Merging Two Strategies for Mixed-Sequence Recognition of Double-Stranded DNA: Pseudocomplementary Invader Probes

Brooke A. Anderson and Patrick J. Hrdlicka*

Department of Chemistry, University of Idaho, Moscow, Idaho 83844-2343, United States

ABSTRACT: The development of molecular strategies that enable recognition of specific double-stranded DNA (dsDNA) regions has been a longstanding goal as evidenced by the emergence of triplex-forming oligonucleotides, peptide nucleic acids (PNAs), minor groove binding polyamides, and—more recently—engineered proteins such as CRISPR/Cas9. Despite this progress, an unmet need remains for simple hybridization-based probes that recognize specific mixed-sequence dsDNA regions under physiological conditions. Herein, we introduce pseudocomplementary Invader probes as a step in this direction. These double-stranded probes are chimeras between pseudocomplementary DNA (pcDNA) and Invader probes, which are activated for mixed-sequence dsDNA-recognition through the introduction of pseudocomplementary base pairs comprised of 2-thiothymine and 2,6-diaminopurine, and +1 interstrand zipper arrangements of intercalator-functionalized nucleotides, respectively. We demonstrate that certain pseudocomplementary Invader probe designs result in very efficient and specific recognition of model dsDNA targets in buffers of high ionic strength. These chimeric probes, therefore, present themselves as a promising strategy for mixed-sequence recognition of dsDNA targets for applications in molecular biology and nucleic acid diagnostics.

INTRODUCTION

Probes capable of recognizing specific mixed-sequence double-stranded DNA (dsDNA) regions have been long-sought-after as they can be developed into tools that enable modulation of gene expression at the transcriptional level, gene editing, and detection of specific genetic signatures. Early examples of dsDNA-targeting probes include triplex-forming oligonucleotides (TFOs) and peptide nucleic acids (PNAs), as well as minor groove binding polyamides. Unfortunately, only a subset of the possible target regions is available for recognition by these probes due to requirements for exclusive purine content (TFOs/PNAs) or short target regions (polyamides). Consequently, significant efforts have been devoted to develop alternative approaches, which has resulted in TFOs and PNAs with reduced target site limitations. More recently, engineered proteins such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs) and— in particular—CRISPR/Cas9 systems, have gained a tremendous amount of attention, despite mounting concerns regarding recognition specificity and cellular delivery. Another class of compounds that has emerged from these efforts are the so-called pseudocomplementary DNA and PNA (pcDNA/ pcPNA), in which a short DNA or PNA duplex is modified to contain pseudocomplementary base pairs between 2-thiothymine and 2,6-diaminopurine. The steric clash between the 2-thio and the 2-amino group perturbs and weakens the hydrogen bonding between these moieties, resulting in a destabilized probe duplex. In contrast, 2-thiothymine and 2,6-diaminopurine form stable base pairs with canonical adenine and thymine, respectively. The difference in thermodynamic stability between probe duplexes and duplexes between individual probe strands and complementary DNA (cDNA) allows for double-duplex invasion of dsDNA target regions under certain conditions (Figure 1b). Thus, pcDNA can recognize terminal target regions, while pcPNA also recognize internal target regions of DNA duplexes, albeit at low ionic strengths. However, a recent study has suggested that recognition of mixed-sequence dsDNA regions by pcPNA may be possible under the highly viscous conditions found in the nucleus.

We, and later others, have pursued an alternative strategy for the construction of energetically activated double-stranded probes for recognition of mixed-sequence dsDNA regions, which is based on forced intercalation of aromatic moieties. Our Invader probes are short DNA duplexes containing +1 interstrand zipper arrangements of intercalator-functionalized nucleotides (Figure 1c; for a definition of the zipper nomenclature, see Experimental Section). This motif, which we denote as an energetic hotspot, forces two intercalators into...
the same region of the duplex, resulting in unwinding and destabilization\textsuperscript{22,23} as the nearest neighbor exclusion principle\textsuperscript{24} is violated. According to this principle, the space between the two nearest base pairs on either side of a bound intercalator, will not be bound by a second intercalator due to limitations in local helix expandability (every intercalation event unwinds the duplex by \( \sim 3.4 \text{ Å} \)),\textsuperscript{25} and/or to avoid disruption of highly stable stacking interactions between the first bound intercalator and neighboring nucleobases.\textsuperscript{26} On the other hand, each of the two strands comprising an Invader probe display very high affinity toward cDNA since the intercalators stack strongly with the neighboring base pairs, acting as molecular glue (Figure 1c).\textsuperscript{22,27} We have previously demonstrated that the differences in thermostability between Invader probes and probe-cDNA duplexes can drive mixed-sequence recognition of isosequential DNA duplexes,\textsuperscript{20,22,27,28} DNA hairpins,\textsuperscript{22} and chromosomal DNA targets.\textsuperscript{29}

Figure 1. (a) Structures of monomers used herein. (b) Representation of energy levels of different dsDNA-targeting probes and the corresponding duplexes with cDNA (only one probe-target duplex is shown). Pseudocomplementary base-pairs are shown in red. Droplets denote intercalators. Note, the large difference in energy between pc-Invader:cDNA duplexes and pc-Invader probe duplexes. (c) Illustration of Invader-mediated recognition of dsDNA target regions.

DOI: 10.1021/jacs.6b00369
J. Org. Chem. 2016, 81, 3333–3346
Initially, 2′-N-(pyren-1-yl)-2′-amino-α-L-LNA (Locked Nucleic Acid) monomers were used to construct the energetic hotspots of Invader probes. However, the challenging synthesis of these building blocks, prompted us to conduct a search for more feasible structural and functional mimics, which, among others, resulted in the identification of 2′-N-(pyren-1-yl)methyl-2′-N-methyl-2′-amino-2-thiouridine monomer X as a next-generation Invader modification (Figure 1a). Access to building blocks that are based on simpler sugar scaffolds has facilitated extensive structure–property relationship studies aiming at improving the recognition efficiency of Invader probes.

In the present study, we set out to study if the dsDNA-recognition potential of Invader probes can be increased further through incorporation of pseudocomplementary base pairs. We hypothesized that these chimeric pseudocomplementary Invader probes will be more energetically labile, yet form even more stable duplexes with dsDNA than either canonical Invader or pcDNA probes, leading to more favorable energetics for dsDNA-recognition (Figure 1b). Toward this end, two different approaches were followed. In the first, we wanted to integrate the intercalator as part of a pseudocomplementary nucleotide, which led to the identification of 2′-N-(pyren-1-yl)methyl-2′-N-methyl-2′-amino-2-thiouridine monomer Y as a target (Figure 1a). Incorporation of this monomer in a +1 interstrand zipper arrangement opposite of conventional 2,6-diaminopurine DNA D monomers would furnish a double-stranded probe with a pseudocomplementary energetic hotspot (DY probes) (Figure 1b). In the second approach, we separated the two key structural features by using conventional 2-thiopyrimidine DNA monomer S and 2,6-diaminopurine DNA monomer D, alongside energetic hotspots comprised of the conventional Invader monomer X (DSX probes) (Figure 1b). We demonstrate that the latter type of probes is particularly interesting for dsDNA-recognition applications.

■ RESULTS AND DISCUSSION

Synthesis of N2′-Pyrene-Functionalized 2-Thiouridine Phosphoramidite. The key 2′-N-(pyren-1-yl)methyl-2′-N-methyl-2′-amino-2-thiouridine phosphoramidite 6 was obtained from known nucleoside 1, following a similar general strategy as was used for the synthesis of 2′-O-[2-(methoxy)ethyl]-2-thiopyrimidine (Scheme 1). Thus, nucleoside 1—obtained in 61% yield over six steps from uridine—was first subjected to a sequence of protecting group manipulations, i.e., 3′-O-acetylation, 5′-O-detritylation and 5′-O-methanesulfonylation, to afford nucleoside 2 in 55% yield over three steps. Prolonged refluxing in anhydrous ethanol in the presence of sodium bicarbonate results in the formation of 2-O-ethyluridine derivative 3 in 52% yield, presumably via a nucleophilic opening of an O2,S′-anhydouridine intermediate. Subsequent O5′-dimethoxytritylation of 3 using standard conditions affords nucleoside 4 in 88% yield, which upon treatment with H2S-saturated pyridine in the presence of 1,1,3,3-tetramethylguanidine provides 2-thiouridine derivative 5 in 82% yield. Treatment of nucleoside 5 with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite and N,N-diisopropylthioamide affords target phosphoramidite 6 in 81% yield, corresponding to an overall yield of ∼17% from nucleoside 1.

Synthesis of Modified ONs. Phosphoramidite 6 was used to incorporate monomer Y into oligodeoxyribonucleotides (ONs) via automated solid-phase DNA synthesis. Extended hand-coupling (15 min) and the use of 4,6-dicyanoimidazole as an activator resulted in stepwise coupling yields of ∼95%. ONs modified with monomer X were synthesized as previously described (15 min coupling, S-[3,5-bis(trifluoromethyl)-phenyl]-1H-tetrazole as an activator, ∼99% coupling yield). The corresponding phosphoramidites of monomers S and D were obtained from commercial sources and incorporated into ONs using the conditions for incorporation of monomer Y (stepwise coupling yields >95%). To prevent desulfurization in ONs modified with Y or S monomers, nucleotide phosphate to

Scheme 1. Synthesis of Target Phosphoramidite 6
phosphate oxidation was performed using tert-butylhydroperoxide/CH3CN/H2O (10 min) rather than the standard aqueous iodine solution. The identity and purity of the modified ONs were established through MALDI-TOF (Table S1) and ion-pair reverse-phase HPLC (>90% purity unless otherwise noted), respectively.

**Thermal Denaturation Properties of Y- or DY-Modified Duplexes.** Pyrrene-functionalized 2-thiothiuracil monomer Y was incorporated into the same 9-mer mixed-sequence ONs that was previously used for evaluation of Invader monomer X.32 Thermal denaturation temperatures (Tm’s) of duplexes between Y-modified ONs and cDNA or rRNA were determined in a medium salt phosphate buffer ([Na+] = 110 mM) and compared relative to unmodified and corresponding X-modified duplexes. The resulting denaturation curves display the expected monophasic sigmoidal transitions (Figure S1). Duplexes between Y1–Y4 and cDNA are significantly more stable than unmodified reference duplexes (ΔTm between +2.5 and +11.5 °C, Table 1), whereas

| ON    | sequence | ΔTm (°C) |
|-------|----------|----------|
| X1    | 5'-GTG A/A TGC | +15.0    |
| X2    | 3'-CAC T/A TAC | +1.5     |
| X3    | 3'-CAC T/A ACG | +15.0    |
| X4    | 3'-CAC T/A ACG | +14.0    |
| Y1    | 5'-GTG A/A TGC | +11.5    |
| Y2    | 3'-CAC T/A ACG | +2.5     |
| Y3    | 3'-CAC T/A ACG | +11.0    |
| Y4    | 3'-CAC A/A ACG | +10.0    |
| DY1   | 5'-GTG A/AT TGC | +16.0    |
| DY2   | 5'-GTG A/AT TGC | +13.0    |
| DY3   | 3'-CAC T/P ACG | +3.5     |
| DY4   | 3'-CAC T/P ACG | +16.0    |
| D1    | 5'-GTG P/T TGC | +1.0     |
| D2    | 5'-GTG P/T TGC | +2.0     |
| D3    | 3'-CAC T/P ACG | +3.0     |
| D4    | 3'-CAC T/P ACG | +5.0     |

Table 1. Thermal Denaturation Temperatures of Duplexes between X-, Y-, DY-, or D-Modified ONs and cDNA

*ΔTm = change in Tm relative to reference duplex DNA1:DNA2 (Tm ≡ 29.5 °C, where DNA1: 5'-GTG ATA TGC and DNA2: 3'-CAC TAT ACG. Tm’s are determined as the maximum of the first derivative of melting curves (A260 vs. T) recorded in medium salt phosphate buffer ([Na+] = 110 mM, [Cl−] = 100 mM, pH 7.0 (NaH2PO4/Na2HPO4)), using 1.0 μM of each strand. Reported Tm’s are averages of at least two measurements within 1.0 °C; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guamin-9-yl DNA monomer, and T = thynin-1-yl DNA monomer. For structures of monomers X, Y, and D, see Figure 1a. *Data previously reported in ref 32.

heteroduplexes with cDNA are much less stable (ΔTm between −6.5 to +2.5 °C, Table S2). ONs in which Y monomers are flanked by 3'-purines result in greater duplex stabilization than ONs with 3'-flanking pyrimidines (e.g., compare ΔTm’s for Y1 and Y3, Table 1). This sequence dependence, along with the prominent DNA selectivity (Table S3), is typical for ONs modified with intercalating pyrene moieties.22,24 Surprisingly, Y-modified ONs form slightly less stable duplexes with cDNA than their X-modified counterparts (compare Tm’s of X1–X4 and Y1–Y4, Table 1), which suggests that the binding modes responsible for the stabilizing contributions of the pyrene and 2-thiouracil moieties are not fully compatible.

To generate Invader probes with pseudocomplementary energetic hotspots, we synthesized ONs in which 2-amino-2'-deoxyadenosine monomers flank monomer Y (i.e., the DY series). Replacing regular 2'-deoxyadenosines with D monomers increases the cDNA/cRNA affinity of Y-modified ONs by 1–5 °C presumably due to stabilization from the extra hydrogen bond in D:T base pairs relative to normal A:T pairs (e.g., compare ΔTm for Y1 and DY1, Table 1 and Table S2). Similar relative increases in cDNA affinity are observed when 2'-deoxyadenosines are replaced with D monomers in otherwise unmodified ONs (see ΔTm for D1–D4 vs cDNA, Table 1), which is in agreement with previous studies.32,43

Next, we set out to study DNA duplexes with different interstrand zipper arrangements of Y monomers and DY segments to identify probe architectures that are more strongly activated for dsDNA-recognition. As expected from our previous studies on Invader probes,32 Y1:Y3, which features a 1+1 interstrand zipper of Y monomers, is much more thermolabile than Y1:Y2 featuring a 1–1 interstrand zipper (Table 2), or duplexes between Y-modified ONs and cDNA (Table 1). The destabilization is likely a consequence of the nearest neighbor exclusion principle25,26 being violated. In line with our hypothesis, introduction of a 2,6-diaminopurine D monomer opposite the pyrene-functionalized 2-thiothiuracil Y monomer, decreases the Tm’s of the duplexes (e.g., compare ΔTm of DY1:DY3 and Y1:Y2, Table 2). Interestingly, the destabilizing effect of the pseudocomplementary base pairs is more pronounced when the Y monomers are positioned in a −1 zipper orientation (drop in Tm of 9.5 °C from Y1:Y2 to DY2:DY3, relative to drop of 2.0 °C from Y1:Y3 to DY1:DY4, Table 2). This indicates that the pseudocomplementary energetic hotspot architecture of DY1:DY4 does not fully harness the activating effects from both structural elements.

The Tm-based conclusions were largely corroborated by the Gibbs free energies associated with duplex formation, which were derived from denaturation curves via line fitting (Table 2).44 Thus: (i) Duplexes between Y-modified ONs and cDNA are much more stable than unmodified reference duplexes (ΔΔG293° between −16 and −7 kJ/mol, first and second ΔΔG° columns, Table 2) due to more favorable enthalpy (ΔΔH between −76 and −44 kJ/mol, Table S7). (ii) In comparison, the corresponding X-modified duplexes are slightly more stable, while the D-modified duplexes are far less stable (e.g., compare ΔΔG293° for Y1:cDNA, X1:cDNA and D1:cDNA, Table 2). (iii) Duplexes between ONs with DY motifs and cDNA are generally less stable than the corresponding Y-modified duplexes (e.g., compare ΔΔG293° for Y1:cDNA and DY2:cDNA, Table 2). This contrasts the Tm-based conclusions (Table 1), but likely reflects the different entropies of these duplexes (Table S8), leading to different temperature dependencies of the Gibbs free energies. (iv) Formation of Y1:Y3 (1+1 zipper) is less thermodynamically favorable than Y1:Y2 (−1 zipper) (third ΔG° column, Table 2). (v) Duplexes with pseudocomplementary energetic hotspots are slightly less stable than the corresponding duplexes featuring only regular energetic hotspots (e.g., compare ΔG° for DY1:DY4 and Y1:Y3, Table 2). The energetic nature of DY1:DY4 is the result of particularly unfavorable enthalpy (ΔΔH = +95 kJ/mol, Table S7), likely reflecting a violation of the nearest neighbor exclusion principle and concomitant formation of destabilizing
pseudocomplementary base pairs. (vi) pc-DNA without any energetic hotspots range between being slightly more stable to slightly less stable than unmodified duplexes (e.g., see $\Delta G^{298}$ for D1:Y2 and Y1:D3, Table 2).

Consequentially, +1 zipper duplex Y1:Y3 is much more energetically activated than −1 zipper duplex Y1:Y2, as gauged by the free energy available for recognition of isosequential dsDNA targets $\Delta G^{298}$ (ON$_4$:ON$_B$) = $\Delta G^{298}$ (ON$_4$;cDNA) + $\Delta G^{298}$ (cDNA:ON$_B$) − $\Delta G^{298}$ (ON$_4$:ON$_B$) − $\Delta G^{298}$ (dsDNA), where ON$_4$:ON$_B$ denotes a double-stranded probe and “dsDNA” is the isosequential dsDNA target for ON$_4$:ON$_B$ (compare $\Delta G^{298}$ for Y1:Y2 and Y1:Y3, Table 2). DY1:DY4, featuring a pseudocomplementary energetic hotspot, is slightly more energetically activated for dsDNA-recognition than Y1:Y3 (compare $\Delta G^{298}$ for DY1:DY4 and Y1:Y3, Table 2). As expected, DY2:Y3, which also features two pseudocomplementary base pairs but has an −1 interstrand zipper arrangement of Y monomers, is far less activated for dsDNA-recognition (compare $\Delta G^{298}$ for DY2:Y3 and DY1:DY4, Table 2). pc-DNA without energetic hotspots are also far less activated for dsDNA-recognition (e.g., compare $\Delta G^{298}$ of Y1:D3 and DY1:DY4, Table 2).

Unexpectedly, DY1:DY4 has lower thermodynamic dsDNA-recognition potential than conventional Invader probe X1:X3 (compare $\Delta G^{298}$ for DY1:DY4 and X1:X3, Table 2), which is due to two factors: (i) intercalation of the pyrene moieties perturbs the local duplex geometry, which weakens the normally stabilizing base pairs between 2,6-diaminopurine:thymine (D:T) and 2-thiouracil:adenine (Y:A), leading to lower-than-expected cDNA-affinity of DY-modified ONs (e.g., compare $\Delta G^{298}$ for X1:cDNA, Y1:cDNA and DY1:cDNA, Table 2), and (ii) +1 interstrand zipper arrangements of nucleotide monomers with intercalating pyrene moieties perturb local duplex geometries, which, in turn, are likely to reduce the steric clash between 2,6-diaminopurine and 2-thiouracil normally occurring in pseudocomplementary base pairs, resulting in less pronounced probe destabilization. It is also possible that the pseudocomplementary base pairs of DY1:DY4 interfere with the forced and destabilizing intercalation of the two pyrene moieties; this is supported by the UV−vis absorption characteristics of DY1:DY4. Normally, DNA duplexes with +1 interstrand zipper motifs of intercalating pyrene-functionalized monomers exhibit markedly blue-shifted pyrene absorption relative to DNA duplexes with other interstrand zipper motifs, which is indicative of reduced pyrene-nucleobase interactions due to a locally perturbed duplex geometry (e.g., compare $\lambda_{\text{max}}$ for X1:X3 relative to X1:Y2, Table 2). The same trend is observed for Y1:Y3 (compare $\lambda_{\text{max}}$ for Y1:Y3 relative to Y1:Y2), but the trend is less pronounced for DY1:DY4 (compare $\lambda_{\text{max}}$ for

**Table 2. Biophysical Properties of X-, Y- or DY-Modified DNA Duplexes***

| ON  | ZP | sequence            | $T_m$ (°C) | $\Delta G^{298}$  | $\Delta G^{298}$ (kJ/mol) | $\lambda_{\text{max}}$ (nm) |
|-----|----|---------------------|------------|-------------------|---------------------------|-----------------------------|
| X1  | -1 | 5′-GTG AXT TGC 3′-CAC YAT TGC | 42.5 | $−65 \pm 1$ | $−54 \pm 1$ | 14 | 352 |
| X2  | +1 | 5′-GTG AXT TGC 3′-CAC YAT TGC | 28.5 | $−65 \pm 1$ | $−64 \pm 1$ | 11 | 352 |
| X3  | +1 | 5′-GTG AXT TGC 3′-CAC YAT TGC | 39.5 | $−61 \pm 1$ | $−52 \pm 1$ | 14 | 353 |

“ZP = zipper. For conditions of thermal denaturation, see Table 1. $\Delta G^{298}$ is measured relative to $\Delta G^{298}$ for DNA1:DNA2 = −45 kJ/mol. $\Delta G^{298}$ (ON$_4$:ON$_B$) = $\Delta G^{298}$ (ON$_4$;cDNA) + $\Delta G^{298}$ (cDNA:ON$_B$) − $\Delta G^{298}$ (ON$_4$:ON$_B$) − $\Delta G^{298}$ (dsDNA). ± denotes standard deviation. Absorption spectra were recorded at T = 10 °C using each strand at 1.0 μM concentration in $T_m$ buffer. Data for X1:X2 and X1:X3 are from ref 32 and are included to facilitate comparison.”
DY1:DY4 relative to DY2:DY4), indicating that DY1:DY4 is not strongly perturbed.

Recognition of DNA Hairpins Using DY Probes. On the basis of the observed \( \Delta G_{298}^{\text{rec}} \) values, we decided to determine the dsDNA-recognition properties of Y1:Y3 and DY1:DY4 relative to benchmark Invader X1:X3 using an electrophoretic mobility shift assay from our earlier studies.\(^{22}\) Thus, a digoxigenin (DIG) labeled DNA hairpin (DH)—comprised of a 9-mer double-stranded mixed-sequence stem, which is linked by a T10 loop—was used as a model dsDNA target (Figure 2a and 2b). Room temperature incubation of DH1 with Y1:Y3, DY1:DY4, or X1:X3 in a HEPES buffer of considerable ionic strength, results in dose-dependent formation of a ternary recognition complex as evidenced by the emergence of a slower migrating band on nondenaturing PAGE gels (Figure 2c).

Nonlinear regression fits of dose–response curves (average of at least three independent experiments; error bars represent standard deviation) (d) illustrating incubation of DH1–DH7 with 200-fold molar excess of X1:X3, Y1:Y3, or DY1:DY4. Experimental conditions for electrophoretic mobility shift assay: separately preannealed targets (34.4 nM) and probes (variable concentrations) were incubated for 12–16 h at ambient temperature in 1X HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl\(_2\), 10% sucrose, 1.4 mM spermine tetrahydrochloride, pH 7.2) and then resolved on 16% nondenaturing PAGE (70 V, 2.5 h, \( \sim 4^\circ\)C) using 0.5x TBE as a running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA); DIG: digoxigenin.

Figure 2. Recognition of DNA hairpins using Invader probes. (a) Illustration of recognition process; (b) sequences and thermal denaturation temperatures of DNA hairpins with isosequential (DH1) or nonisosequential stems (DH2–DH7); underlined nucleotides indicate sequence deviations relative to probes; (c) representative electrophoretograms of recognition of DH1 using 1- to 500-fold molar excess of Y1:Y3 or DY1:DY4; (d) dose–response curves (average of at least three independent experiments; error bars represent standard deviation); (e) electrophoretograms illustrating incubation of DH1–DH7 with 200-fold molar excess of X1:X3, Y1:Y3, or DY1:DY4. Experimental conditions for electrophoretic mobility shift assay: separately preannealed targets (34.4 nM) and probes (variable concentrations) were incubated for 12–16 h at ambient temperature in 1X HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl\(_2\), 10% sucrose, 1.4 mM spermine tetrahydrochloride, pH 7.2) and then resolved on 16% nondenaturing PAGE (70 V, 2.5 h, \( \sim 4^\circ\)C) using 0.5x TBE as a running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA); DIG: digoxigenin.
nucleotide sequence at one position relative to the Invader probes (Figure 2b). No recognition was observed, demonstrating that Invader-mediated dsDNA-recognition proceeds with excellent binding specificity (Figure 2e).

A Change in Strategy: pcDNA with Energetic Hotspots (DSX Probes). The above results suggest that incorporation of pseudocomplementary energetic hotspots—i.e., 5′-YD-3′ (3′-DY-5′) cassettes—provide less of a benefit for dsDNA-recognition than originally anticipated. At this stage, we hypothesized that the two structural elements that activate Invader and pcDNA probes for dsDNA-recognition—i.e., the intercalator-functionalized nucleotides forming the energetic hotspots, and the pseudocomplementary base pairs between 2,6-diaminopurine and 2-thiouracil—need to be spatially separated in order to harness their full benefits. Accordingly, a series of 13-mer DSX-modified Invader probes were designed, i.e., double-stranded probes featuring energetic hotspots comprised of conventional Invader building block X, along with pseudocomplementary base pairs between regular 2,6-diaminopurine D and 2-thiouracil S monomers. The following probes were synthesized: (i) two different DSX-modified Invader probes, in which the energetic hotspot either is next to or one nucleotide away from two pseudocomplementary base pairs (DSX1:DSX2 and DSX3:DSX4), (ii) an Invader probe comprised of regular X monomers (X5:X6), (iii) an Invader probe with a single pseudocomplementary energetic hotspot (DY5:DY6), and (iv) three pcDNA probes, each containing two directionally spaced, regular pseudocomplementary base pairs (SD1:SD2, SD3:SD4 and SD5:SD6) (Table 3).

First, \( T_m \)'s for duplexes between individual probe strands and cDNA (Table 3, first two \( T_m \) columns) or cRNA (Table S9) were determined. In line with observations from the 9-mer series, X-modified ONs form highly thermostable duplexes with cDNA (\( T_m = 11−12°C \) and less thermostable duplexes with cRNA (\( T_m = 3°C \)). In comparison, ONs with DY motifs display slightly higher cRNA affinity (\( T_m \) for DY5:DY6 = 4.5−5.0°C, Table S9) and slightly lower cDNA affinity (\( T_m = 10°C \), Table 3), again suggesting that the normally stabilizing base-pairs between 2,6-diaminopuridine:thymine and 2-thiouracil:adenine are weakened by proximal intercalation of the pyrene moieties. Encouragingly, DSX-modified ONs display significantly increased cDNA and cRNA affinity relative to X-modified ONs (\( T_m,cDNA = 14−15°C \), Table 3; \( T_m,cRNA = 7.0−8.5°C \), Table S9), suggesting that separation of the intercalators and the modified nucleobases is beneficial for binding affinity. In comparison, regular pcDNA strands form far less stable duplexes with cDNA (\( T_m,cDNA = 2.5−4.0°C \), Table 3).

Benchmark Invader probe X5:X6 and DY5:DY6 are both relatively thermostable (\( T_m = −0.5°C \) and −1.5°C, respectively, Table 3). DSX probes are slightly less stable (\( T_m = −2.0°C \) and −3.0°C for DSX1:DSX2 and DSX3:DSX4, respectively, Table 3). However, comparison with the corresponding pcDNA probes suggests that the full destabilizing effect of the pseudocomplementary base pairs still is not fully realized with these probe architectures (e.g., compare \( T_m \) of −8.0, −0.5, and −3.0 for SD5:SD6, X5:X6 and DSX3:DSX4, respectively, Table 3). The above \( T_m \)-based conclusions are substantiated by the Gibbs free energies for formation of duplexes (Table 3). As a result of these stability trends, DSX probes are significantly more thermodynamically activated for dsDNA-recognition than X5:X6, DY5:DY6, or any of the regular pcDNA (trend in \( ΔG^{298} \) values: DSX3:DSX4 < DSX1:DSX2 < X5:X6 < DY5:DY6 < SD5:SD6 < SD3:SD4 < SD1:SD2, Table 3).

Recognition of DNA Hairpins Using Energetically Activated 13-mer Probe Duplexes. On the basis of the

### Table 3. Thermal Denaturation and Thermodynamic Properties of X-, DY-, DSX-, and DS-Modified Duplexes

| ON sequence | \( ΔT_m \) (°C) | \( ΔG^{298} \) (kJ/mol) |
|-------------|----------------|--------------------------|
| **X5:X6**   | 2.5−4.0°C      |                           |
| **DY5:DY6** | 4.5−5.0°C      |                           |
| **DSX1:DSX2** | 14−15°C      |                           |
| **DSX3:DSX4** | 7.0−8.5°C      |                           |

\( ΔT_m = \) change in \( T_m \) relative to reference duplexes DNA3:DNA4 (\( T_m = 37.5°C \)), where DNA3: 5′-GGTA TATATA GCC, DNA4: 3′-CCAT ATATAT CCG. \( ΔG^{298} \) is measured relative to \( ΔG^{298} \) for DNA3:DNA4 = −61 kJ/mol. For definition of \( ΔG^{298} \), see Table 2. "±" denotes standard deviation. For experimental conditions, see Table 1. For structures of monomers X, Y, D and S, see Figure 1.
observed $\Delta G_{298}^{\text{rec}}$ values, we decided to evaluate the dsDNA-targeting efficiency of DSX1:DSX2, DSX3:DSX4, X5:X6, and DY5:DY6 using a similar electrophoretic mobility shift assay as used in the 9-mer series (Figure 3a). Thus, a DIG-labeled DNA hairpin (DH8)—comprised of a 13-mer double-stranded mixed-sequence stem that is linked by a T10 loop—was used as a model dsDNA target. Incubation of DH8 with the various 13-mer Invader probes results in dose-dependent formation of a slower moving band on nondenaturing PAGE gels (Figure 3b). As expected from the initial 9-mer studies, the parent Invader X5:X6 recognizes DH8 more effectively at low probe concentrations than DY5:DY6, which has a pseudocomplementary energetic hotspot (Figure 3c). Gratifyingly, Invader probes with separated pseudocomplementary base pairs and energetic hotspots display improved recognition efficiency (see curves for DSX1:DSX2 and DSX3:DSX4, respectively, Figure 3c), which follows the observed trend in $\Delta G_{298}^{\text{rec}}$ values. It is particularly noteworthy that the use of as little as 1.0 mol equiv of DSX3:DSX4 results in $\sim 20\%$ recognition of DH8, especially when considering that optimal Invader design normally calls for incorporation of multiple energetic hotspots.33 This suggests that spatial separation of pseudocomplementary base pairs and energetic hotspots is a promising principle for the design of efficient dsDNA-targeting Invader probes.

In the present study, we have only evaluated AT-rich DNA targets. Although there is an absence of suitable pseudocomplementary base pairs for CG-steps,46 we have previously shown that energetic hotspots can be comprised of intercalator-functionalized monomers that are based on any of the natural nucleobases (although there is a preference for pyrimidine monomers).30 It is, therefore, likely possible to design DSX-like probes against DNA targets with a higher GC-content than studied herein.

Lastly, binding specificity was studied by incubating the Invader probes with DNA hairpins DH9–DH14, which differ in the nucleotide sequence at one position in the stem region relative to the probes (Figure 4a). Excellent discrimination of the nontarget DNA hairpins is observed, even when using a 200-fold molar excess of X5:X6, DY5:DY6 or DSX1:DSX2 (Figure 4b). Similarly, high-affinity Invader probe DSX3:DSX4 only results in trace recognition of DH10 and DH14. To gain further insight into the underlying reasons for the excellent binding fidelity, we evaluated individual probe strands against singly mismatched single-stranded DNA targets (Tables S10–S12). Excellent mismatch discrimination is generally observed. In agreement with the observations from the dsDNA-recognition experiments, the discrimination is least efficient when DSX3 or DSX4 are hybridized with single-stranded targets that have a sequence corresponding to the target region.
in DH10 and DH14 (\(T_m\)’s only reduced by 5–7 °C, relative to matched duplexes, Table S12). The binding of Invader probes to noncomplementary dsDNA targets would accordingly entail formation of two mismatched and destabilized probe-target duplexes, which is energetically prohibitive in most cases. Moreover, like other structured probes, the double-stranded Invader probes are also likely to exhibit improved binding specificity due to a “stringency clamping” effect.47,48 Thus, pseudocomplementary Invader probes allow for strong and highly specific binding to mixed-sequence dsDNA target regions at ionic conditions.

### CONCLUSION

A synthetic route to 2′-N-(pyren-1-yl)methyl-2′-amino-2′-deoxy-2′-N-methyl-2-thiouridine phosphoramidite 6 has been developed (~10% overall yield over 13 steps from uridine). ONs modified with the corresponding Y monomer display high affinity toward cDNA (\(\Delta T_m\) up to +11.5 °C). Incorporation of 2-aminoadenosine monomer D next to the Y monomer further increases cDNA affinity. Double-stranded probes with “pseudocomplementary hotspots” (i.e., 5′-YD:3′-DY-5′ cassettes) are thermolabile and activated for recognition of dsDNA targets, but less so than regular Invader probes with hotspots comprised of 2′-N-(pyren-1-yl)methyl-2′-N-methyl-2′-aminouridine monomer X. In other words, close proximity of the two structural elements that normally activate pseudocomplementary DNA and Invader probes for dsDNA-recognition—i.e., the weak base pairs between 2,6-diaminopurine and 2-thiouracil, and the destabilizing +1 interstrand zipper arrangements of intercalating pyrene moieties—does not provide a clear design benefit. In contrast, DSX probes, in which the two destabilizing structural motifs are separated, are strongly activated for recognition of mixed-sequence dsDNA targets. This was confirmed in studies with model dsDNA targets as efficient recognition was demonstrated to occur with excellent specificity. Thus, the use of chimeric pseudocomplementary Invader probes presents itself as a promising strategy for mixed-sequence recognition of dsDNA for applications in molecular biology and nucleic acid diagnostics, especially since we have recently demonstrated that conventional Invader probes can recognize target regions in chromosomal DNA.33

### EXPERIMENTAL SECTION

3′-O-Acetyl-2′-amino-2′-deoxy-5′-O-methanesulfonyl-2′-N-methyl-2′-N-(pyren-1-ylmethyl)uridine (2). Nucleoside 1A (3.40 g, 4.39 mmol) was coevaporated with anhydrous 1,2-dichloroethane (2 × 30 mL), redissolved in anhydrous pyridine (55 mL) and cooled to 0 °C. To this was added 4-dimethylaminopyridine (DMAP, 55 mg, 0.44 mmol) and acetic anhydride (1.25 mL, 13.18 mmol). After stirring at ambient temperature for 12 h, the reaction mixture was diluted with EtOAc (150 mL) and washed with \(\text{H}_2\text{O}\) (80 mL) and saturated aqueous \(\text{NaHCO}_3\) (80 mL). The organic layer was evaporated to near dryness and coevaporated with absolute EtOH:toluene (2:1 v/v, 3 × 30 mL). The resulting residue (~3.5 g) was suspended in AcOH:H\(\text{H}_2\text{O}\) (4:1 v/v, 55 mL) and the reaction mixture was stirred overnight at room temperature. Solvents were evaporated off and the residual residue was purified by silica gel column chromatography (0–5% MeOH/CH\(_2\text{Cl}_2\) v/v) to afford O5′-hydroxy derivative (~1.8 g) as a white solid material. This material was coevaporated with anhydrous pyridine:CH\(_2\text{Cl}_2\) (1:1 v/v, 2 × 30 mL), redissolved in anhydrous...
pyridine:CH₂Cl₂ (1:1 v/v, 44 mL) and cooled to −20 °C (ice/salt). Methanesulfonyl chloride (0.75 mL, 9.62 mmol) was added dropwise over 30 min and the reaction mixture was stirred at −20 °C for 2 h more. The reaction mixture was diluted with CH₂Cl₂ (80 mL) and washed with saturated aqueous NaHCO₃ (50 mL). The aqueous layer was back-extracted with CH₂Cl₂ (3 × 15 mL) and the organic layers were combined and evaporated to dryness, followed by drying with 3 Å silica gel and dilution with absolute EtOH/toluene (2:1 v/v, 3 × 30 mL). The resulting residue was purified by silica gel column chromatography (0–3% MeOH/CH₂Cl₂ v/v) to afford nucleoside 2 (1.43 g, 55%), as a white foam. Rf = 0.4 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 512.1985 ([M + Na]+, C₃₀H₂₇N₃O₇Na⁺, Calc. 512.2009).

The reaction mixture was stirred at room temperature for 3.5 h at which point ice-cold EtOH (1.0 mL) was added. The reaction mixture was diluted with CH₂Cl₂ (80 mL) and washed with saturated aqueous NaHCO₃ (50 mL). The aqueous layer was back-extracted with CH₂Cl₂ (3 × 20 mL) and the combined organic layers were evaporated to dryness and coevaporated with absolute EtOH/toluene (2:1 v/v, 3 × 15 mL). The resulting residue was purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v) to afford nucleoside 5 (0.35 g, 82%) as a white foam. Rf = 0.8 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS m/z 812.2765 ([M + Na]+, C₄₈H₄₃N₃O₆SNa⁺, Calc. 812.2797). Rf = 0.4 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 682.2675 ([M + Na]+, C₃₂H₂₃N₃O₅Na⁺, Calc. 682.2695).

The solution was transferred, using an argon-flushed syringe, to a precooled flask containing nucleoside 4 (0.44 g, 54% mmol) and the reaction mixture was allowed to reach room temperature. After stirring under an argon atmosphere for 72 h, EtOAc (100 mL) was added and the organic layer was washed with saturated aqueous NaHCO₃ (50 mL) and H₂O (50 mL). The aqueous layer was back-extracted with CH₂Cl₂ (3 × 20 mL) and the combined organic layers were evaporated to dryness and coevaporated with absolute EtOH/toluene (2:1 v/v, 3 × 15 mL). The resulting residue was purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v) to afford nucleoside 5 (0.35 g, 82%) as a white foam. Rf = 0.8 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS m/z 812.2765 ([M + Na]+, C₄₈H₄₃N₃O₆SNa⁺, Calc. 812.2797). Rf = 0.4 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 682.2675 ([M + Na]+, C₃₂H₂₃N₃O₅Na⁺, Calc. 682.2695).

The reaction mixture was stirred at room temperature for 3.5 h at which point ice-cold EtOH (1.0 mL) was added. The reaction mixture was diluted with CH₂Cl₂ (80 mL) and washed with saturated aqueous NaHCO₃ (50 mL). The aqueous layer was back-extracted with CH₂Cl₂ (3 × 20 mL) and the combined organic layers were evaporated to dryness and coevaporated with absolute EtOH/toluene (2:1 v/v, 3 × 15 mL). The resulting residue was purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v) to afford nucleoside 5 (0.35 g, 82%) as a white foam. Rf = 0.8 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS m/z 812.2765 ([M + Na]+, C₄₈H₄₃N₃O₆SNa⁺, Calc. 812.2797). Rf = 0.4 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 682.2675 ([M + Na]+, C₃₂H₂₃N₃O₅Na⁺, Calc. 682.2695).

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succinyl-linked LCAA-CPG (long chain alkyl amine controlled pore glass) columns with a pore size of 500 Å, and standard protocols for incorporation of $A^0$, $C^0$, $G^0$, and $T$ DNA phosphoramidites. The following hand-coupling conditions were used for incorporation of the corresponding phosphoramidites of monomers $X$, $Y$, $S$ (NH/0-3-toluyl protected) and $D$ (bis(4-isobutylaminomethylphosphoryl)-protected) (capping time; activator; coupling yield): $X$ (15 min; 5-3,5-bis(trifluoromethyl)phenyl)-1H-tetrazole; 99%), $Y$ (15 min; 4,5-dicyanoimidazol; 95%) and $S$ and $D$ (15 min; 4,5-dicyanoimidazol; 99%). Modified phosphoramidites were used at 50-fold molar excess and 0.05 M concentration in CH$_3$CN. Extended oxidation (45 s) with standard 0.05 M aqueous iodine was used for $D1$-$D4$ and $X1$-$X6$. Extended oxidation (2 × 5 min oxidation with an acetonitrile wash between oxidations) using a tert-butylhydroperoxide/CH$_3$CN/H$_2$O solution (10/87.5, v/v/v) was used for all ONs containing $S$ and $Y$ modifications to prevent desulfurization. Cleavage from solid support and removal of protecting groups was accomplished by treatment with 32%aq. ammonia (55 °C, 16–24 h). ONs were purified in the DMT-on mode via ion-pair reverse phase HPLC (C$_18$ column) using a 0.05 M triethylammonium acetate, 2,4,6-trihydroxyacetophenone (THAP) as matrices. Purity was verified using ImageJ software. The efficiency of DNA recognition was determined as the intensity ratio between the recognition complex band and the total lane. An average of three independent measurements is reported along with standard deviations. Nonlinear regression was used to fit data points from dose–response experiments, using a script written for the “Solver” module in Microsoft Office Excel.

**Definition of Zipper Nomenclature.** The following nomenclature describes the relative arrangement between two pyrene-function-alized monomers positioned on opposing strands in a duplex: the number $n$ describes the distance measured in number of base pairs and has a positive value if a monomer is shifted toward the 5′-side of its own 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 own strand relative to a second reference monomer on the other strand.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00369.

General experimental section; NMR spectra for new compounds; MS data for new modified ONs; representative $T_m$ curves; additional thermal denaturation, UV–vis absorption, steady-state fluorescence emission, thermodynamic parameter and dsDNA recognition data. (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: hrducka@uidaho.edu.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This study was supported by Award Number GM088697 from the National Institute of General Medical Sciences, National Institutes of Health and by Grant No. 12658-I from the University of Idaho Student Grant Program. We thank Dr. Alex Blumenfeld (Dept. Chemistry, Univ. Idaho) and Dr. Lee Blumenfeld (Dept. Chemistry, Univ. Idaho) for their contributions to this work.
Deobald (EBI Murdock Mass Spectrometry Center, Univ. Idaho) for assistance with NMR and mass spectrometric analysis, and Prof. Carolyn Hovde (Food Science, Univ. Idaho) for access to gel documentation stations. Dr. Dale C. Guenther and Ms. Grace H. Anderson (Dept. Chemistry, Univ. Idaho) are thanked for making X-modified ONs available for this study.

**DEDICATION**

This article is dedicated to the memory of Prof. Leszek Czuchajowski (Univ. Idaho): Pole, poet, and professor.

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