‴), 8.22 (m, 1H, $J_{4''',5'''}$ 9.0 Hz, H-4‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.6 Hz, H-7‴), 7.81 (d, 1H, $J_{5,6}$ 8.2 Hz, H-6), 7.73 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 8.0 Hz, H-3″,5″), 7.50 (d, 2H, $J_{2'',3''} = J_{5'',6''}$ 8.0 Hz, H-2″,6″), 7.39–7.30 (m, 4H), 7.25 (m, 5H), 6.90 (m, 4H) (Dmt), 6.00 (d, 1H, $J_{1',2'}$ 3.6 Hz, H-1′), 5.81 (m, 2H, ^2J 9.6 Hz, OC*H*$_2$N), 5.46–5.40 (d, 2H, H-5, 3′-O*H*), 4.80 (m, 2H, ^2J 13.0 Hz, OC*H*$_2$Ar), 4.32 (m, 1H, H-3′), 4.09 (m, 2H, H-2′,4′), 3.74 (s, 6H, OC*H*$_3$), 3.40–3.26 (m, 2H, H-5′), 1.12 (s, 9H, CC*H*$_3$); ^{13}C NMR (126 MHz; DMSO-d_6) δ 176.68, 161.04, 158.22 (2C), 150.06, 144.57, 139.87, 139.19, 135.42, 135.08, 131.49 (2C), 131.15, 131.04, 130.84, 130.56, 129.85 (2C), 129.80 (2C), 129.66, 128.90, 128.47, 128.00 (2C), 127.79 (2C), 127.75 (2C), 127.29, 126.88, 126.83, 126.09, 126.04, 125.00, 124.88, 123.73, 123.47, 121.62, 116.78, 113.34 (4C), 100.75, 95.19, 88.32, 88.16, 86.10, 83.18, 80.69, 70.84, 68.62, 64.70, 62.65, 55.11 (2C), 38.35, 26.71 (3C). MALDI HRMS *m/z* 997.3708 [M+Na]$^+$ (calcd for $C_{61}H_{54}N_2NaO_{10}^+$ 997.3671).

4.2.5. General procedure for the preparation of the phosphoramidites ***6a,b***

The starting nucleoside derivative **5a,b** (471 mg, 0.48 mmol) was evaporated with anhydrous DCM (2 × 15 mL), dissolved in anhydrous DCM (20 mL), diisopropylammonium tetrazolide (121 mg, 0.7 mmol) and bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine (431 μL, 0.89 mmol) were added under argon, and the mixture was stirred for 16 h. After conversion of the starting compound was complete (monitoring by TLC (10% acetone–toluene + 0.5% pyridine (v/v/v))), the mixture was diluted with EtOAc (100 mL) and washed with a saturated solution of $NaHCO_3$ (2 × 100 mL) and brine (150 mL). The organic layer was dried over Na_2SO_4, evaporated and the residue was purified by chromatography on silica gel using 10% acetone in toluene, containing 0.5% pyridine (v/v/v).

*4.2.5.1. 3′-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[3-(pyren-1-ylethynyl)benzyl]-uridine (**6a**).* Yield 514 mg (94%), yellow foam. R_f 0.39, 0.36 (10% acetone–toluene + 0.5% pyridine, v/v/v). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.61 (m, 1H, H-10‴), 8.40–8.31 (m, 4H, H-3‴,6‴,8‴,9‴), 8.29–8.22 (m, 3H, H-2‴,4‴,5‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.7 Hz, H-7‴), 7.90 (m, 1H, H-6), 7.75 (m, 1H, H-2″), 7.70 (m, 1H, H-5″), 7.48–7.42 (m, 2H, H-4″,6″), 7.41–7.21 (m, 9H), 6.89 (m, 4H) (Dmt), 6.01 (m, 1H, H-1′), 5.78 (m, 2H, OC*H*$_2$N), 5.39 (m, 1H, H-5), 4.88–4.73 (m, 2H, OC*H*$_2$Ar), 4.52 (m, 1H, H-3′), 4.34–4.18 (m, 2H, H-2′,4′), 3.86–3.50 (m, 10H, OC*H*$_3$, POC*H*$_2$, PNC*H*), 3.44–3.32 (m, 2H, H-5′), 2.76 (m, 0.88H), 2.65 (m, 1.12H) (C*H*$_2$CN), 1.12–0.96 (m, 21H, CC*H*$_3$, CHC*H*$_3$). ^{31}P NMR (202.4 MHz; DMSO-d_6) δ 149.36 (0.41P), 148.52 (0.59P). ESI HRMS *m/z* 1197.4773 [M+Na]$^+$ (calcd for $C_{70}H_{71}N_4NaO_{11}P^+$ 1197.4749).

*4.2.5.2. 3′-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-5′-O-(4,4′-dimethoxytrityl)-2′-O-[4-(pyren-1-ylethynyl)benzyl]-uridine (**6b**).* Yield 536 mg (97%), yellow foam. R_f 0.49, 0.45 (10% acetone–toluene + 0.5% pyridine, v/v/v). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.62 (d, 1H, *J* 9.2 Hz, H-10‴), 8.39 (m, 3H, H-6‴,8‴,9‴), 8.33 (d, 1H, *J* 7.9 Hz, H-3‴), 8.27 (m, 2H) (H-2‴,5‴), 8.23 (m, 1H, $J_{4''',5'''}$ 8.8 Hz, H-4‴), 8.14 (apparent t, 1H, $J_{6''',7'''} = J_{7''',8'''}$ 7.6 Hz, H-7‴), 7.86 (m, 1H, H-6), 7.72 (m, 2H, H-3″,5″), 7.47 (m, 2H, H-2″,6″), 7.42–7.22 (m, 9H), 6.90 (m, 4H) (Dmt), 5.99 (m, 1H, H-1′), 5.80 (m, 2H, OC*H*$_2$N), 5.42 (m, 1H, H-5), 4.87–4.73 (m, 2H, OC*H*$_2$Ar), 4.50 (m, 1H, H-3′), 4.30–4.17 (m, 2H, H-2′,4′), 3.85–3.52 (m, 10H, OC*H*$_3$, POC*H*$_2$, PNC*H*), 3.44–3.32 (m, 2H, H-5′), 2.76 (m, 0.95H), 2.65 (m, 1.05H) (C*H*$_2$CN), 1.18–0.96 (m, 21H, CC*H*$_3$, CHC*H*$_3$). ^{31}P NMR ([D$_6$]DMSO, 202.4 MHz) δ 149.36 (0.41P), 148.52 (0.59P). ^{31}P NMR (202.4 MHz; DMSO-d_6) δ 149.36 (0.41P), 148.52 (0.59P). ESI HRMS *m/z* 1197.4752 [M+Na]$^+$ (calcd for $C_{70}H_{71}N_4NaO_{11}P^+$ 1197.4749).

4.2.6. Synthesis, purification and analyses of oligonucleotides used in this study

The oligonucleotide synthesis was carried out on a BiosSet ASM-800 instrument in a 100 nmol scale using standard manufacturer's protocols. The coupling step time for the modified phosphoramidites was extended to 5 min. Oligonucleotides were isolated using 20% denaturing (7 M urea) PAGE in Tris-borate buffer, pH 8.3, electoeluted by EluTrap (Whatman) and purified by Akta Purifier RP-HPLC (Phenomenex Jupiter C18 column 4.6 × 250 mm, linear gradient of MeCN 0 → 60% during 30 min). Concentrations of PEPy labeled probes were measured as described.[12l] MALDI-TOF mass spectra of oligonucleotides were recorded on Bruker Ultraflex mass spectrometer in positive ion mode; a mixture (1:1 v/v) of 2,6-dihydroxyacetophenone (40 mg/mL in MeOH) and ammonium hydrogen citrate (80 mg/mL in water) was used as a matrix. Measured masses of the oligomers (calculated masses *m/z* for [M+H]$^+$): **DT-WT**: 6147 (6147); **DT-A43G**: 6163 (6162); **DT-A43C**: 6124 (6122); **DT-A44G**: 6148 (6147); **RT-WT**: 6455 (6452); **RT-A43G**: 6468 (6468); **RT-A43C**: 6429 (6428); **RT-A44G**: 6452 (6452);

DP-U^{9}U^{10}: 6724 (6724); **DP-U^{9}U^{10}**: 6725 (6724); **DP-U$_p$^{9}U$_m$^{10}**: 6724 (6724); **DP-U$_p$^{9}U$_p$^{10}**: 6722 (6724); **DP-U^{10}U^{11}**: 6720 (6724); **DP-U^{10}U^{11}**: 6723 (6724); **DP-U^{10}U^{11}**: 6719 (6724); **DP-U$_p$^{10}U$_p$^{11}**: 6721 (6724); **MP-U^{9}U^{10}**: 7197 (7194); **MP-U^{9}U^{10}**: 7196 (7194); **MP-U^{9}U^{10}**: 7196 (7194); **MP-U^{9}U^{10}**: 7194 (7194); **MP-U^{10}U^{11}**: 7196 (7194); **MP-U^{10}U^{11}**: 7194 (7194); **MP-U^{10}U^{11}**: 7196 (7194); **MP-U^{10}U^{11}**: 7195 (7194).

4.2.7. Fluorescence steady-state emission studies

Fluorescence spectra were obtained in a medium salt buffer (100 mM NaCl, 10 mM Na-phosphate, 0.1 mM EDTA, pH 7.0) using a PerkinElmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller. For recording of fluorescence spectra 0.2 μM concentrations of the ss probe or corresponding duplex were used; excitation wavelength 360 nm.

4.2.8. Structure modeling

Modified nucleotide geometries were optimized at B3LYP 6-31G(3d, 2f) level followed by point atomic charge calculation from ESP. Last one was derived on same level of theory with R.E.D. tool based on resp approach.[25] Molecular topology with optimized coordinates was built by Acpype.[26] Parameters for modified residues was built by merging parameters from parmbsc0 uridine and developed set of parameters for 1-phenylethynylpyrene based on 1-(3(or 4)-methoxyphenyl)-ethynylpyrene as a template. Initial structures of all duplexes were prepared with X3DNA package.[27] Classical geometry optimization was performed for each duplex individually. Explicit solvent simulations in parmbsc0 force field[28] were performed at $T = 300$ K under control of velocity rescaling thermostat,[29] with isotropic constant-pressure boundary conditions under the control of the Berendsen algorithm of pressure coupling[30] with application of particle mesh Ewald method for long-range electrostatics interactions (PME).[31] A triclinic box of the TIP4P[32] water molecules was added around the RNA to a depth 15 Å on each side of the solute. Negative charges of systems were neutralized by adding of sodium cations, resulting in ~0.15 M concentration of the ions. We used two temperature coupling groups in the simulations. The first one is modified RNA duplex and the second one – water with ions. Analysis was carried out by tools from GROMACS 5.0 software package[33] and with python scripts. Overlap values were obtained as number of contacts of each carbon in pyrene ring with carbons in other pyrene ring in distance equal or less than 3.6 A.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2017.04.045.

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