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Synthesis and biophysical characterization of oligonucleotides modified with O2'-alkylated RNA monomers featuring substituted pyrene moieties†

Saswata Karmakar^a, Tyler Horrocks^{a,b}, Bradley C. Gibbons^{a,b}, Dale C. Guenther^a, Raymond Emehiser^a, and Patrick J. Hrdlicka^a

^aDepartment of Chemistry, University of Idaho, Moscow, ID-83844, USA

^bDepartment of Chemistry, Brigham Young University-Idaho, ID-83440, USA

Abstract

Over the past three decades, a wide range of pyrene-functionalized oligonucleotides have been developed and explored for potential applications in material science and nucleic acid diagnostics. Our efforts have focused on their possible use as components of Invader probes, i.e., DNA duplexes with +1 interstrand zipper arrangements of intercalator-functionalized nucleotides. We have previously demonstrated that Invader probes based on 2'-*O*-(pyren-1-yl)methyl-RNA monomers are energetically activated for sequence-unrestricted recognition of chromosomal DNA targets under non-denaturing conditions. As part of ongoing efforts towards delineating structure-property relationships and optimizing Invader probes, we report the synthesis and biophysical characterization of oligodeoxyribonucleotides (ONs) modified with 2'-*O*-(7-*neo*-pentylpyren-1-yl)methyl-uridine monomer **V** and 2'-*O*-(7-*tert*-butyl-1-methoxypyren-5-yl)methyl-uridine monomer **Y**. ONs modified with monomer **V** display increased DNA affinity (T_m up to +10.5 °C), while **Y**-modified ONs display lower DNA affinity and up to 22-fold increases in fluorescence emission upon RNA binding. Although these monomers display limited potential as building blocks for Invader probes, their photophysical properties render them of interest for diagnostic RNA-targeting applications.

TOC FIGURE

Oligonucleotides modified with 2'-*O*-(7-*neo*-pentyl-pyren-1-yl)methyl-uridine or 2'-*O*-(7-*tert*-butyl-1-methoxy-pyren-5-yl)methyl-uridine display prominent increases in pyrene fluorescence upon binding with complementary RNA (up to 22-fold).

Electronic supplementary information (ESI) available: General experimental section; protocol for synthesis of 7-*neo*-pentyl-1-pyrenemethanol and 7-*tert*-butyl-1-methoxy-5-pyrenemethanol; MS data for modified ONs; representative thermal denaturation curves; additional denaturation and thermodynamic data; additional absorption and steady-state fluorescence emission spectra; background discussion concerning the ΔG_{rec}^{293} term; additional dsDNA-recognition experiments; NMR spectra for labels and nucleosides.

Conflicts of interest. There are no conflicts of interest to declare.

Introduction

Major efforts have been devoted to the development of molecules that recognize specific double-stranded DNA (dsDNA) regions. These efforts are fueled by a need for tools that enable gene regulation, editing, and detection. Pioneering approaches included triplex-forming oligonucleotides, triplex-forming peptide nucleic acids (PNAs), and minor-groove binding polyamides.^{1–5} Subsequent developments aimed to partially overcome the inherent polypurine target site limitation of triplex-forming probes but were still rooted in triplex formation.^{6–12} A limited number of probes – which include pseudo-complementary (pc) DNA,¹³ pc-PNA^{14–16}, γ -PNA,¹⁷ and acpc-PNA¹⁸ – can recognize mixed-sequence dsDNA targets via invasion of Watson-Crick base-pairs. However, pcDNA only allows for recognition of terminal target regions, pc- and γ -PNA require low salinity for optimal strand invasion, and acpc-PNA has only been studied in a single context using fluorophore-modified and destabilized dsDNA targets. These circumstances, along with their challenging synthesis and restricted availability, have likely impeded the widespread popularization of these probes. In contrast, CRISPR/Cas RNA-guided endonucleases have shown an unparalleled ability to target and edit genomic DNA. Accordingly, CRISPR/Cas-systems are tremendously popular, even though the need to expand their targeting scope (i.e., reduce or eliminate PAM sequence restrictions), improve binding specificity, and develop viable delivery strategies for *in vivo* applications, is well-known.¹⁹ Presumably, future improvements of CRISPR/Cas-systems will address some of these challenges, but oligomer-based hybridization probes that display efficient, sequence-unrestricted, and error-free recognition of dsDNA targets are likely to offer several advantages, including simpler handling and delivery, more facile large-scale production, and lower cost.

Some years ago, our laboratory introduced an alternative class of oligonucleotide-based probes for sequence-unrestricted recognition of dsDNA.^{20,21} These so-called *Invader probes* are short DNA duplexes that are modified with +1 interstrand zipper arrangements of intercalator-functionalized nucleotides, which inherently renders the probes energetically activated for recognition of complementary dsDNA regions (Figure 1 – upper panel). The unique probe architecture forces the two intercalating moieties of the modified monomers to compete for the same inter-base-pair region, resulting in partial unwinding and destabilization of the probe duplex near these energetic hotspot,^{22,23} as the nearest-neighbor exclusion principle²⁴ is violated. The principle asserts that intercalation is anti-cooperative at adjacent sites due to limitations in local helix expandability (every intercalation event unwinds the duplex by ~ 3.4 Å) and to prevent disruption of stabilizing stacking interactions between a first intercalating moiety and surrounding base-pairs.^{25,26} In contrast, the two

strands comprising the energetically activated Invader probe duplex display very high affinity toward complementary DNA (cDNA) since duplex formation with cDNA results in strongly stabilizing stacking interactions between the intercalator and flanking base-pairs (Figure 1 – upper panel). The greater stability of the duplexes formed between individual probe strands and cDNA, as compared to the Invader probe duplex and the dsDNA target region, provides the thermodynamic driving force for dsDNA-recognition.²⁷

We originally used N2'-pyrene-functionalized-2'-amino- α -L-LNA (Locked Nucleic Acid) monomers as the key building blocks of Invader probes,^{20,21} but subsequently discovered that synthetically more readily available 2'-*O*-(pyren-1-yl)methyl-RNA monomers^{28,29} can serve as functional surrogates.²² We have used Invader probes based on 2'-*O*-(pyren-1-yl)methyl-RNA monomers for detection of mixed-sequence dsDNA sequences specific to food pathogens³⁰ and gender-specific chromosomal DNA targets in interphase and metaphase nuclei from bovine kidney cells under non-denaturing conditions,³¹ underscoring the potential of Invader probes for diagnostic applications. In parallel, we have carried out optimization studies aiming at further enhancing the dsDNA-recognition efficiency of Invader probes. Along these lines, we have examined the impact of different nucleobases,²⁹ sugar skeletons,^{22,32,33} linkers,²² intercalator sizes,^{33,34} sugar-intercalator orientations,²³ backbones,³⁵ modification patterns³¹ and additional destabilizing elements.^{36,37}

In the present study, we set out to evaluate O2'-pyrene-functionalized RNA monomers with bulky substituents on the pyrene moieties as potential building blocks for Invader probes. Informed by energy-minimized structures from prior force-field calculations on Invader probes based on 2'-*O*-(pyren-1-yl)methyl-RNA monomers,²³ we speculated that probe duplexes with +1 interstrand zipper arrangements of monomers with bulky substituents on the 7-position of the pyrene moiety will experience additional steric clashes, stacking perturbation, and destabilization (Figure 1 – middle panel). At the same time, we speculated that substituents on the 7-position might be able to point into the minor groove of duplexes between individual probe strands and cDNA with limited effect on duplex stability (Figure 1 – middle panel), thereby increasing the thermodynamic driving force for dsDNA-recognition. Based on expected synthetic availability (see ESI† for discussion), we set out to synthesize the corresponding phosphoramidites of 2'-*O*-(7-*neo*-pentylpyren-1-yl)methyl-uridine monomer **V** and 2'-*O*-(7-*tert*-butyl-1-methoxy-5-pyren-1-yl)methyl-uridine monomer **Y** (Figure 1 – lower panel). The synthesis, biophysical characterization, and cDNA/cRNA/dsDNA-binding properties of oligodeoxyribonucleotides (ONs) modified with these monomers is reported herein.

Results and Discussion

Synthesis of O2'-pyrene-functionalized uridine phosphoramidites.

Target phosphoramidites **4V** and **4Y** were synthesized in a similar manner as the corresponding 2'-*O*-(pyren-1-yl)methyl-uridine phosphoramidite²⁸ (Scheme 1). Thus, substituted tris(pyrenyl)methyl borates - generated in situ³⁹ through addition of 7-*neo*-pentyl-1-pyrenemethanol (Scheme S1†) or 7-*tert*-butyl-1-methoxy-5-pyrenemethanol (Scheme S2†) to borane-tetrahydrofuran - were reacted with O2,O2'-anhydrouridine **1**⁴⁰ in hot DMSO to afford nucleosides **2V** and **2Y** in low, but acceptable yields. We have

previously explored alternative synthetic strategies to O2'-pyrene-functionalized uridine phosphoramidites including routes proceeding via O3',O5'-protected uridines but have found the present strategy to be equally efficient and more practical.⁴¹ Subsequent O5'-dimethoxytritylation using standard conditions afforded nucleosides **3V** and **3Y** in 64% and 70% yield, respectively, which were then treated with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite and Hünig's base to provide target phosphoramidites **4V** and **4Y** in 81% and 80% yield, respectively.

Synthesis of modified oligodeoxyribonucleotides.

Phosphoramidites **4V** and **4Y** were used to incorporate monomers **V** and **Y** into oligodeoxyribonucleotides via machine-assisted solid-phase DNA synthesis using 4,5-dicyanoimidazole as an activator and extended hand-coupling (15 min), which resulted in stepwise coupling yields of ~97–99% for the modified monomers. The identity and purity of the modified ONs was established via MALDI-TOF (Table S1†) and ion-pair reverse phase HPLC (>90% purity), respectively. ONs modified with the reference 2'-*O*-(pyren-1-yl)methyl-uridine monomer **U** were available from previous studies.^{23,28} The pyrene-functionalized monomers were studied in two 9-mer ON sequences that we use routinely for evaluation of potential Invader building blocks. ONs containing a single incorporation in the 5'-GBG ATA TGC context are denoted **U1**, **V1**, and **Y1**, with similar conventions applying for the **B2-B6** series (Table 1). Reference DNA and RNA strands are denoted **D1**, **D4**, **R1**, and **R4** (see footnote, Table 1).

Thermal denaturation properties.

Melting temperatures (T_m 's) of duplexes between **B1-B6** and complementary DNA or RNA targets were determined from thermal denaturation experiments performed in medium salt phosphate buffers ($[Na^+] = 110$ mM). The resulting denaturation curves display monophasic sigmoidal transitions as expected (Figure S1†). The effect upon incorporating monomer **V** into DNA duplexes ranges from slightly destabilizing to highly stabilizing (T_m /modification = -1.5 °C to $+10.5$ °C, Table 1), whereas the introduction of **Y** monomers results in duplexes that are less stable (T_m /mod = -6.5 °C to $+6.5$ °C, Table 1). Higher T_m -values are observed when the pyrene-functionalized monomers are flanked by a 3'-purine, which is indicative of a 3'-directional intercalative binding mode of the pyrene moiety⁴² (e.g., compare T_m /mod values for **B2** and **B4**-series, Table 1). DNA duplexes modified with the reference 2'-*O*-(pyren-1-yl)methyl-uridine monomer **U** are much more stable, which suggests that the substituted pyrene moieties of monomers **V** and **Y** interfere with duplex formation. This may be due to steric clashes between the substituents and the backbone, less efficient pyrene-nucleobase stacking interactions, and/or disruption of the stabilizing hydration spines that surround DNA duplexes.

Duplexes between **V**- or **Y**-modified ONs and cRNA are strongly destabilized (T_m /mod between -0.5 °C and -12.0 °C, Table 1), with the monomer trend being **Y** (most destabilizing) < **V** < **U** (least destabilizing). Prominent DNA-selectivity, defined as T_m /mod (DNA-RNA) = T_m /mod (vs DNA) - T_m /mod (vs RNA) > 0 °C, is observed for **V**- or **Y**-modified ONs (T_m /mod (DNA-RNA) between 5.5 °C and 14.5 °C, Table S2†), which further points towards intercalative binding modes for the substituted pyrene

moieties, as intercalators typically favor the less compressed *B*-type helix geometry of DNA:DNA duplexes.⁴³

The binding specificities of singly modified ONs (**B2**-series) were determined against DNA and RNA strands with mismatched nucleotides opposite of the pyrene-functionalized monomers. **V2** and **Y2** generally display less efficient discrimination of mismatched DNA and RNA targets than reference strand **D1**, especially when mismatched pyrimidines are opposite the pyrene-functionalized monomer (Table 2 and S3†).

Optical spectroscopy.

UV-vis absorption and steady-state fluorescence emission spectra of **V**- and **Y**-modified ONs were recorded under non-deoxygenated conditions in the presence or absence of cDNA/cRNA and compared to corresponding spectra involving **U**-modified ONs. Intercalation of pyrene moieties is known to promote bathochromic shifts of pyrene absorption peaks^{42,44} and nucleobase-mediated quenching of pyrene fluorescence.^{29,42,45} **V**-modified ONs generally display prominent bathochromic shifts upon duplex formation ($\lambda_{\text{max}} = 0\text{--}6$ nm, Tables 3 and S5†, Figure S2†), with larger shifts being observed upon cDNA binding. In contrast, **Y**-modified ONs only display minor shifts upon cDNA/cRNA binding (Tables 3 and S5†) but the absorption bands are broad with little structure and caution must be exercised when interpreting these results (Figure S3†). We note that the absorption maxima of the functionalized pyrene moieties of monomers **V** and **Y** are red-shifted by ~ 5 nm relative to the reference 2'-*O*-(pyren-1-yl)methyl-uridine monomer **U**, presumably due to electronic substituent effects (Table S5).

Steady-state fluorescence emission spectra ($\lambda_{\text{ex}} = 350$ nm; $T = 5$ °C) of **V**-modified ONs and the corresponding duplexes with cDNA/cRNA display two major vibronic bands at $\lambda_{\text{em}} = 385 \pm 3$ nm and 405 ± 4 nm, along with a small shoulder at $\sim 420 \pm 4$ nm (Figure 2 and S5†). **Y**-modified ONs and their corresponding duplexes with DNA/RNA display similar features, except that the two main vibronic bands are shifted to $\lambda_{\text{em}} = 390 \pm 3$ nm and 410 ± 3 nm, with a shoulder at $\sim 428 \pm 2$ nm (Figure 2 and S6†). The emission intensity of **V**-modified ONs decreases by 60–80% upon cDNA binding, whereas moderate-to-prominent increases are observed upon cRNA binding (1.1- to 6.7-fold, Figure 3). Binding of **Y**-modified ONs to cDNA results in emission changes that vary from $\sim 70\%$ reduction to 1.8-fold increases, whereas cRNA binding consistently results in prominent emission increases ranging from 2.1- to 22-fold greater than the single-stranded probes (Figure 3). The larger relative increases in emission observed upon cRNA binding with **Y**-modified ONs relative to **V**-modified ONs are mainly due to more efficient quenching of the single-stranded probes rather than more emissive duplexes. Emission is most intense with **V4/Y4** (5'-ABC-3'), followed by **V2/Y2** and **V5/Y5** (5'-ABA-3'), and weakest with **V1/Y1** (5'-GBG-3') and **V3/Y3** (5'-ABG-3'), indicating that the nature of the surrounding nucleotides influences the fluorescence properties of the pyrene-functionalized monomers.

The following observations suggest that the substituted pyrene moieties of the **V**- and **Y**-modified ONs monomers, on balance, are more likely to intercalate into the base-stack upon cDNA binding, and more likely to occupy extrahelical positions upon cRNA binding: 1)

pronounced DNA-selectivity (i.e., $T_m/\text{mod (DNA-RNA)} \gg 0^\circ\text{C}$); 2) more pronounced bathochromic shifts upon cDNA binding (with **V**-modified ONs); 3) duplexes with cRNA more emissive than duplexes with cDNA; and 4) higher I_1/I_3 peak intensity ratios upon duplex formation with cRNA than with cDNA (Figures 2, S5† and S6†), suggesting that the pyrene moieties are located in a more polar microenvironment with the former, and a more hydrophobic microenvironment with the latter.⁴⁶ In contrast, ONs modified with the reference 2'-*O*-(pyren-1-yl)methyl-uridine **U** monomer result in decreased emission with both cDNA and cRNA (50–80% and 20–80%, respectively, Figure 3), indicating that intercalative binding modes are more prevalent with the unsubstituted pyrene moiety. This is further corroborated by the more pronounced bathochromic shifts (Table 3) and lower I_1/I_3 peak intensity ratios observed upon cRNA binding of **U**-modified ONs relative to **V**-/ **Y**-modified ONs ($I_1/I_3 \sim 1.1$, ~ 1.3 and ~ 1.6 , respectively, Figures 2 and S4-S6†).⁴⁷

The photophysical properties of **Y**-modified ONs render them of interest for further exploration, especially since **U**-modified ONs have been explored for a range of potential applications in material science and diagnostics.⁴¹

Properties of double-stranded probes with interstrand zippers of pyrene-functionalized monomers.

Owing to our interest in intercalator-functionalized nucleotides as possible building blocks of dsDNA-targeting Invader probes, we set out to study the thermal denaturation, thermodynamic, and photophysical properties of double-stranded probes with different interstrand zipper arrangements of **V** or **Y** monomers and compare them to the corresponding **U**-modified probes. As expected from our prior studies,^{20–23,28–37} duplexes with +1 interstrand zipper arrangements of **V** or **Y** monomers display significantly lower T_m 's than duplexes with other interstrand monomer arrangements⁴⁸ (compare T_m 's for **B2:B5** relative to the other double-stranded probes, Table 4). The distinct properties of +1 zipper probes are also reflected in the blue-shifted pyrene absorption maxima (compare λ_{max} for **B2:B5** relative to other probe duplexes, Table 4) and – for **V2:V5** – pyrene-pyrene excimer emission (Figure S7†). As discussed previously, the modified pyrene moieties of the **V** and **Y** monomers likely compete for the same inter-base-pair region within the probe duplex when positioned in +1 interstrand zipper arrangements due to their 3'-intercalative binding modes, resulting in a violation of the nearest neighbor exclusion principle.^{24–26} In double-stranded probes with other interstrand zipper arrangements of **V** or **Y** monomers, the pyrene moieties are unlikely to compete for the same inter-base-pair region and accordingly exert little direct influence on each other. Consequently, **B2:B5** duplexes display prominent *thermal advantage* (TA) defined as $TA(\text{ONX:ONY}) \equiv T_m(\text{ONX:cDNA}) + T_m(\text{cDNA:ONY}) - T_m(\text{ONX:ONY})$, where **ONX:ONY** is a duplex with an interstrand zipper arrangement of monomers, while other probe duplexes do not (compare TA values for **B2:B5** and other double-stranded probes, Table 4). Probes with large positive TA values are inherently activated for recognition and double-duplex invasion of isosequential dsDNA targets. Unfortunately, **V2:V5** and **Y2:Y5** display significantly lower TA values than reference probe **U2:U5** (23.5 °C, 19.5 °C, and 29.5 °C, respectively, Table 4), which indicates a lower dsDNA-recognition potential.

Thermodynamic parameters, determined through analysis of thermal denaturation profiles via the van't Hoff method,⁴⁹ largely corroborate the above T_m -based findings and conclusions (Tables 4, S6† and S7†). Hence, formation of duplexes between individual probe strands and cDNA is generally associated with more favorable changes in Gibbs free energy than formation of the corresponding unmodified DNA duplexes, with **U**- and **Y**-modified strands typically resulting in the most and least stabilized duplexes, respectively (e.g., compare $G^{293} = -5, -4$ and ~ 0 kJ/mol for duplexes between **U1**, **V1**, or **Y1** and cDNA, respectively, Table 4). The increased stability of duplexes between **U**- or **V**-modified ONs and cDNA is predominantly due to less unfavorable entropic factors, whereas no clear pattern is evident for duplexes entailing **Y**-modified ONs (Tables S6 and S7†). The double-stranded **B2:B5** probes are far less stable than duplexes between individual probe strands and cDNA (observe the third G^{293} column, Table 4) due to markedly less favorable enthalpy (Table S6†), presumably as stacking interactions are perturbed. Hence, **B2:B5** probes display a favorable thermodynamic gradient for recognition of isosequential dsDNA targets defined as $\Delta G_{rec}^{293}(\text{ONX:ONY}) = G^{293}(\text{ONX:cDNA}) + G^{293}(\text{cDNA:ONY}) - G^{293}(\text{ONX:ONY}) - G^{293}(\text{dsDNA})$ (i.e., observe ΔG_{rec}^{293} for **V2:V5** $\ll 0$ kJ/mol, Table 4).⁵⁰ However, the thermodynamic driving force is more prominent for reference probe **U2:U5**. Double-stranded probes with other interstrand arrangements of pyrene-functionalized monomers **V** or **Y**, display minimal potential for recognition of isosequential dsDNA targets (ΔG_{rec}^{293} between -7 and $+3$ kJ/mol, Table 4).

Recognition of dsDNA model targets.

On the basis of the observed T_A and ΔG_{rec}^{293} trends, we set out to evaluate the dsDNA-recognition efficiency of +1 zipper probes **V2:V5** and **Y2:Y5**, whilst comparing them to reference Invader probe **U2:U5**. Towards this end, we utilized an electrophoretic mobility shift assay that we previously developed and validated (Figure 4a).²² A digoxigenin (DIG) labeled DNA hairpin (DH) – comprised of a 9-mer double-stranded stem region with the same core sequence as the **B2:B5** probes and a T_{10} loop that connects the two strands at one terminus – served as a model dsDNA target. Successful recognition of **DH1** by **B2:B5** probes is expected to result in the formation of a ternary complex with lower electrophoretic mobility than **DH1** on native PAGE gels. The unimolecular nature of **DH1** stabilizes the stem region and reduces fraying at the free end ($T_m = 57.0$ °C for **DH1** vs. $T_m = 29.5$ °C for the corresponding DNA duplex **D1:D4** without the connecting T_{10} loop). This renders **DH1** as a more challenging target than the corresponding, isosequential linear dsDNA duplex.

Room temperature incubation of **DH1** with up to 500-fold molar excess of **V2:V5** or **Y2:Y5** did not result in formation of recognition complexes, whereas reference Invader probe **U2:U5** displayed dose-dependent recognition of **DH1** as previously reported (Figure 4b).²² To determine if the absence of **DH1**-recognition with **V2:V5** and **Y2:Y5** is due to inadequate binding affinity or slow kinetics, we performed experiments in which **DH1** was annealed (i.e., heated to 90 °C, followed by cooling) in the presence of a 500-fold molar excess of

V2:V5 and **Y2:Y5**. The resulting gel electrophoretograms only show traces of recognition complex formation (Figure S8†), suggesting that the recognition complexes are not sufficiently stable under the assay conditions. Accordingly, monomers **V** and **Y** featuring additionally substituted pyrene moieties have limited potential as Invader probe modifications, thus refuting our initial hypothesis.

Conclusion.

Although the two O2'-pyrene-functionalized uridine monomers reported herein have limited potential as building blocks for dsDNA-targeting Invader probes, they display other interesting biophysical properties. Thus, ONs modified with 2'-O-(7-*neo*-pentyl-pyren-1-yl)methyl-uridine monomer **V** result in moderate-to-prominent increases in T_m of duplexes with cDNA (T_m = up to 10.5 °C/mod), bathochromic shifts of pyrene absorption peaks upon cDNA binding consistent with intercalation, and increased fluorescence emission upon cRNA binding (1.1- to 6.7-fold increases). Conversely, ONs modified with 2'-O-(7-*tert*-butyl-1-methoxy-pyren-5-yl)methyl-uridine monomer **Y** display lower cDNA affinity but more pronounced pyrene fluorescence upon cRNA binding (increases ranging between 2.1- to 22-fold). Monomer **Y** presents itself as an interesting building block for RNA diagnostic applications, given that pyrene-functionalized ONs⁵¹ – and ONs modified with 2'-O-(pyren-1-yl)methyl RNA monomers in particular⁴¹ – have been extensively explored for a wide range of applications in material science and diagnostics.

Experimental Section

O2'-Alkylation protocol for the preparation of 2V and 2Y (description for ~4.4 mmol scale).—The appropriate pyrenemethanol, solid NaHCO₃, and 1 M borane-tetrahydrofuran complex were placed in a pressure tube, suspended in anhydrous DMSO, and stirred under an argon atmosphere at room temperature until effervescence ceased (~10 min). At this point, O2,O2'-anhydrouridine **1**⁴⁰ was added (quantities of substrates and reagents specified below), the pressure tube was purged with argon and sealed, and the mixture was heated at ~140 °C until analytical TLC indicated full conversion (~3h). After cooling to room temperature, the reaction mixture was poured into water (~50 mL), stirred for 30 min, and diluted with EtOAc (~100 mL). After separation of the layers, the organic phase was washed with water (4 × 50 mL), evaporated to dryness, and the resulting crude purified by silica gel column chromatography (2–4%, MeOH in CH₂Cl₂, v/v) to yield a residue, which was further purified by precipitation from refluxing methanol (30 min) to afford nucleoside **2** (yields specified below).

2'-O-(7-*neo*-pentylpyren-1-yl)methyl-uridine (2V).—O2,O2'-Anhydrouridine **1** (0.40 g, 1.77 mmol), 7-*neo*-pentyl-1-pyrenemethanol (Scheme S1†, 1.07 g, 3.54 mmol), NaHCO₃ (30 mg, 0.35 mmol), borane-tetrahydrofuran (0.88 mL, 0.88 mmol), and anhydrous DMSO (4 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **2V** (190 mg, 20 %) as a white solid material. R_f = 0.4 (10 % MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 551.2159 ([M+Na]⁺, C₃₁H₃₂N₂O₆Na⁺, Calc. 551.2153); ¹H NMR (DMSO-*d*₆): δ 11.30 (br s, 1H, ex, NH), 8.33–8.36 (d, 1H, J = 9.3 Hz, Py), 8.20–8.23 (d, 1H, J = 7.8 Hz, Py), 8.12–8.15 (m, 3H, Py), 8.06–8.08 (m, 3H, Py), 7.84 (d, 1H, J =

8.3 Hz, H6), 6.05 (d, 1H, J = 5.2 Hz, H1'), 5.50–5.52 (dd, 1H, J = 8.3 Hz, 1.5 Hz, H5), 5.41–5.44 (d, 1H, J = 11.4 Hz, CH₂Py), 5.35 (d, 1H, ex, J = 5.7 Hz, 3'-OH), 5.26–5.30 (d, 1H, J = 11.4 Hz, CH₂Py), 5.11 (t, 1H, ex, J = 5.2 Hz, 5'-OH), 4.26–4.30 (m, 1H, H3'), 4.18–4.21 (m, 1H, H2'), 3.95–3.98 (m, 1H, H4'), 3.64–3.69 (m, 1H, H5'), 3.57–3.62 (m, 1H, H5'), 2.93 (s, 2H, CH₂C(CH₃)₃), 1.00 (s, 9H, (CH₃)₃CCH₂); ¹³C NMR (DMSO-*d*₆) δ 162.9, 150.6, 140.2 (C6), 137.5, 131.2, 130.4, 130.2, 129.8, 128.5, 127.32 (Py), 127.30 (Py), 127.22 (Py), 127.18 (Py), 126.7 (Py), 124.3 (Py), 124.0, 123.4 (Py), 122.3, 101.7 (C5), 86.2 (C1'), 85.4 (C4'), 80.9 (C2'), 69.8 (CH₂Py), 68.5 (C3'), 60.5 (C5'), 49.7 (CH₂C(CH₃)₃), 31.8, 29.3 ((CH₃)₃C). A trace impurity of DMSO was observed at 2.54 ppm and 40.4 ppm in the ¹H NMR and ¹³C NMR spectra, respectively.

2'-O-(7-*tert*-butyl-1-methoxy-5-yl)methyl-uridine (2Y).—O₂,O₂'-

Anhydrouridine **1** (0.40 g, 1.77 mmol), 7-*tert*-butyl-1-methoxy-5-pyrenemethanol (Scheme S2†, 1.13 g, 3.54 mmol), NaHCO₃ (0.03 g, 0.35 mmol), borane-tetrahydrofuran (0.88 mL, 0.88 mmol), and anhydrous DMSO (4 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **2Y** (0.20 g, 21 %) as a white solid material. R_f = 0.4 (10 % MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 567.2114 ([M+Na]⁺, C₃₁H₃₂N₂O₇-Na⁺, Calc. 567.2102); ¹H NMR (DMSO-*d*₆): δ 11.28 (br s, 1H, ex, NH), 8.40 (d, 1H, J = 1.5 Hz, Py-H6), 8.30–8.33 (d, 1H, J = 9.2 Hz, Py-H10), 8.25 (d, 1H, J = 1.5 Hz, Py-H8), 8.18 (d, 1H, J = 8.5 Hz, Py-H3), 8.10–8.12 (d, 1H, J = 9.2 Hz, Py-H9), 8.06 (s, 1H, Py-H4), 7.82 (d, 1H, J = 8.1 Hz, H6), 7.71 (d, 1H, J = 8.5 Hz, Py-H2), 6.10 (d, 1H, J = 5.5 Hz, H1'), 5.45–5.47 (d, 1H, J = 8.1 Hz, H5), 5.39 (d, 1H, ex, J = 6.0 Hz, 3'-OH), 5.29–5.32 (d, J = 11.9 Hz, CH₂Py), 5.14–5.17 (d, 1H, J = 11.9 Hz, CH₂Py), 5.10 (t, 1H, ex, J = 5.0 Hz, 5'-OH), 4.33–4.37 (m, 1H, H3'), 4.24–4.27 (m, 1H, H2'), 4.15 (s, 3H, CH₃O), 3.97–4.00 (m, 1H, H4'), 3.57–3.68 (m, 2H, H5'), 1.51 (s, 9H, Me₃C); ¹³C NMR (DMSO-*d*₆) δ 162.8, 153.4 (Py-C1), 150.6, 148.9 (Py-C7), 140.2 (C6), 131.1 (Py-C8a), 130.0 (Py-C5), 129.6 (Py-C5a), 127.4 (Py-C4), 126.8 (Py-C9), 126.0 (Py-C3), 124.6 (Py-C3a1), 123.6 (Py-C3a), 122.7 (Py-C5a1), 121.3 (Py-C8), 120.2 (Py-C10), 118.9 (Py-C10a), 118.6 (Py-C6), 108.5 (Py-C2), 101.8 (C5), 86.1 (C1'), 85.4 (C4'), 80.3 (C2'), 70.3 (CH₂Py), 68.6 (C3'), 60.6 (C5'), 56.1 (CH₃O), 35.0 (CMe₃), 31.6 (Me₃C). A trace impurity of DMSO solvent was identified at 40.4 ppm in the ¹³C NMR spectrum. The HMBC spectrum is fully consistent with the proposed substitution pattern of the pyrene moiety of **2Y**. For full discussion, see the ESI†.

O5'-DMTr-protection protocol for the preparation of 3V and 3Y (description for ~1 mmol scale).—Nucleoside **2** was co-evaporated twice with anhydrous pyridine and re-dissolved in anhydrous pyridine (quantities specified below). To this was added 4,4'-dimethoxytrityl chloride (DMTrCl) and catalytic *N,N*-dimethyl-4-aminopyridine (DMAP), and the reaction mixture was stirred at rt under an argon atmosphere for ~14 h when analytical TLC indicated complete conversion. The reaction mixture was diluted with CH₂Cl₂ (40 mL) and the organic phase was sequentially washed with water (2 × 30 mL) and sat. aq. NaHCO₃ (2 × 50 mL). The organic phase was evaporated to near dryness and the resulting crude was co-evaporated with abs. EtOH and toluene (2:1, v/v, 3 × 3 mL) and purified by silica gel column chromatography (0–5%, MeOH in CH₂Cl₂, v/v) to afford nucleoside **3** (yield specified below).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(7-*neo*-pentylpyren-1-yl)methyl-uridine (3V).—

Nucleoside **2V** (130 mg, 0.25 mmol), DMTrCl (143 mg, 0.37 mmol), DMAP (~3 mg) and anhydrous pyridine (2.5 mL) were mixed, reacted, worked up, and purified as described above to afford **3V** (130 mg, 64 %) as pale yellow foam. $R_f = 0.6$ (5%, MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 853.3467 ([M+Na]⁺, C₅₂H₅₀N₂O₈·Na⁺, Calc. 853.3459); ¹H NMR (DMSO-*d*₆) δ 11.37 (s, 1H, ex, NH), 8.37–8.39 (d, 1H, $J = 9.3$ Hz, Py), 8.19–8.22 (d, 1H, $J = 7.8$ Hz, Py), 8.07–8.17 (m, 6H, Py), 7.64 (d, 1H, $J = 8.3$ Hz, H₆), 7.32–7.34 (m, 2H, DMTr), 7.24–7.28 (m, 2H, DMTr), 7.18–7.22 (m, 5H, DMTr), 6.81–6.87 (m, 4H, DMTr), 6.03 (d, 1H, $J = 4.2$ Hz, H1'), 5.45–5.48 (d, 1H, $J = 11.9$ Hz, CH₂Py), 5.43 (d, 1H, ex, $J = 6.5$ Hz, 3'-OH), 5.35–5.39 (d, 1H, $J = 11.9$ Hz, CH₂Py), 5.15 (d, 1H, $J = 8.3$ Hz, H₅), 4.34–4.39 (m, 1H, H₃'), 4.21–4.24 (m, 1H, H₂'), 4.06–4.09 (m, 1H, H₄'), 3.71 (s, 3H, CH₃O), 3.69 (s, 3H, CH₃O), 3.30–3.33 (m, 1H, H₅' – partial overlap with H₂O signal), 3.21–3.25 (m, 1H, H₅'), 2.94 (CH₂(CH₃)₃), 1.00 (s, 9H, (CH₃)₃C); ¹³C NMR (DMSO-*d*₆) δ 162.8, 158.09, 158.07, 150.4, 144.5, 140.0 (C₆), 137.5, 135.4, 135.0, 131.1, 130.5, 130.2, 129.79, 129.72 (DMTr), 129.66 (DMTr), 128.5, 127.8 (DMTr), 127.6 (DMTr), 127.4 (Py), 127.31 (Py), 127.25 (Py), 127.20 (Py), 126.7 (DMTr), 126.6 (Py), 124.3 (Py), 124.0, 123.3 (Py), 122.4, 113.20 (DMTr), 113.18 (DMTr), 101.4 (C₅), 87.1 (C1'), 85.9, 83.1 (C4'), 80.6 (C2'), 69.9 (CH₂Py), 68.7 (C3'), 62.8 (C5'), 55.0 (CH₃O), 49.7 (CH₂C(CH₃)₃), 31.8, 29.3 ((CH₃)₃CCH₂).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(7-*tert*-butyl-1-methoxyppyren-5-yl)methyl-uridine (3Y).—

Nucleoside **2Y** (180 mg, 0.33 mmol), DMTrCl (193 mg, 0.49 mmol), DMAP (~3 mg), and anhydrous pyridine (6 mL), were mixed, reacted, worked up, and purified as described above to afford **3Y** (195 mg, 70 %) as pale yellow foam. $R_f = 0.6$ (5%, MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 869.3441 ([M+Na]⁺, C₅₂H₅₀N₂O₉·Na⁺, Calc. 869.3409); ¹H NMR (DMSO-*d*₆): δ 11.35 (s, 1H, ex, NH), 8.43 (d, 1H, $J = 2.0$ Hz, Py), 8.30–8.33 (d, 1H, $J = 9.1$ Hz, Py), 8.27 (d, 1H, $J = 2.0$ Hz, Py), 8.11–8.17 (m, 3H, Py), 7.67 (d, 1H, $J = 8.6$ Hz, Py), 7.58 (d, 1H, $J = 8.1$ Hz, H₆), 7.27–7.30 (m, 2H, DMTr), 7.13–7.22 (m, 7H, DMTr), 6.79–6.82 (m, 2H, DMTr), 6.74–6.77 (m, 2H, DMTr), 6.10 (d, 1H, $J = 4.7$ Hz, H1'), 5.50 (d, 1H, ex, $J = 6.2$ Hz, 3'-OH), 5.35–5.38 (d, 1H, $J = 11.9$ Hz, CH₂Py), 5.22–5.26 (d, 1H, $J = 11.9$ Hz, CH₂Py), 5.06 (d, 1H, $J = 8.1$ Hz, H₅), 4.43–4.48 (m, 1H, H₃'), 4.26–4.29 (m, 1H, H₂'), 4.10–4.14 (m, 4H, CH₃OPy, H₄'), 3.71 (s, 3H, CH₃O), 3.69 (s, 3H, CH₃O), 3.28–3.32 (m, 1H, H₅' – overlap with H₂O signal), 3.19–3.22 (m, 1H, H₅'), 1.51 (s, 9H, Me₃C); ¹³C NMR (DMSO-*d*₆): δ 162.7, 158.04, 157.99, 153.4, 150.4, 149.5, 148.9, 144.3, 140.1 (C₆), 135.3, 134.9, 131.1, 129.8, 129.68 (DMTr), 129.66, 129.5 (DMTr), 127.8 (DMTr), 127.6 (Py, DMTr), 126.7 (Py), 126.6 (DMTr), 126.1 (Py), 124.7, 123.6, 122.7, 121.4 (Py), 120.3 (Py), 118.9, 118.6 (Py), 113.14 (DMTr), 113.10 (DMTr), 108.5 (Py), 101.4 (C₅), 86.8 (C1'), 85.9, 83.2 (C4'), 79.8 (C2'), 70.5 (CH₂Py), 68.9 (C3'), 63.0 (C5'), 56.1 (CH₃OPy), 54.96 (CH₃O), 54.95 (CH₃O), 35.0, 31.6 (Me₃C).

O3'-Phosphitylation protocol for the preparation of 4V and 4Y (description for ~1 mmol scale).—

Nucleoside **3** (quantities of substrates and reagents specified below) was co-evaporated with anhydrous 1,2-dichloroethane (4 mL) and re-dissolved in anhydrous CH₂Cl₂. To this was added *N,N*-diisopropylethylamine (DIPEA) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCl-reagent) and the reaction mixture

was stirred at room temperature until analytical TLC indicated complete conversion (~3h). At this point, abs. EtOH (2 mL) and CH₂Cl₂ (20 mL) were sequentially added and the organic phase was washed with sat. aq. NaHCO₃ (10 mL), evaporated to near dryness, and the resulting residue purified by silica gel column chromatography (40–70% EtOAc in petroleum ether, v/v) to afford the corresponding phosphoramidite **4** (yields specified below).

3'-O-(2-Cyanoethoxy(diisopropylamino)phosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2'-O-(7-*neo*-pentylpyren-1-yl)methyl-uridine (4V).—Nucleoside **3V** (110 mg, 0.13 mmol), DIPEA (94 μ L, 0.53 mmol), PCI-reagent (60 μ L, 0.26 mmol), and anhydrous CH₂Cl₂ (3 mL) were mixed, reacted, worked up, and purified as described above to afford phosphoramidite **4V** (110 mg, 81 %) as a white foam. R_f = 0.8 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 1053.4526 ([M+Na]⁺, C₆₁H₆₇N₄O₉P·Na⁺, Calc. 1053.4538); ³¹P NMR (CDCl₃) δ 150.2, 150.1.

3'-O-(2-Cyanoethoxy(diisopropylamino)phosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2'-O-(7-*tert*-butyl-1-methoxypyren-5-yl)methyl-uridine (4Y).—Nucleoside **3Y** (150 mg, 0.18 mmol), DIPEA (126 μ L, 0.71 mmol), PCI-reagent (79 μ L, 0.35 mmol), and anhydrous CH₂Cl₂ (10 mL) were mixed, reacted, worked up, and purified as described above to afford phosphoramidite **4Y** (148 mg, 80 %) as a white foam. R_f = 0.8 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 1069.4515 ([M+Na]⁺, C₆₁H₆₇N₄O₉P·Na⁺, Calc. 1069.4487); ³¹P NMR (CDCl₃) δ 149.50, 149.46.

Synthesis and purification of ONs.—V- or Y-modified ONs were synthesized on an automated DNA synthesizer using 0.2 μ mol scale succinyl linked LCAA-CPG (long chain alkyl amine controlled pore glass) support with a pore size of 500 Å. Standard protocols for incorporation of DNA phosphoramidites were used. A ~50-fold molar excess of modified phosphoramidites in anhydrous acetonitrile (at 0.05 M), along with extended oxidation (45 s) and coupling times (activator: 0.01 M 4,5-dicyanoimidazole, 15 min) were used during hand-couplings, which resulted in stepwise coupling yields of **4V** and **4Y** between 97–99%. ONs were cleaved from solid support and protecting groups removed with 32% aq. ammonia (55 °C, 24 h). The crude ONs were purified via ion-pair reverse phase HPLC (XTerra MS C18 column) using a 0.05 M triethylammonium acetate - water/acetonitrile gradient, followed by detritylation (80% aq. AcOH), and precipitation (NaOAc/NaClO₄/acetone, –18 °C for 12–16 h). The identity of the modified ONs was established by MALDI-MS analysis recorded in positive ions mode on a Quadrupole Time-Of-Flight Tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K) using anthranilic acid as a matrix. ON purity (>90%) was verified by ion-pair reverse phase HPLC running in analytical mode. ONs modified with the parent 2'-O-(pyren-1-yl)methyl-uridine monomer **U** were available from previous studies.^{23,28}

Protocol - thermal denaturation studies.—ON concentrations were estimated using the following extinction coefficients for DNA (OD/ μ mol): G (12.01), A (15.20), T (8.40), C (7.05); for RNA (OD/ μ mol): G (13.70), A (15.40), U (10.00), C (9.00); (OD/ μ mol): for pyrene⁵² (22.4). Strands were mixed and denatured by heating to 70–85 °C, followed by

cooling to the starting temperature of the experiment. Quartz optical cells with a path length of 1.0 cm were used. Thermal denaturation temperatures (T_m 's) of duplexes (1.0 μ M final concentration of each strand) were measured on a Cary 100 UV/VIS spectrophotometer equipped with a 12-cell Peltier temperature controller and determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs. T) recorded in medium salt buffer (T_m buffer: 100 mM NaCl, 0.1 mM EDTA, and pH 7.0 adjusted with 10 mM Na_2HPO_4 and 5 mM Na_2HPO_4). The temperature of the denaturation experiments ranged from at least 15 $^\circ\text{C}$ below T_m to 20 $^\circ\text{C}$ above T_m (although never below 3 $^\circ\text{C}$). A temperature ramp of 0.5 $^\circ\text{C}/\text{min}$ was used in all experiments. Reported T_m -values are averages of two experiments within ± 1.0 $^\circ\text{C}$.

Protocol - absorption spectra.—Absorption spectra (range 300–400 nm) were recorded at 5 $^\circ\text{C}$ using the same samples (i.e., each strand used at 1.0 μ M in T_m buffer) and instrumentation as in the thermal denaturation experiments.

Protocol - steady-state fluorescence emission spectra.—Steady-state fluorescence emission spectra of modified ONs and duplexes were recorded in thermal denaturation buffer (each strand used at 1.0 μ M) and obtained as an average of five scans using an excitation wavelength of $\lambda_{\text{ex}} = 350$ nm, excitation slit 5.0 nm, emission slit 2.5 nm, and a scan speed of 600 nm/min. Experiments were conducted at 5 $^\circ\text{C}$ to maximize duplex formation. Deoxygenation was deliberately not applied to the samples since the scope of the work was to determine fluorescence enhancement under aerated conditions prevailing in various bioassays. Fluorescence quantum yields were not determined for V- or Y-modified probes and their duplexes with cDNA/cRNA in this preliminary study.

Protocol - thermodynamic parameters.—Thermodynamic parameters were determined through baseline fitting of denaturation curves (van't Hoff method) using software provided with the UV-Vis spectrophotometer. Bimolecular reactions, two-state melting behavior, and constant heat capacity were assumed.⁴⁹ Values are reported as an average from two different melting curves, each analyzed three times, to minimize errors arising from baseline choice.

Definition of interstrand zipper nomenclature.—The following nomenclature describes the relative arrangement between two monomers positioned on opposing strands in probe duplexes. The number n describes the number of base pairs between the two monomers, and has a positive value if a monomer is shifted toward the 5'-side of its strand relative to a second reference monomer on the other strand. Conversely, n has a negative value if a monomer is shifted toward the 3'-side of its strand relative to a second reference monomer on the other strand.

Protocol - recognition of DNA hairpins.—The electrophoretic mobility shift assay was essentially performed as previously reported.³⁴ Thus, unmodified DNA hairpin **DH1** was obtained from commercial sources and used without further purification. **DH1** was 3'-DIG-labeled using the 2nd generation DIG Gel Shift Kit (Roche Applied Bioscience) as recommended by the manufacturer. The DIG-labeled **DH1** obtained in this manner, was

diluted and used directly in the recognition experiments. Pre-annealed probes (90 °C for 2 min, cooled to room temperature over 15 min) and DIG-labeled **DH1** (34.4 nM) were mixed and incubated in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 10% sucrose, 1.44 mM spermine tetrahydrochloride, pH 7.2) for 15 h at room temperature. The reaction mixtures were then diluted with 6x DNA loading dye (Fermentas) and loaded onto a 12% non-denaturing polyacrylamide gel. Electrophoresis was performed using constant voltage (~70 V) for 3 h at ~4 °C (internally circulating ice-cold water). Gels were blotted onto a positively charged nylon membrane (Roche Applied Bioscience) using constant voltage with external cooling (100V, ~4 °C). The membranes were exposed to anti-digoxigenin-AP F_{ab} fragments as recommend by the manufacturer of the DIG Gel Shift Kit, transferred to a hybridization jacket, and incubated with the substrate (CSPD) in detection buffer for 10 min at 37 °C. The chemiluminescence of the formed product was captured on X-ray film, which was developed using an X-Omatic 1000A X-ray film developer (Kodak). The resulting bands were quantified using a BioRad ChemiDoc MP Imaging System. The percentage of dsDNA recognition was calculated as the intensity ratio between the recognition complex band and the total lane. An average of three independent experiments is reported along with standard deviations (±).

Supplementary Material

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Figure 1.

Upper panel: Sequence-unrestricted recognition of double-stranded DNA targets using Invader probes (droplets denote intercalators). Middle panel: Top view of central region of energy-minimized structures for an Invader probe (left) and a probe-cDNA duplex (right) based on 2'-*O*-(pyren-1-yl)methyl RNA monomers.^{23,38} Arrows indicate the approximate location of substituents on the 7-position. The following color code is used - sugar phosphate backbone (red), pyrene moieties (blue), and nucleobases (green). Residues in pale colors are in the background. Adapted with permission from reference 23 (used with permission from

the Royal Society of Chemistry). Bottom panel: Structures of O2'-pyrene-functionailized uridine monomers studied herein.

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Figure 2.

Steady-state fluorescence emission spectra of single-stranded probes (SSPB2 and B5 and the corresponding duplexes with cDNA/cRNA. Spectra were recorded at $T = 5^\circ\text{C}$ using $\lambda_{\text{ex}} = 350\text{ nm}$ and each strand at $1.0\text{ }\mu\text{M}$ concentration in T_{m} buffer. Corresponding spectra for B1, B3 and B4 probes are shown in Figures S4-S6†. Spectra for U2 and U5 have been previously published in reference 23 (used with permission from the Royal Society of Chemistry) and are included to facilitate comparison.

Figure 3.

Fluorescence intensity of **B1–B5** and the corresponding duplexes with cDNA or cRNA targets as measured at $\lambda_{em} = 381, 383$ and 389 nm respectively. Hybridization-induced increases/decreases – defined as the intensity ratio between a duplex and single-stranded probe (SSP) – are listed above corresponding histograms. Spectra were recorded at $T = 5$ °C using $\lambda_{ex} = 350$ nm and each strand at 1.0 mM concentration in T_m buffer.

Figure 4. (a) Illustration of the assay used to monitor Invader-mediated recognition of model dsDNA targets. (b) Representative gel electropherograms illustrating dose-response experiment between **DH1** and 5- to 500-fold molar excess of **V2:V5**, **Y2:Y5** or **U2:U5**. DIG-labeled **DH1** (5'-GTGATATGC-T₁₀-GCATATCAC-3') was incubated with the specified probes in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.2, 10% sucrose, 1.44 mM spermine tetrahydrochloride) for 15 h at room temperature.

Scheme 1.

Synthesis of O2'-pyrene-functionalized uridine phosphoramidites **4V** and **4Y**. DMTr = 4,4'-dimethoxytrityl; PCl reagent = 2-cyanoethyl-*N,N'*-diisopropylchlorophosphoramidite; U = uracil-1-yl.

Table 1.

Thermal denaturation temperatures (T_m 's for duplexes between **B1-B6** and complementary DNA or RNA (cDNA/cRNA)).^a

ON	Duplex	B =	T_m [T_m /mod] (°C)					
			+cDNA			+cRNA		
			U ^b	V	Y	U ^b	V	Y
B1	5'-GBG ATA TGC		34.5 [+5.0]	32.0 [+2.5]	28.5 [-1.0]	24.5 [-2.0]	14.5 [-12.0]	16.5 [-10.0]
B2	5'-GTG ABA TGC		42.5 [+13.0]	40.0 [+10.5]	36.0 [+6.5]	30.5 [+4.0]	24.5 [-2.0]	21.5 [-5.0]
B3	5'-GTG ATA BGC		37.5 [+8.0]	31.0 [+1.5]	31.5 [+2.0]	26.5 [±0.0]	22.5 [-4.0]	17.0 [-9.5]
B4	3'-CAC BAT ACG		33.0 [+3.5]	28.0 [-1.5]	23.0 [-6.5]	20.0 [-4.5]	17.0 [-7.5]	14.0 [-10.5]
B5	3'-CAC TAB ACG		42.5 [+13.0]	38.0 [+8.5]	35.0 [+5.5]	27.0 [+2.5]	24.0 [-0.5]	23.0 [-1.5]
B6	3'-CAC BAB ACG		43.5 [+7.0]	-	30.5 [+0.5]	24.0 [-0.3]	-	12.5 [-6.0]

^a T_m = change in T_m 's relative to unmodified reference duplexes **D1:D4** (T_m = 29.5 °C), **D1:R4** (T_m = 26.5 °C), and **R1:D4** (T_m = 24.5 °C) where **D1**: 5'-[DNA]-GTG ATA TGC, **D4**: 3'-[DNA]-CAC TAT ACG, **R1**: 5'-[RNA]-GUG AUA UGC, and **R4**: 3'-[RNA]-CAC UAU ACG. T_m 's determined as the maximum of the first derivative of melting curves (A_{260} vs T) recorded in medium salt buffer ([Na⁺] = 110 mM, [Cl⁻] = 100 mM, pH 7.0 (NaH₂PO₄/Na₂HPO₄)), using 1.0 μM of each strand. T_m 's are averages of at least two measurements within 1.0 °C; For structures of monomers U, V, and Y, see Figure 1.

^b Data from reference 28 are included to facilitate comparison.

Table 2.Discrimination of mismatched DNA targets by **V2**, **Y2** and reference strands.^a

ON	Sequence	B =	DNA: 3'-CAC TBT ACG			
			T_m [°C]		T_m [°C]	
			A	C	G	T
D1	5'-GTG ATA TGC		29.5	-16.5	-9.5	-17.0
U2 ^b	5'-GTG AUA TGC		42.5	-13.5	-5.5	-7.0
V2	5'-GTG AVA TGC		40.0	-12.5	-4.5	-10.5
Y2	5'-GTG AYA TGC		36.0	-14.0	-7.0	-6.0

^aFor conditions of thermal denaturation experiments, see Table 1. T_m -values of fully complementary duplexes are shown in bold. T_m = change in T_m relative to fully complementary DNA:DNA duplex.

^bData from reference 28 are included to facilitate comparison.

Table 3.

Average bathochromic shifts of pyrene absorption bands in the 345–360 nm upon hybridization of **U/V/Y**-modified ONs with cDNA or cRNA^a

B =	average λ_{max} (nm)					
	U		V		Y	
	+cDNA	+cRNA	+cDNA	+cRNA	+cDNA	+cRNA
	3.4±1.1	2.6±0.9	3.0±2.4	1.4±1.1	0.2±0.8	0.8±0.8

^aMeasurements were performed at 5 °C in T_{M} buffer using a spectrophotometer and quartz optical cells with a 1.0 cm path length. $\pm$ denotes standard deviation. For the full data set, see Table S5†.

^bBased on data from reference 28.

Table 4.

Properties of U/V/Y-modified probe duplexes.^a

ON	ZP	Sequence	<i>T_m</i> (°C)	<i>T_A</i> (°C)	<i>G</i> ²⁹³ [<i>G</i> ²⁹³] (kJ/mol)			ΔG_{rec}^{293} (kJ/mol)	λ_{max} (nm)
					upper ON vs cDNA	lower ON vs cDNA	probe duplex		
<i>b</i> _{U1} U5	+4	5'-GUG ATA TGC 3'-CAC TAU ACG	47.5	±0.0	-46±0 [-5]	-52±0 [-11]	-55±1 [-14]	-2	352
<i>b</i> _{U1} U4	+2	5'-GUG ATA TGC 3'-CAC UAT ACG	31.5	6.5	-46±0 [-5]	-46±0 [-5]	-45±1 [-4]	-6	350
<i>b</i> _{U2} U5	+1	5'-GTG AUA TGC 3'-CAC TAU ACG	26.5	29.0	-55±0 [-14]	-52±0 [-11]	-38±1 [+3]	-28	348
<i>b</i> _{U2} U4	-1	5'-GTG AUA TGC 3'-CAC UAT ACG	39.5	6.5	-55±0 [-14]	-46±0 [-5]	-51±1 [-10]	-9	351
<i>b</i> _{U3} U5	-1	5'-GTG ATA UGC 3'-CAC TAU ACG	45.5	5.0	-50±0 [-9]	-52±0 [-11]	-55±1 [-14]	-6	352
<i>b</i> _{U3} U4	-3	5'-GTG ATA UGC 3'-CAC UAT ACG	40.5	0.5	-50±0 [-9]	-46±0 [-5]	-53±1 [-12]	-2	352
V1 V5	+4	5'-GVG ATA TGC 3'-CAC TAV ACG	39.0	1.5	-45±0 [-4]	-49±0 [-8]	-47±1 [-6]	-6	359
V1 V4	+2	5'-GVG ATA TGC 3'-CAC VAT ACG	35.0	-4.5	-45±0 [-4]	-41±1 [±0]	-46±1 [-5]	+1	359
V2 V5	+1	5'-GTG AVA TGC 3'-CAC TAV ACG	25.0	23.5	-55±0 [-14]	-49±0 [-8]	-40±1 [+1]	-23	355
V2 V4	-1	5'-GTG AVA TGC 3'-CAC VAT ACG	37.0	5.0	-55±0 [-14]	-41±1 [±0]	-50±1 [-9]	-5	358
V3 V5	-1	5'-GTG ATA VGC 3'-CAC TAV ACG	39.0	0.5	-46±0 [-5]	-49±0 [-8]	-47±1 [-6]	-7	359
V3 V4	-3	5'-GTG ATA VGC 3'-CAC VAT ACG	31.0	1.5	-46±0 [-5]	-41±1 [±0]	-43±1 [-2]	-3	359
Y1 Y5	+4	5'-GYG ATA TGC 3'-CAC TAY ACG	36.0	-2.0	-41±1 [±0]	-49±0 [-8]	-46±1 [-5]	-3	356
Y1 Y4	+2	5'-GYG ATA TGC 3'-CAC YAT ACG	27.5	-5.5	-41±1 [±0]	-38±1 [+3]	-41±1 [±0]	+3	354
Y2 Y5	+1	5'-GTG AYA TGC 3'-CAC TAY ACG	22.0	19.5	-50±0 [-9]	-49±0 [-8]	N/A	N/A	352
Y2 Y4	-1	5'-GTG AYA TGC 3'-CAC YAT ACG	26.5	3.0	-50±0 [-9]	-38±1 [+3]	-42±1 [-1]	-5	356
Y3 Y5	-1	5'-GTG ATA YGC 3'-CAC TAY ACG	34.0	3.0	-44±1 [-3]	-49±0 [-8]	-46±1 [-5]	-6	356
Y3 Y4	-3	5'-GTG ATA YGC 3'-CAC YAT ACG	25.0	±0.0	-44±1 [-3]	-38±1 [+3]	-38±1 [+3]	-3	357

^aZP = zipper. For conditions of thermal denaturation and absorbance experiments, see Table 1 and Table 3, respectively. For definitions of *T_A* and ΔG_{rec}^{293} , see main text. *G*²⁹³ is measured relative to *G*²⁹³ for **D1:D4** = -41 kJ/mol. "±" denotes standard deviation. N/A = lack of a clear lower baseline precluded determination of this value.

^bData from reference 23 are included to facilitate comparison.

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