Stabilizing or Destabilizing Oligodeoxynucleotide Duplexes Containing Single 2′-Deoxyuridine Residues with 5-Alkynyl Substituents

Thomas Kottysch, Carolin Ahlborn, Frank Brotzel, and Clemens Richert*[a]

Abstract: The 5-position of pyrimidines in DNA duplexes offers a site for introducing alkynyl substituents that protrude into the major groove and thus do not sterically interfere with helix formation. Substituents introduced at the 5-position of the deoxyuridine residue of dU:dA base pairs may stabilize duplexes and reinforce helices weakened by a low G/C content, which would otherwise lead to false negative results in DNA chip experiments. Here we report on a method for preparing oligonucleotides with a 5-alkynyl substituent at a 2′-deoxyuridine residue by on-support Sonogashira coupling involving the fully assembled oligonucleotide. A total of 25 oligonucleotides with 5-alkynyl substituents were prepared. The substituents either decrease the UV melting point of the duplex with the complementary strand or increase it by up to 7.1 °C, compared with that of the unmodified control duplex. The most duplex-stabilizing substituent, a pyrenylbutyramidopropyne moiety, is likely to intercalate but does not prevent sequence-specific base pairing of the modified deoxyuridine residue or the neighboring nucleotides. It also increases the signal for a target strand when employed on a small oligonucleotide microarray. The ability to tune the melting point of a DNA dodecamer duplex with a single side chain over a temperature range of >11 °C may prove useful when developing DNA sequences for biomedical applications.

Keywords: alkynes · cross-coupling · DNA recognition · nucleobase modification · oligonucleotides

Introduction

Since oligodeoxynucleotides have become routinely available through chemical syntheses,[1] the number of applications for this class of biomacromolecules has increased steadily. Antisense[2] and antigene therapies,[3] as well as immunostimulation through oligonucleotides containing the dinucleotide CG,[4] are applications for oligonucleotides established in the last three decades. Among the applications that have caused the most massive demand for synthetic oligodeoxynucleotides, however, are the amplification of DNA by polymerase chain reaction[5] with oligonucleotide primers and the parallel detection of complementary strands by oligonucleotide microarrays (DNA chips).[6]

Sequence-specific formation of Watson–Crick helices between probe strands immobilized on the surface and target strands in solution is the principle underlying molecular recognition on DNA chips. The ability of probe strands to form duplexes with the target depends on the rate of duplex formation, the stability of the duplex, and the dissociation rate during the washing process. Unfortunately, duplex stability depends on G/C content. Probes rich in A and T form weak duplexes, which can cause false negative results in chip-based experiments.[7] Therefore, DNA chips with modified probes are beginning to emerge,[8,9] and efforts are being made to develop isostable DNA, that is, modified DNA where A:T and T:A base pairs are strengthened and/or G:C and C:G base pairs are weakened,[10] such that the stability of the resulting DNA duplexes of a given length is independent of their sequence.[11] For 2′-deoxyadenosine residues in oligonucleotides, advances have been made toward analogues whose affinity for thymidine residues in DNA duplexes is similar to that of 2′-deoxyguanosine for 2′-deoxycytidine residues.[12] With thymidine as the modified residue, increased base-pairing stability has been achieved for peptide nucleic acid strands.[13] Other modifications involving the nucleobases in DNA that affect duplex stability have recently been reviewed.[14]

From the possible sites for introducing chemical modifications in thymidine that may reinforce T:A base pairs, the 5-position was chosen for the present study, since alkynyl groups at this position, such as a propyne group replacing

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the methyl group, are known to stabilize duplexes.[15,16] Ethyne spacers at the 5-position of a 2′-deoxyuridine residue place substituents in the spacious major groove of DNA duplexes (Figure 1). The rigid spacer thus positions these substituents into the solvent, from where they may fold back to interact with the DNA but should not sterically interfere with duplex formation. One would therefore expect such ethynyl-linked substituents not to destabilize duplexes but either to stabilize them or to leave duplex stability unaffected. Here we report on a number of substituents linked to 2′-deoxyuridine residues through ethynyl linkers that substantially stabilize or destabilize DNA duplexes.

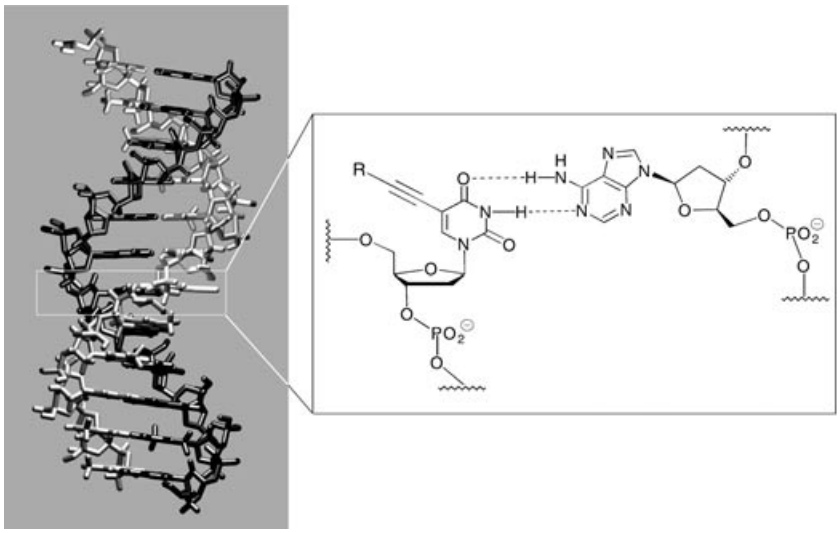


Figure 1. B-form DNA double helix, where the site of incorporation of an ethynyl substituent at the 5-position of a 2′-deoxyuridine residue is highlighted. The coordinates were generated by using Macromodel,[43] and the figure was produced with VMD software.[43] The inset shows the base pair with the deoxyuridine residue featuring the alkynyl substituent.

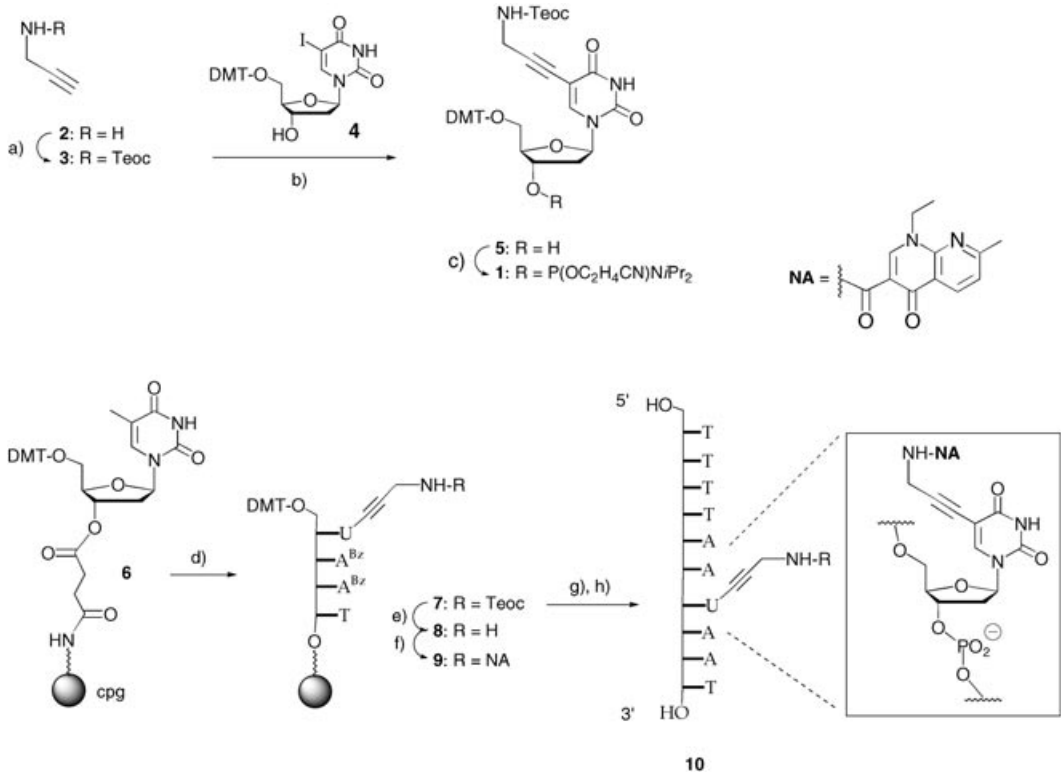
Results and Discussion

The synthetic work started with the preparation of phosphoramidite **1** (Scheme 1), which is suitable for incorporation into oligodeoxynucleotides by automated DNA synthesis. Propargylamine (**2**) was N-protected with a Teoc group and the protected alkyne **3**[17] was employed in a Sonogashira coupling[18,19] with iodonucleoside **4**.[20] Other methods to introduce aminopropargyl chains are known,[21] and so are other protecting groups,[22] but these were deemed less suitable for the present case. The 3′-alcohol **5** was converted into **1** in 52% overall yield from **4**. Starting from a commercial cpg loaded with thymidine (**6**), protected tetramer **7** was prepared by automated DNA synthesis. Several conditions for removing the Teoc group without cleaving the DNA from the cpg or dissolving the cpg altogether were tested, and the synthetic success of these conditions was evaluated by analyzing MALDI-TOF mass spectra of crude mixtures obtained after treatment with aqueous ammonia. Both HF/pyridine and tris(dimethylamino)sulfonium trimethylsilyl difluoride led to a softening of the support, after 30 and 10 min, respectively, a result suggesting degradation of the cpg. Furthermore, even after incomplete conversion, MALDI-TOF mass spectra of crude products from reactions with these fluoride sources showed approximately 20–30% of a side product whose mass was indicative of the formation of ethyloxycarbonyl-protected propargylamine side chains. This was interpreted as the result of a cleavage of the Si–C bond with subsequent protonation at the carbon center. The ethyloxycarbonyl-protected side product was also found as a low-level side product when Teoc deprotection of **7** was performed with TBAF (1 M in THF) for 5 min, but no loss of integrity of the cpg was apparent under these conditions, so amine **8** could be acylated with activated nalidixic acid, a quinolone previously shown to stabilize oligonucleotide duplexes when appended to 3′-terminal 2′-amino-2′-deoxyuridine residues.[23] The resulting acylated tetramer **9** was extended by automated DNA synthesis and deprotected to give decamer **10**.

The UV melting point of the duplex of **10** and the unmodified complementary DNA strand 5′-ATTATTAAAA-3′ (**11**), was below 15 °C at a concentration of 1 M NaCl in sodium citrate buffer (pH 7), whereas that of control duplex TTTTAATAAT:ATTATTAAAA (**12**:**11**) was 21 °C. This suggested i) that the substituent at the 5-position of the deoxyuridine residue of **10** has a substantial destabilizing, rather than stabilizing effect[24] and ii) that a duplex sequence of higher melting point was needed to measure the effect of alkyne substituents. Furthermore, it was desirable to develop a synthesis suitable for higher throughput, in order to study the unexpected duplex destabilization that the substituents protruding from the 5-position of deoxyuridine residues can have.

Accordingly, the route shown in Scheme 2 was developed. It generates oligonucleotide dodecamers with three deoxycytidine residues. The melting point of unmodified control duplex CTTTTCTTTCTT:AAGAAAGAAAAG (**13**:**14**) was found to be 43.2 °C at 1 M NaCl (Table 1). This is sufficiently high to allow detection of significant destabilizing effects without leaving the experimentally accessible temperature window. Furthermore, melting points obtained with this sequence may be compared with those containing other modifications.[25,26] Finally, the pyrimidine residues in the sequence ensure a sufficiently low steric bulk of the DNA on the controlled pore glass to allow for on-support reactions with sterically demanding reagents.

The synthesis of the dodecamers again started from commercial, thymidine-loaded cpg **6**, which was extended by automated phosphoramidite syntheses (Scheme 2). At the sixth position from the 3′-terminus, a 2′-deoxy-5-iodouridine



Scheme 1. a) Teoc-OC_6H_4-NO_2, NEt_3, 60%; b) [$Pd(PPh_3)_4$], NEt_3, CuI, 82%; c) NCC_2H_4O-P(N-*i*Pr_2)$_2$, DIPAT, 63%; d) DNA synthesis with the phosphoramidite protocol, including **1**; e) TBAF; f) NA-OH, HBTU, HOBt, DIEA; g) DNA synthesis; h) NH_4OH.

Table 1. UV melting points of duplexes between unmodified (**13**) or modified (**19a**–**w**, **y**, **z**) oligonucleotides and the complementary strand 5′-AA-GAAAGAAAAG-3′ (**14**).

Sequence[a]	10 mM NaCl[b]			100 mM NaCl[b]			1 M NaCl[b]		
	T_m [°C][c]	ΔT_m [°C]	hyperchr. [%][d]	T_m [°C][c]	ΔT_m [°C]	hyperchr. [%][d]	T_m [°C][c]	ΔT_m [°C]	hyperchr. [%][d]
13	19.0±0.7	–	30	39.0±0.6	–	34	43.2±0.6	–	31
19a	<15	–	–	22.3±1.0	−16.7	[e]	24.6±1.1	−18.6	[e]
	<15	–	–	39.5±0.4	0.5	[e]	42.5±0.2	−0.7	[e]
19b	<15	–	–	35.4±1.1	−3.6	29	38.5±0.8	−4.7	27
19c	<15	–	–	37.9±1.0	−1.1	28	40.4±0.8	−2.8	26
19d	15.4±0.5	−3.6	20	37.5±1.0	−1.5	24	41.3±0.8	−1.9	24
19e	17.2±0.6	−1.8	24	37.7±1.1	−1.3	28	41.6±0.9	−1.6	27
19f	16.8±0.9	−2.2	26	38.8±0.8	−0.2	27	41.8±0.5	−1.4	26
19g	16.0±0.4	−3.0	22	38.6±1.0	−0.4	25	42.5±0.8	−0.7	25
19h	17.7±0.7	−1.3	27	39.9±0.8	0.9	32	42.8±0.8	−0.4	33
19i	19.0±1.0	0.0	23	38.9±0.7	−0.1	27	42.9±0.7	−0.3	25
19j	17.1±0.4	−1.9	20	39.1±0.7	0.1	26	42.9±0.8	−0.3	24
19k	19.6±0.6	0.6	28	38.2±0.5	−0.8	33	43.1±0.5	−0.1	30
19l	20.3±0.7	1.3	29	39.0±0.7	0.0	32	43.7±0.8	0.5	29
19m	20.3±0.6	1.3	26	39.4±0.7	0.4	31	43.9±0.6	0.7	28
19n	18.4±0.7	−0.6	27	39.8±1.0	0.8	31	43.9±0.8	0.7	27
19o	21.2±0.7	2.2	36	40.0±0.5	1.0	40	44.2±0.7	1.0	31
19p	19.2±0.7	0.2	28	40.4±0.8	1.4	31	44.2±0.7	1.0	29
19q	19.3±1.0	0.3	27	40.7±1.0	1.7	30	44.4±0.8	1.2	28
19r	18.3±0.7	−0.7	30	40.4±0.7	1.4	33	44.5±0.4	1.3	31
19s	19.6±0.9	0.6	28	41.4±0.8	2.4	31	44.5±0.8	1.3	30
19t	20.0±1.1	1.0	26	40.5±0.8	1.5	30	44.7±0.7	1.5	28
19u	20.1±0.8	1.1	24	40.6±0.7	1.6	30	45.0±0.7	1.8	28
19v	20.7±0.8	1.7	33	41.7±0.8	2.7	30	45.5±0.7	2.3	30
19w	20.3±0.6	1.3	28	42.9±0.6	3.9	31	45.6±0.5	2.4	29
19y	<15	–	–	37.6±0.7	−1.4	23	40.4±0.7	−2.8	23
19z	25.6±0.5	6.6	24	46.1±0.4	7.1	31	49.6±0.5	6.4	30

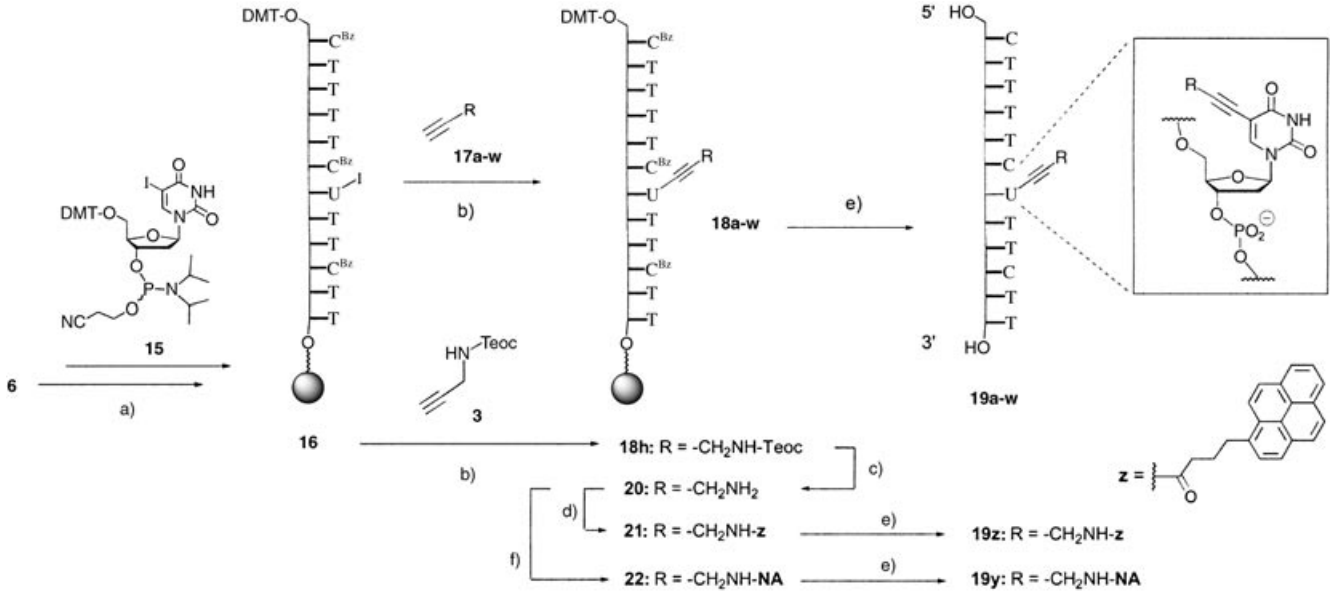
[a] Sequences are 5′-CTTTTCU(**a–w/y/z**)TTCTT-3′, where U(**a–w/y/z**) denotes the deoxyuridine residue alkynyl substituted at the 5-position. [b] Experiments performed in 10 mM PIPES buffer (pH 7) with 10 mM $MgCl_2$ and with the appropriate concentration of NaCl (10 mM, 100 mM, or 1 M). [c] Average of four melting points ± the standard deviation; ΔT_m = the difference in melting point relative to that of the unmodified control duplex. [d] Hyperchr. = the average of hyperchromicity at 260 nm upon duplex dissociation. [e] Two transitions with varying hyperchromicity observed; see Figure S29 in the Supporting Information for details.

residue was incorporated by using **15**. Unlike earlier synthetic approaches,[27,28] the present protocol does not require interrupting the DNA synthesis for the Sonogashira coupling; instead, this reaction is performed on the fully assembled oligomer **16**. A total of 23 different substituents were initially introduced on-support, by using a mixture of the respective terminal alkynes (**17a–w**), $[Pd(PPh_3)_2Cl_2]$, PPh_3, and CuI in THF/NEt_3 for 5 h at room temperature. The "side chains" of the alkynes (**a–w**) are shown in Scheme 3. The side chain **m** was introduced by coupling 3-(trimethylsilyl)-propyne and desilylating with TBAF, analogously to the conversion of **18h** to **20** (see below). Steroid side chains **b** and **f** were included in the study, since steroid–DNA interactions have recently been described that increase duplex stability and base-pairing fidelity.[29] The alkynes leading to side chains **l** and **q** were used as racemic mixtures and the corresponding oligonucleotides were prepared as mixtures of two diastereomers. The stoichiometry of the reagents for the Sonogashira coupling was optimized with ethynylbenzene as the alkynyl substrate. An excess of CuI over the palladium catalyst, a substoichiometric quantity of triphenylphosphine, and a twofold excess of triethylamine over the alkyne gave the best results, as determined by MALDI-TOF mass spectrometry of crude products.

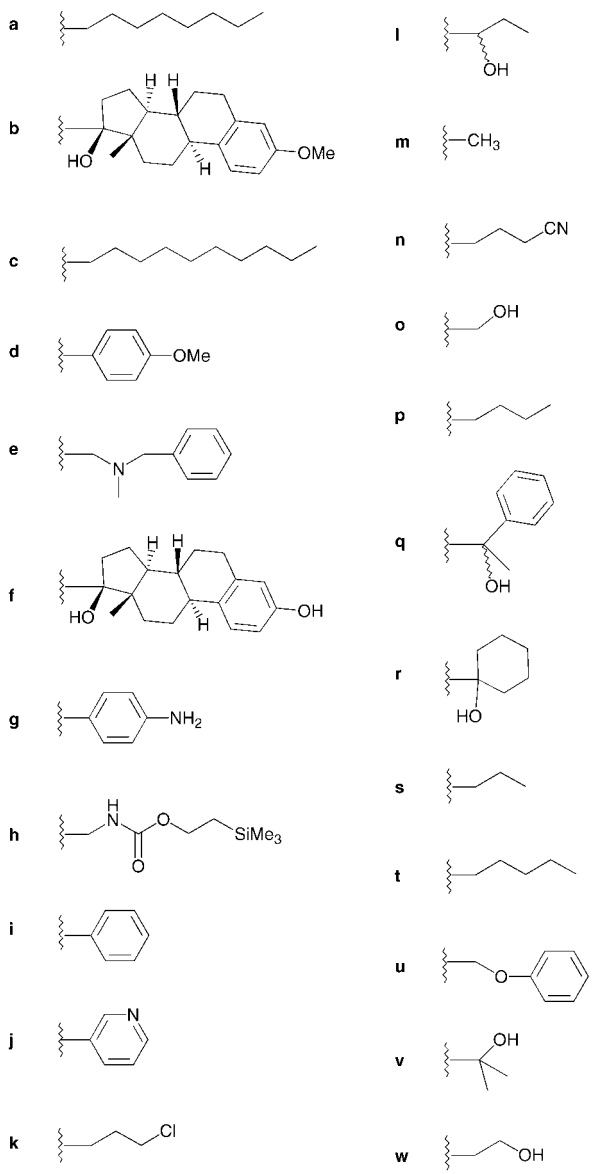
Deprotection of oligonucleotides **18a–w** with aqueous ammonia at room temperature for 14 h gave **19a–w**. All oligonucleotides were purified by HPLC and obtained in an overall yield of 17–53%, as determined by integration of the HPLC traces of the crude products. The average overall yield of the solid-phase syntheses was approximately 30%, a result validating the on-support synthesis method, which greatly increased the efficiency of the screening. After careful relyophilization to remove traces of the volatile HPLC buffer, samples were prepared for UV melting experiments with complementary DNA strand **14**. Since the alkynyl substituents of **19l** and **19q** were introduced with the racemic mixtures that are commercially available, the melting points were also determined with the combined diastereomers. All other oligonucleotides are single isomers. Interestingly, **19k** was isolated as the chloride, a fact indicating that the alkyl halide survives the strongly basic/nucleophilic deprotection conditions.

The melting points of the duplexes between **19a–w** and **14** were found to vary over a wide range (Table 1). Both destabilization and increases of up to 3.9°C were observed, with a shallow dependence on the salt concentration of the solution. The values reported are the mean of four melting curves each. The consistently high hyperchromicities indicated that full duplexes were formed in each case, even for the duplex with the lowest melting point (Figure 2). Only for the duplex **19a**:**14** were two transitions of variable relative intensity observed (see Figure S29 in the Supporting Information). Neither size nor lipophilicity are discernibly correlated with the differences in the UV melting points (ΔT_m values) observed. Compound **19m**, containing propynyl deoxyuridine, the compound whose alkynyl modification is well known,[15,16] gave small melting-point increases in agreement with what could be expected based on the literature. Unexpected, however, was the effect of chain length on duplex stability for the oligonucleotides with other *n*-alkyl substituents. The octyl chain (**a**) gives, in the first transition, a ΔT_m value of up to −18.6°C, the decyl chain (**c**) gives no more than −4.6°C melting-point depression, a butyl chain (**p**) gives a minimal increase in melting point, but propyl (**s**) and pentyl chains (**t**) give ΔT_m values of between +1 and +2.4°C, depending on the salt concentration. The hydroxyethyl group (**w**) was identified as the most duplex-stabilizing side chain in this series, both at medium and high salt concentrations, with a ΔT_m value of up to +3.9°C at 100 mM NaCl concentrations. This substituent is one of the smallest moieties tested.

The synthetic work was then extended to oligonucleotides with acylated propargylamine side chains. These were prepared by coupling Teoc-protected **3** to **16**, deprotecting **18h**



Scheme 2. a) DNA synthesis with standard phosphoramidites and **15**; b) $[Pd(PPh_3)_2Cl_2]$, CuI, NEt_3, PPh_3; c) TBAF; d) pyrene butyric acid, HBTU, HOBt, DIEA; e) NH_4OH; f) NA-OH, HBTU, HOBt, DIEA.



Scheme 3. Side chains of alkynyl groups employed in this study.

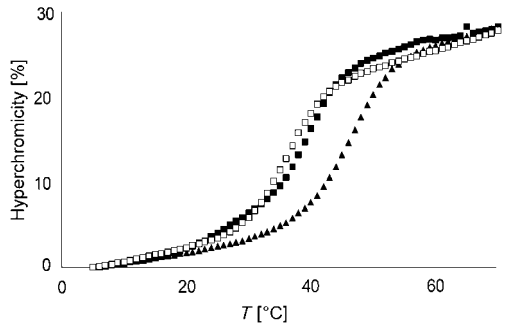


Figure 2. Melting curves of selected oligonucloetide duplexes. **19b**:**14** (□); **19m**:**14** (■); **19z**:**14** (▲). Experimental conditions: 1.6 μM strand concentration, 10 mM PIPES buffer (pH 7) with 100 mM NaCl and 10 mM $MgCl_2$.

with TBAF to form **20**, and acylating either with 4-(pyren-1-yl)butyric acid (pyrene butyric acid) to obtain **21** or with nalidixic acid to produce **22**. Deprotection with aqueous ammonia produced **19y** and **19z** (Scheme 2). As with the side chains **a**–**w**, a large difference in melting point was observed for the duplexes of the two acylated oligonucleotides with target strand **14**. Whereas pyrene-bearing **19z** gave a markedly increased melting point with a ΔT_m value of up to +7.1 °C, nalidixoyl oligonucleotide **19y** lowered the melting point by up to −2.8 °C (Table 1). For **19y**:**14**, the hyperchromicity associated with duplex dissociation was significantly lower than that determined for **19z**:**14**, a result suggesting that the quinoline substituent may have a disrupting effect on the base-pairs, as described for the related 5′-appended acyl groups.[30]

Since the 6.4–7.1 °C melting point increase observed for the pyrenyl derivative **19z** is high for a single modification, tests were performed to determine whether the duplex stabilization is sufficient to reinforce the duplex as much as a C:G base pair at the same site in the sequence. Accordingly, unmodified duplex 5′-CTTTTC*C*TTCTT-3′:5′-AAGAA*G*-GAAAAG-3′ (**23**:**24**) was prepared, which contains such a base pair at the same position as the alkynyl-substituted uracil:adenine base pair in **19z**:**14**. The melting points of **23**:**24** are 23.1 °C at 10 mM NaCl, 44.0 °C at 100 mM NaCl, and 46.8 °C at 1 M NaCl concentrations. They are thus only 3.6–5.0 °C higher than those of the control duplex **13**:**14** (Table 1). In other words, the pyrene-containing substituent **z** more than compensates for the weaker base pairing in a T:A base pair compared with a C:G base pair in this sequence context.

Since a recent NMR spectroscopy study on a duplex containing a pyrenyl-substituted cytidine nucleoside that intercalates in a DNA duplex reported a peak broadening for the 5′-neighboring base pair,[31] we decided to study the effect of our pyrenyl residue on base-pairing selectivity. If our modification also substantially enhances the dynamics (by inducing an increased breathing rate), it should lower mismatch discrimination at the site of modification and at the neighboring positions of the duplex **19y**:**14**. Accordingly, the melting points of duplexes with single mismatches in the target strand, both at the site of modification and at the neighboring base pairs, were determined. These are compiled in Table 2. Gratifyingly, the melting point decrease caused by a single mismatch was at least 8.6 °C in the presence of the pyrenyl unit for all duplexes studied, a result suggesting that canonical base pairs are being formed with satisfactory fidelity. Force-field minimization of duplexes with different starting conformations led to structures where the pyrenyl moiety was intercalated, even if the pyrene ring was outside an intact Watson–Crick helix at the beginning of the calculation. A typical structure is shown in Figure 3. It can be discerned that the linker between the 5-position of the deoxyuridine and the pyrene ring is of a proper length for intercalation.

Next, exploratory experiments with DNA microarrays prepared on aldehyde-bearing glass slides were performed with an oligonucleotide probe carrying the pyrenyl substituent. For these, lysine-loaded cpg **25**[32] was extended to

Table 2. UV melting points of duplexes of **13** or **19z** and target strands with or without a mismatched nucleobase.

[NaCl][a]	Oligonucleotide[b]	Target strand[b]	T_m [°C][c]	ΔT_m [°C]	hyperchr. [%][d]
150 mM	CTTTTCTTTCTT (**13**)	AAGAAA**A**GAAAAG (**14**)	35.9±0.7	–	27
		AAGAA**C**GAAAAG	22.0±0.7	−13.9	24
		AAGAA**G**GAAAAG	25.3±0.6	−10.6	21
		AAGAA**T**GAAAAG	22.4±0.9	−13.5	24
		AAGA**C**AGAAAAG	22.7±0.9	−13.2	34
		AAGAAA**T**AAAAG	20.5±0.8	−15.4	25
	CTTTTCU*TTCTT (**19z**)	AAGAAA**A**GAAAAG (**14**)	41.8±0.7	–	27
		AAGAA**C**GAAAAG	29.2±0.8	−12.6	25
		AAGAA**G**GAAAAG	33.0±1.0	−8.8	26
		AAGAA**T**GAAAAG	34.3±0.8	−7.5	22
		AAGA**C**AGAAAAG	30.9±0.7	−10.9	22
		AAGAAA**T**AAAAG	23.0±0.6	−18.8	22
1 M	CTTTTCTTTCTT (**13**)	AAGAAA**A**GAAAAG (**14**)	43.9±0.6	–	28
		AAGAA**C**GAAAAG	30.2±0.7	−13.7	26
		AAGAA**G**GAAAAG	33.1±0.9	−10.8	25
		AAGAA**T**GAAAAG	31.0±0.7	−12.9	27
		AAGA**C**AGAAAAG	30.4±0.8	−13.5	28
		AAGAAA**T**AAAAG	28.3±0.7	−15.6	29
	CTTTTCU*TTCTT (**19z**)	AAGAAA**A**GAAAAG (**14**)	49.2±0.5	–	26
		AAGAA**C**GAAAAG	36.4±0.8	−12.8	23
		AAGAA**G**GAAAAG	39.2±0.7	−10.0	25
		AAGAA**T**GAAAAG	40.6±0.7	−8.6	23
		AAGA**C**AGAAAAG	38.4±0.9	−10.8	23
		AAGAAA**T**AAAAG	30.4±0.6	−18.8	23

[a] Experiments performed with 1.5±0.3 μM strand concentration in 10 mM sodium phosphate buffer (pH 7) with the appropriate concentration of NaCl (150 mM or 1 M). [b] Sequences are given from the 5′- to the 3′-terminus; U* denotes a 5′-modified deoxyuridine. [c] Average of four melting points ± one standard deviation; ΔT_m = the difference in melting point relative to that of the perfectly matched duplex. [d] Hyperchr. = the average of hyperchromicity at 260 nm upon duplex dissociation from four curves.

Figure 3. Hypothetical structure of the duplex **19z**:**14**, as obtained by force-field minimization with Macromodel, from the starting point of a conformation where the pyrenyl moiety is extrahelical and the base pairs are continuously stacked.

mixed-sequence tetradecamer **26** and the 5-iodo group of the modified uridine was coupled to **3** on-support to give **27** (Scheme 4). MALDI-TOF mass spectra of an analytical sample obtained through deprotecting **27** showed approximately 70% conversion during the on-support Sonogashira coupling involving this longer mixed-sequence oligomer. Deprotection with TBAF gave **28**, whose acylation with the residue of pyrene butyric acid produced **29**. Conventional deprotection and HPLC purification gave **30**, which was immobilized on slides passified with an amino tri(ethylene glycol) methyl ether by reductive amination, as described for other modified oligonucleotides.[32] Unmodified control tetradecamer **32** was immobilized on neighboring spots. Both sequences were spotted in duplicate to study reproducibility. Incubation with fluorescent target strand Cy3-ATCGCAGTCAACCA (**33**),[32] followed by washing and scanning with a chip reader, produced the fluorescence image shown in Figure 4. An increase in fluorescence signal can clearly be discerned for the spots displaying **31** on their surface.

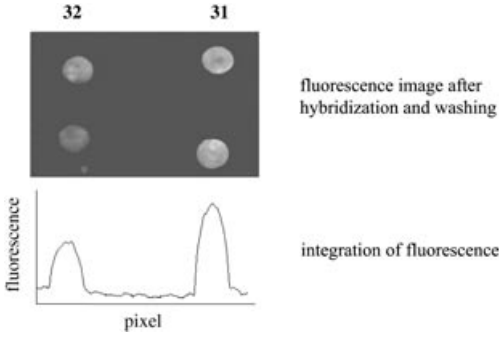
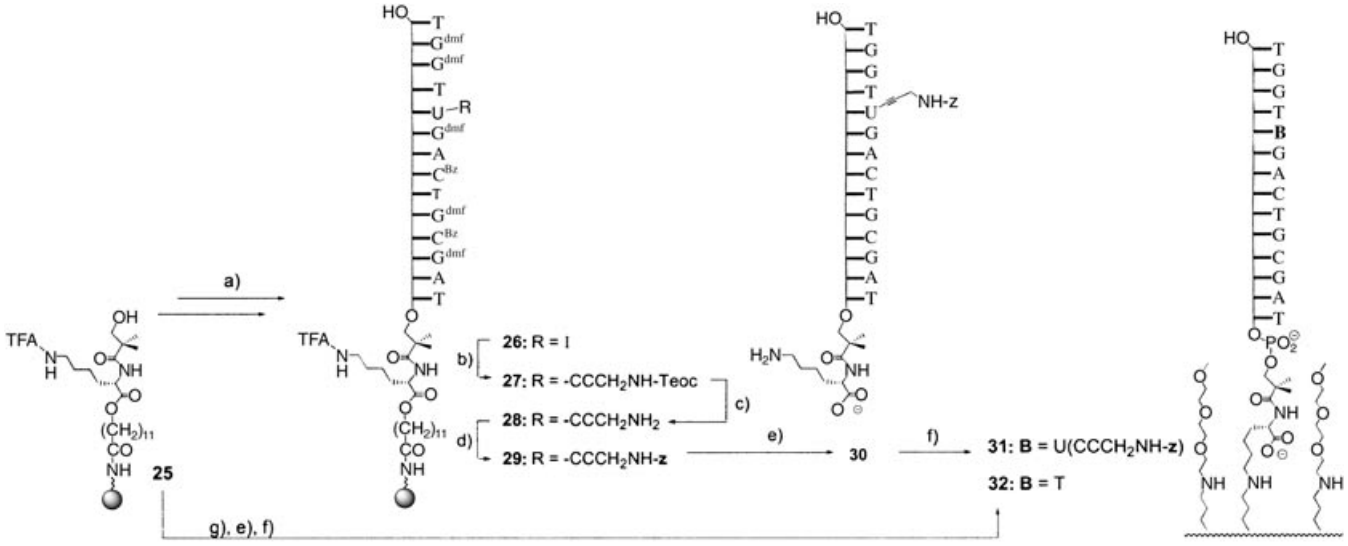


Figure 4. Fluorescence of a DNA microarray displaying spots with immobilized DNA strands **31** (right) or **32** (left), after incubation with fluorophore-labeled complementary strand Cy3-ATCGCAGTCAACCA (**33**) and washing. Spots were generated in duplicate to test for reproducibility. Shown below the fluorescence image is the integration over the slices containing the respective peak pair.

On a synthetic level, the current route provides an example of a successful on-support derivatization involving a fully assembled oligonucleotide. For screening, this is desirable, as it avoids the time-consuming synthesis of individual nucleosides and their phosphoramidite building blocks. Furthermore, side-chain functionalities incompatible with subsequent phosphoramidite couplings do not need to be protected, a measure that is required when interrupting syntheses



Scheme 4. a) DNA synthesis with standard phosphoramidites and **15**; b) **3**, $[Pd(PPh_3)_2Cl_2]$, CuI, NEt_3, PPh_3; c) TBAF; d) pyrene butyric acid, HBTU, HOBt, DIEA; e) NH_4OH; f) immobilization on background-passified aldehyde slides; g) DNA synthesis with standard phosphoramidites.

for cross-coupling.[27] Duplex-stabilizing side chain **w**, for example, would not have been compatible with the extension of the DNA chain in a synthesis interrupted at the modified nucleoside without a protecting group. Also, among the different cross-coupling reactions suitable for DNA,[33] the Sonogashira coupling arguably uses the mildest conditions, a fact making it suitable for constructing sequences containing otherwise fragile substituents at position 5.

On the level of the structure and stability of the folded DNA structures, the current work shows how strong an effect alkynyl substituents at the 5-position of a single deoxyuridine can have on a DNA duplex. This is interesting, since the alkynyl substituents attached to this position of the deoxyuridine residue do not sterically block any portion of the backbone or nucleobases in the duplex (Figure 1). Instead, the rigid ethynyl linker directs them straight into the solvent, where they protrude from the major groove. Closely related ethenyl substituents are even known to be tolerated by polymerases when introduced to 2′-deoxyuridine 5′-triphosphate.[34]

How then is the destabilization of duplexes with substituents such as **a**, **b**, or **c** to be explained? Most probably, both sides of the equilibrium that UV melting curves report on (the single-stranded or nonduplex state and the duplex state) must be considered. Both destabilizing the duplex state and stabilizing the single-stranded state may shift the equilibrium to the nonduplex side. Since duplex-destabilizing substituents such as **a**, **b**, and **c** are lipophilic, they may stabilize a folded structure of single strands where they are buried in the interior. The more polar groups, such as the phosphodiester anions, would presumably be placed on the exterior of the folded or micellar structure. If so, such substituents could not only provide an opportunity to tune the stability of duplexes but the presumed folded structures could also prove useful for formulating DNA for biomedical applications. For example, a side chain structurally similar to **a**, but chemically labile, could be protecting during transport but release a high-affinity form of the oligonucleotide at the desired site of deployment. Disulfide units are structurally similar to an ethylene unit, but are readily cleaved intracellularly, where a reducing environment is found. A conversion of the disulfide derivative of **a** could thus lead to a shape mimic of **t** with a terminal thiol group replacing the methyl group.

Furthermore, the duplex-stabilizing substituents identified, most notably **z**, have the potential to lead to hybridization probes with affinity-enhancing replacements for thymidine residues. With these, isostable DNA,[11] where any sequence gives a similar melting point, may be more readily generated. Oligonucleotide microarrays (DNA chips) with increased fidelity may be obtained when using isostable DNA probes, since with probe oligonucleotides whose affinity for a target strand is independent of the sequence, stringent hybridization conditions can be developed for an entire chip. Under these stringent conditions, target strands with a single mismatch may not be well bound by any of the probes immobilized. To ensure high sensitivity for such arrays, it is desirable to reinforce T:A and A:T base pairs when generating isostable DNA, rather than to destabilize C:G and G:C base pairs.

The details of the molecular recognition are also worth discussing. Propynyl groups have been found to strengthen base pairs in DNA duplexes by providing additional stacking interactions with neighboring base pairs, increasing the polarizability of the nucleobases, and possibly through an effect on the hydration in the minor groove.[35] Larger alkynyl groups may further stabilize duplexes by directly bridging the two strands of a helix. A detailed comparison of duplexes where one or several dA, dC, dG, or dU residues were derivatized with propynyl groups showed that, with a melting point increase of 0.75–1.5 °C per modification, the deoxyuridine residues give the smallest effect.[12b] This indicates that for these residues the development of stabilizing derivatives is particularly challenging. The ΔT_m value for the pro-

pynyl substituent (**m**) in the current sequence context (0.4–1.3 °C, Table 1) is in the lower range of what is found for other sequences, a fact indicating that a homopyrimidine sequences may be particularly difficult to stabilize. This may be due to the fact that neither of the direct neighbors is a purine, which presents a larger surface for stacking. Also, since a single modification is being studied, co-operative stabilizations, involving several alkynyl groups,[16] must be absent. Therefore, other sequences might experience more stabilization by the pyrenyl substituent **z**.

For substituent **z**, which is likely to intercalate, the duplex-stabilizing effect may be due to stacking interactions with the bases forming the intercalation site. More rigidly attached pyrenes give smaller melting-point increases[36] and so do pyrenes attached through the 2′-position.[37] Maybe the linker employed in the current work is particularly favorable for duplex stabilization because it has the right length combined with a sufficient degree of rigidity through its ethynyl and amido portions. Also, the more rigid substituent **z** does not seem to favor alternative structures, since the lower entropy of duplex formation for **19z**:**14** relative to that for the control duplex (Table 3) indicates increased preorganization towards duplex formation of the single-stranded state. In this respect, the effect is similar to that of propynyl groups, which have been reported to have a rigidifying effect on the single-stranded state.[38]

Table 3. Thermodynamic parameters for duplex dissociation at 100 mM NaCl,[a] derived from fits to the UV melting data performed with the program Meltwin.

Duplex	$\Delta H°$ [kcal mol^{-1}]	$\Delta S°$ [cal mol^{-1} K^{-1}]	$\Delta G°$ [kcal mol^{-1}]
13:**14**	105.6	307.9	10.1
19z:**14**	82.7	230.2	11.3

[a] The experimental conditions are the same as those given in Table 1.

Conclusion

In conclusion, the current results show that 5-alkynyl substituents provide an opportunity to tune the melting point of a duplex over a wide range. The most duplex-stabilizing pyrenyl substituent **z**, when attached to a deoxyuridine residue, more than compensates for the weakness of a T:A base pair relative to the strength of a C:G base pair. The substituent can be installed through on-column reactions and the duplex-stabilizing effect is also observed on a DNA microarray. Therefore, there is hope that substituents of the type studied here can be used to fine tune the affinity of oligonucleotides towards their target and eventually help to create DNA chips with isostable probes that engage their target strands under uniformly stringent conditions. Such DNA chips should have higher fidelity than the current models with unmodified probe strands.

Experimental Section

Abbreviations: Boc = *tert*-butyloxycarbonyl, Bz = benzoyl, cpg = controlled pore glass, Cy3 = indodicarbocyanine label, Dp = 3-hydroxy-2,2-dimethylpropionic acid residue, DIEA = *N*,*N*-diisopropylethylamine, DIPAT = *N*,*N*-diisopropylammonium tetrazolide, dmf = *N*,*N*-dimethylformamidino group, DMF = *N*,*N*-dimethylformamide, DMT = dimethoxytrityl group, HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, MES = 2-(*N*-morpholino)-ethanesulfonic acid, PBS = phosphate-buffered saline, PIPES = piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), SDS = sodium dodecylsulfate, SSC = saline sodium citrate, TBAF = tetrabutylammonium fluoride, Teoc = 2-(trimethylsilyl)ethoxycarbonyl group, TFA = trifluoroacetic acid residue, T_m = UV melting point of DNA duplex, U* = 5-modified deoxyuridine.

General: Anhydrous solvents were obtained over molecular sieves and were used without further purification. Unless otherwise noted, reagents were from Acros (Geel, Belgium), Aldrich/Fluka/Sigma (Deisenhofen, Germany), or Merck (Darmstadt, Germany). The building block Boc-Lys-(TFA)-OH and the reagents HOBt and HBTU were from Advanced ChemTech (Louisville, KY) and were used without purification. Reagents for DNA synthesis, including 4,5-dicyanoimidazole activator, and phosphoramidites (dA^{Bz}, dC^{Bz}, T, dG^{dmf}) were from Proligo (Hamburg, Germany), except for dG^{dmf}-loaded cpg, which was from ABI (Warrington, UK). Underivatized long-chain alkyl amine cpg (loading: 77.5 μmol g^{-1}) was from Controlled Pore Glass Inc. (Lincoln Park, NJ). The phosphoramidite of 2′-deoxy-5′-dimethoxytrityl-5-iodouridine was from Glen Research (Sterling, VA). Oligonucleotides were purified by reversed-phase HPLC, with a gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0) and detection at 260 nm, by using Nucleosil C4 columns for modified oligonucleotides and C18 columns for unmodified oligonucleotides (both 250 × 4.6 mm; Macherey–Nagel, Düren, Germany). Yields of oligonucleotides were determined from the integration of the HPLC trace of the crude products. The integration was not corrected for the absorbance caused by the solvent front. Extinction coefficients for oligonucleotides were calculated through linear combination of the extinction coefficients of the nucleotides and are uncorrected for hyperchromicity effects. For modified oligonucleotides, the extinction coefficients were calculated as the sum of the extinction coefficient of the unmodified oligonucleotide and the extinction coefficient of the incorporated alkyne. Aldehyde-coated glass slides were from Genetix Ltd. (New Milton, UK). For hybridization experiments, PBS solution (pH 7), 20-fold concentrated SSC solution (pH 7.4), and MES buffer (pH 6.3) were prepared by using standard protocols.[39] MALDI-TOF spectra were acquired on a Bruker REFLEX IV spectrometer in negative, linear mode, by using the software XACQ 4.0.4 and XTof 5.1. The MALDI matrix mixtures (2:1 *v*/*v*) for oligonucleotides were 2,4,6-trihydroxyacetophenone (0.2 M in ethanol) and diammonium citrate (0.1 M in water). Calculated masses are average masses but *m*/*z* peaks found are those of the unresolved pseudomolecular ions ([*M*−H]$^-$). The accuracy of mass determination with the external calibration used is approximately ±0.1 %. DNA sequences are given from the 5′- to the 3′-terminus; U* in a sequence denotes a 5-modified-deoxyuridine and Dp represents a 3-hydroxy-2,2-dimethylpropionic acid residue. Molecular modeling was performed with MacroModel 3D GLX.[43]

***N*-(2-Trimethylsilyl)ethoxycarbonylpropargylamine (3)**: A stirred solution of 2-(trimethylsilyl)ethyl *p*-nitrophenyl carbonate (3.00 mmol, 0.85 g) in triethylamine (15 mL) was treated with propargylamine (**2**, 4.20 mmol, 0.23 g, 268 μL). The mixture was left for 16 h in the dark at room temperature. Triethylamine and excess propargylamine were removed under reduced pressure and the yellow oily crude was purified by column chromatography (silica gel, CH_2Cl_2/MeOH (step gradient 20:1→4:1)) to give **3** as a slightly yellow oil (0.33 g, 1.79 mmol, 60 %). Spectroscopic data were in agreement with the literature.[17]

2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(2-trimethylsilyl)ethoxycarbonyl-3-aminopropynyl]uridine (5): NEt_3 (42 μL, 304 μmol) and DMF (1 mL) were added to a mixture of 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine[20] (**4**, 100.7 mg, 153 μmol), *N*-(2-trimethylsilyl)ethoxycarbonylpropargylamine (**3**, 58 mg, 300 μmol), CuI (7.24 mg, 38.0 μmol), and triphenylphosphine (17.6 mg, 15.2 μmol). The resulting solution was stirred under argon for 2 d at room temperature. The solvent was removed in vacuo and the residue was purified by chromatography (silica gel,

CH_2Cl_2/MeOH/NEt_3 30:1:0.3) to yield **5** (90.5 mg, 125 μmol, 82%) as a slightly yellow solid. $R_f = 0.5$ ($CHCl_3$/MeOH 9:1); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.0$ (s, 9H), 0.91 (t, 2H, $J = 8.9$ Hz), 1.82 (br s, 1H), 2.21 (m, 1H), 2.60 (m, 2H), 3.35 (m, 2H), 3.79 (s, 6H), 3.81 (t/overlapping m, 2H), 4.09 (m, 3H), 4.58 (m, 1H), 6.31 (t, 1H, $J = 6.7$ Hz), 6.84 (m, 4H), 7.05–7.58 (m, 9H), 8.12 (s, 1H), 9.05 (br s, 1H) ppm; FAB MS (3-NBA matrix): m/z: 750 $[M+Na]^+$, 303 $[DMT^+]$.

2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(2-trimethylsilyl)ethoxycarbonyl-3-aminopropynyl]uridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylamino phosphoramidite) (1): Protected aminopropynyluridine **5** (187 mg, 259 μmol) and DIPAT (23.2 mg, 136 μmol) were taken up in CH_3CN (0.74 mL) and treated with 2-cyanoethyl-bis(diisopropylamino)phosphoramidite (0.118 mL, 372 μmol) under argon and with stirring. After complete conversion of the starting material (followed by TLC, $CHCl_3$/MeOH 9:1, R_f (**1**) = 0.6), the reaction mixture was partitioned between dichloromethane (5 mL) and sat. $NaHCO_3$ (5 mL). The aqueous phase was reextracted twice with dichloromethane (5 mL each) and the combined organic phases were dried over Na_2SO_4. The volume was reduced to 1 mL in vacuo and the solution was dripped into petroleum ether (10 mL); this was followed by thorough mixing. The supernatant was aspirated and the residue was purified by chromatography (silica gel, column prepared with CH_2Cl_2/NEt_3 9:1 and eluted with CH_2Cl_2/NEt_3 99:1) to yield **1** as a colorless foam (168 mg, 164 μmol, 63%). ^{31}P NMR (101 MHz, [D$_6$]DMSO): $\delta = 150.9$, 151.4 ppm; FAB MS (3-NBA matrix): m/z: 950 $[M+Na]^+$, 303 $[DMT^+]$.

DNA synthesis: Oligonucleotides were synthesized by using a polypropylene reaction chamber for DNA synthesis (Prime Synthesis, Aston, PA). For the chain-elongation steps, the β-cyanoethyl phosphoramidites were employed in an 8909 Expedite DNA synthesizer (system software 2.01) with the standard protocol for 1 μmol syntheses. Modified phosphoramidites were coupled for 6 min. The synthesis of support **25** followed a published protocol.[40]

General protocol for cleavage of oligonucleotides from solid support (general protocol A): The cpg loaded with the oligonucleotide was briefly dried (0.1 Torr) and was treated with ammonium hydroxide (sat. aqueous NH_3, 1 mL) in a polypropylene vessel. After 16 h at room temperature, the supernatant was aspirated and the solid support was washed with water (2 × 0.3 mL). The aqueous solutions were combined and excess ammonia was removed with a gentle stream of compressed air. The solution was filtered (pore size 0.2 μm) and used directly for HPLC purification.

General protocol for the removal of DMT groups from oligonucleotides purified "trityl on" (general protocol B): Modified oligonucleotides were synthesized and HPLC-purified with the DMT group intact ("trityl on"), except for compounds **19a**, **19c**, **19k**, **19n**, and **19o**. A solution of the oligonucleotide (approximately 200 nmol) in water (0.5 mL) was treated with aqueous acetic acid (80% *v*/*v*, 5 mL) for 15 min. After washing with diethyl ether (3 × 0.7 mL), the aqueous layer was lyophilized and the residue was taken up in water (50 μL) to generate a stock solution for UV melting experiments.

General protocol for on-support Sonogashira coupling (general protocol C): The following procedure is for coupling **17q** to **16** and is representative. The cpg loaded with the iododeoxyuridine-containing oligonucleotide (**16**, 2 mg, approximately 50 nmol loading) was dried (0.1 Torr) for 1 h in a polypropylene vessel. A slurry of $[Pd(Ph_3P)_2Cl_2]$ (2.9 mg, 3.6 μmol) and **17q** (11.8 mg, 81 μmol) in THF (210 μL) was added, followed by a solution of PPh_3 (0.29 mg, 1.1 μmol) in THF (30 μL). A solution of copper iodide (0.82 mg, 4.3 μmol) and triethylamine (20 μL, 142 μmol) in THF (20 μL) was then added to the slurry. The reaction was allowed to proceed for 16 h at room temperature with shaking. The supernatant was aspirated and the cpg bearing the modified oligonucleotide was washed with DMF, a solution of triethylamine in DMF (1% *v*/*v*), a solution of the sodium salt of ethylenedithiocarbamic acid in DMF (0.5% *v*/*v*), and methanol (250 μL of each). The support was then dried at 0.1 Torr.

5′-CTTTTCU*TTCTT-3′ (19a): After completion of the DNA synthesis, 1-decyne (**17a**) was treated with cpg **16** (2.1 mg, 53 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 56$ min. Removal of the DMT group by using general protocol B gave **19a** (25%); HPLC (trityl-off, CH_3CN, gradient: 0% for 5 min to 20% in 60 min): $t_R = 46$ min; MALDI-TOF MS: m/z: calcd for $C_{126}H_{168}N_{27}O_{79}P_{11}$: 3665.5; found: 3667.8.

5′-CTTTTCU*TTCTT-3′ (19b): After completion of the DNA synthesis, 17-ethynyl-3-(*O*-methyl)estradiol (**17b**; 23.5 mg, 70 μmol) was treated with cpg **16** (2.2 mg, 55 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 57$ min. Removal of the DMT group by using general protocol B gave **19b** (20%). MALDI-TOF MS: m/z: calcd for $C_{137}H_{176}N_{27}O_{81}P_{11}$: 3837.7; found: 3834.2.

5′-CTTTTCU*TTCTT-3′ (19c): After completion of the DNA synthesis, 1-dodecyne (**17c**, 15 μL, 70 μmol) was treated with cpg **16** (1.9 mg, 47.5 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 53$ min. Removal of the DMT group by using general protocol B gave **19c** (21%). HPLC (trityl-off, CH_3CN, gradient: 0% for 5 min to 15% in 45 min): $t_R = 49$ min; MALDI-TOF MS: m/z: calcd for $C_{128}H_{182}N_{27}O_{79}P_{11}$: 3693.6; found: 3691.2.

5′-CTTTTCU*TTCTT-3′ (19d): After completion of the DNA synthesis, 4-ethynylanisole (**17d**, 10.2 μL, 70 μmol) was treated with cpg **16** (3.3 mg, 83 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 50% in 25 min): $t_R = 22$ min. Removal of the DMT group by using general protocol B gave **19d** (28%). MALDI-TOF MS: m/z: calcd for $C_{125}H_{158}N_{27}O_{80}P_{11}$: 3659.4; found: 3659.1.

5′-CTTTTCU*TTCTT-3′ (19e): After completion of the DNA synthesis, benzylmethylpropargylamine (**17e**, 12.0 μL, 71 μmol) was treated with cpg **16** (2.7 mg, 68 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 20% in 5 min to 50% in 25 min): t_R = 22 min. Removal of the DMT group by using general protocol B gave **19e** (27%). MALDI-TOF MS: m/z: calcd for $C_{127}H_{163}N_{28}O_{79}P_{11}$: 3686.5; found: 3686.0.

5′-CTTTTCU*TTCTT-3′ (19f): After completion of the DNA synthesis, 17-ethynylestradiol (**17f**, 25.4 mg, 70 μmol) was treated with cpg **16** (2.0 mg, 50 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient 0% for 5 min to 30% in 60 min): $t_R = 54$ min. Removal of the DMT group by using general protocol B gave **19f** (25%). MALDI-TOF MS ($C_{136}H_{174}N_{27}O_{81}P_{11}$): calcd: 3823.7; found: 3820.9.

5′-CTTTTCU*TTCTT-3′ (19g): After completion of the DNA synthesis, 4-ethynylaniline (**17g**, 13 μL, 70 μmol) was treated with cpg **16** (3.0 mg, 75 nmol loading) according to general protocol C and then deprotection according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 48$ min. Removal of the DMT group by using general protocol B gave **19g** (37%). MALDI-TOF MS: m/z: calcd for $C_{124}H_{157}N_{28}O_{79}P_{11}$: 3644.4; found: 3643.5.

5′-CTTTTCU*TTCTT-3′ (19h): After completion of the DNA synthesis, Teoc-protected propargylamine **17h** (14 μL, 140 μmol) was treated with cpg **16** (2.2 mg, 55 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 56$ min. Removal of the DMT group by using general protocol B gave **19h** (39%). MALDI-TOF MS: m/z: calcd for $C_{125}H_{167}N_{28}O_{81}P_{11}Si$: 3726.6; found: 3721.4.

5′-CTTTTCU*TTCTT-3′ (19i): After completion of the DNA synthesis, phenylacetylene (**17i**, 7.7 μL, 70 μmol) was treated with cpg **16** (3.2 mg, 80 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 35% in 10 min to 90% in 10 min): $t_R = 20$ min. Removal of the DMT group by using general protocol B gave **19i** (23%). MALDI-TOF MS: m/z: calcd for $C_{124}H_{156}N_{27}O_{79}P_{11}$: 3629.4; found: 3626.5.

5′-CTTTTCU*TTCTT-3′ (19j): After completion of the DNA synthesis, 3-ethynylpyridine (**17j**, 7.3 mg, 71 μmol) was treated with cpg **16** (2.8 mg, 70 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 5% in 30 min): $t_R = 42$ min. Removal of the DMT group by using general protocol B gave **19j** (17%). MALDI-TOF MS: m/z: calcd for $C_{123}H_{155}N_{28}O_{79}P_{11}$: 3630.4; found: 3630.4.

5′-CTTTTCU*TTCTT-3′ (19k): After completion of the DNA synthesis, 5-chloro-1-pentyne (**17k**, 7.6 μL, 70 μmol) was treated with cpg **16** (2.0 mg, 50 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 50$ min. Removal of the DMT group by using general protocol B gave **19k** (34%). HPLC (trityl-off, CH_3CN, gradient: 0% for 5 min to 15% in 45 min): $t_R = 35$ min; MALDI-TOF MS: *m/z*: calcd for $C_{121}H_{157}N_{27}O_{79}P_{11}Cl$: 3629.8; found: 3629.9.

5′-CTTTTCU*TTCTT-3′ [(±)-19l]: After completion of the DNA synthesis, (±)-1-pentyn-3-ol (**(±)-17l**, 6.1 μL, 70 μmol) was treated with cpg **16** (1.8 mg, 45 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 46$ min. Removal of the DMT group by using general protocol B gave (±)-**19l** (36%). MALDI-TOF MS: *m/z*: calcd for $C_{121}H_{158}N_{27}O_{80}P_{11}$: 3611.4; found: 3607.6.

5′-CTTTTCU*TTCTT-3′ (19m): After completion of the DNA synthesis, 3-(trimethylsilyl)-1-propyne (**17m**, 10.5 μL, 70 μmol) was treated with cpg **16** (3.1 mg, 78 nmol loading) according to general protocol C and then desilylated with TBAF in THF, as described below for the conversion of **18h** to **20**. Oligonucleotide **19m** was obtained by treating the solid support thus obtained according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 38$ min. Removal of the DMT group by using general protocol B gave **19m** (27%); MALDI-TOF MS: *m/z*: calcd for $C_{119}H_{154}N_{27}O_{79}P_{11}$: 3566.3; found: 3568.3.

5′-CTTTTCU*TTCTT-3′ (19n): After completion of the DNA synthesis, 5-cyano-1-pentyne (**17n**, 7.4 μL, 71 μmol) was treated with cpg **16** (2.6 mg, 65 nmol loading) according to general protocol C and then deprotected with general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 50% in 25 min): $t_R = 20$ min. Removal of the DMT group by using general protocol B gave **19n** (35%). HPLC (trityl-off, CH_3CN, gradient: 0% for 5 min to 15% in 45 min): $t_R = 34$ min; MALDI-TOF MS: *m/z*: calcd for $C_{122}H_{157}N_{28}O_{79}P_{11}$: 3620.4; found: 3619.7.

5′-CTTTTCU*TTCTT-3′ (19o): After completion of the DNA synthesis, propargylalcohol (**17o**, 4.2 μL, 70 μmol) was treated with cpg **16** (2.4 mg, 60 nmol loading) according to general protocol C and then deprotected with general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 46$ min. Removal of the DMT group by using general protocol B gave **19o** (31%). HPLC (trityl-off, CH_3CN, gradient: 0% for 5 min to 15% in 45 min): $t_R = 30$ min; MALDI-TOF MS: *m/z*: calcd for $C_{119}H_{154}N_{27}O_{80}P_{11}$: 3583.3; found: 3582.2.

5′-CTTTTCU*TTCTT-3′ (19p): After completion of the DNA synthesis, 1-hexyne (**17p**, 8.1 μL, 70 μmol) was treated with cpg **16** (1.9 mg, 48 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 48$ min. Removal of the DMT group by using general protocol B gave **19p** (31%). MALDI-TOF MS: *m/z*: calcd for $C_{122}H_{160}N_{27}O_{79}P_{11}$: 3609.4; found: 3609.3.

5′-CTTTTCU*TTCTT-3′ (19q): After completion of the DNA synthesis, **17q** (11.8 mg, 81 μmol) was treated with cpg **16** (2 mg, 50 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 50$ min. Removal of the DMT group by using general protocol B gave **19q** (53%). MALDI-TOF MS: *m/z*: calcd for $C_{126}H_{160}N_{27}O_{80}P_{11}$: 3673.4; found: 3672.8.

5′-CTTTTCU*TTCTT-3′ (19r): After completion of the DNA synthesis, 1-ethynylcyclohexanol (**17r**, 9.1 μL, 70 μmol) was treated with cpg **16** (3.3 mg, 83 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 50% in 30 min): $t_R = 27$ min. Removal of the DMT group by using general protocol B gave **19r** (40%). MALDI-TOF MS: *m/z*: calcd for $C_{124}H_{162}N_{27}O_{80}P_{11}$: 3651.4; found: 3650.2.

5′-CTTTTCU*TTCTT-3′ (19s): After completion of the DNA synthesis, 1-pentyne (**17s**, 6.9 μL, 70 μmol) was treated with cpg **16** (1.8 mg, 45 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 47$ min. Removal of the DMT group by using general protocol B gave **19s** (21%). MALDI-TOF MS: *m/z*: calcd for $C_{121}H_{158}N_{27}O_{79}P_{11}$: 3595.4; found: 3596.6.

5′-CTTTTCU*TTCTT-3′ (19t): After completion of the DNA synthesis, 1-heptyne (**17t**, 9.2 μL, 70 μmol) was treated with cpg **16** (2.7 mg, 68 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 55% in 30 min): $t_R = 27$ min. Removal of the DMT group by using general protocol B gave **19t** (43%). MALDI-TOF MS: *m/z*: calcd for $C_{123}H_{162}N_{27}O_{79}P_{11}$: 3623.4; found: 3620.7.

5′-CTTTTCU*TTCTT-3′ (19u): After completion of the DNA synthesis, 3-phenoxy-1-propyne (**17u**, 9.1 μL, 70 μmol) was treated with cpg **16** (3.6 mg, 90 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 55% in 30 min): $t_R = 27$ min. Removal of the DMT group by using general protocol B gave **19u** (20%). MALDI-TOF MS: *m/z*: calcd for $C_{125}H_{158}N_{27}O_{80}P_{11}$: 3659.4; found: 3657.0.

5′-CTTTTCU*TTCTT-3′ (19v): After completion of the DNA synthesis, 2-methyl-3-butyn-2-ol (**17v**, 6.9 μL, 70 μmol) was treated with cpg **16** (1.8 mg, 45 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 50$ min. Removal of the DMT group by using general protocol B gave **19v** (17%). MALDI-TOF MS: *m/z*: calcd for $C_{121}H_{158}N_{27}O_{80}P_{11}$: 3611.4; found: 3610.8.

5′-CTTTTCU*TTCTT-3′ (19w): After completion of the DNA synthesis, 3-butyn-1-ol (**17w**, 5.3 μL, 70 μmol) was treated with cpg **16** (3.3 mg, 83 nmol loading) according to general protocol C and then deprotected according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 47$ min. Removal of the DMT group by using general protocol B gave **19w** (6.8%). MALDI-TOF MS: *m/z*: calcd for $C_{120}H_{157}N_{27}O_{80}P_{11}$: 3597.4; found: 3597.9.

5′-CTTTTCU*TTCTT-3′ (19y): After completion of the DNA synthesis, *N*-(2-trimethylsilyl)ethoxycarbonyl propargylamine (**3**, 24 μL, 240 μmol) was treated with cpg **16** (4.0 mg, 100 nmol loading) to give **18h** according to general protocol C. The support was treated with a solution of TBAF (200 μmol, 1 M solution) in THF (200 μL) for 15 min. Support **20** was then washed with THF, DMF, and methanol (2×1 mL each) and dried (0.1 Torr). A mixture of nalidixic acid (46.4 mg, 200 μmol), HOBt (27.0 mg, 200 μmol), and HBTU (68.2 mg, 180 μmol) in DMF (600 μL) was treated with DIEA (80 μL, 60.4 mg, 230 μmol). After 2 min, the resulting solution was added to support **20**. The slurry was shaken for 60 min. After removal of the supernatant, solid support **21** was washed with THF, DMF, and methanol (1 mL each) and dried at 0.1 Torr. The DMT group was removed by treating with deblock solution for DNA synthesizers (3% trichloroacetic acid in CH_2Cl_2, 500 μL). Compound **19y** (26%) was then liberated from the solid support by using general protocol A. HPLC (CH_3CN, gradient 0% for 5 min to 30% in 60 min): $t_R =$ 38 min; MALDI-TOF MS: *m/z*: calcd for $C_{131}H_{165}N_{30}O_{81}P_{11}$: 3796.5; found: 3798.3.

5′-CTTTTCU*TTCTT-3′ (19z): Support-bound intermediate **20** was prepared as described above for compound **19y**. A mixture of 1-pyrene butyric acid (37.1 mg, 129 μmol), HOBt (19.1 mg, 141 μmol), and HBTU (45.7 mg, 121 μmol) in DMF (500 μL) was treated with DIEA (48 μL, 36.2 mg, 48 μmol). After 2 min, the resulting solution was added to cpg **20** (10.2 mg, approximately 260 nmol loading). The reaction mixture was shaken for 60 min to produce **21**. After removal of the supernatant, the solid support was washed with THF, DMF, and methanol (1 mL each) and dried. The DMT-protected precursor of **19z** was cleaved from the solid support according to general protocol A. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 60 min): $t_R = 31$ min. The DMT group was removed by using general protocol B to give **19z** (9%). MALDI-TOF MS: *m/z*: calcd for $C_{139}H_{169}N_{28}O_{80}P_{11}$: 3853.7; found: 3853.4.

5′-TGGTU*GACTGCGAT-Dp-Lys-3′ (30): *N*-(2-Trimethylsilyl)ethoxycarbonyl propargylamine (**3**, 28 μL, 280 μmol) was treated with cpg **26** (9.2 mg, 110 nmol loading) according to general protocol C. The subsequent steps (removal of Teoc group, coupling of **17z**, and final deprotection with cleavage from the support) were performed as described above for **19z**. HPLC (trityl-on, CH_3CN, gradient: 0% for 5 min to 30% in 55 min): $t_R = 45$ min. The DMT group was removed by using general protocol B to give **30** (4%). MALDI-TOF MS: *m/z*: calcd for $C_{171}H_{211}N_{53}O_{92}P_{14}$: 4924.8; found: 4923.9.

5′-TGGTTGACTGCGAT-Dp-Lys-3′ (32) and 5′-Cy3-ATCGCAGTCAACCA-3′ (33): These sequences, used for DNA chip experiments, were prepared as previously reported.[32]

DNA chip generation: The protocol for preparing DNA chips is similar to that reported previously.[32] Briefly, spots of paraffin wax separately heated to 130 °C were applied to selected areas of aldehyde-modified glass slides. After the wax solidified, the background was passified with a solution of 6 mM 3,6,9-trioxadecylamine and 3.7 mM $NaBH_3CN$ in PBS (5 mL) for 6.5 h at room temperature. The slide was washed with 1× SSC/0.2% SDS and water, and then dried. The wax was removed with CH_2Cl_2 and the slide was rinsed with more CH_2Cl_2 and ethanol, and then dried under a stream of argon. The lysine-terminated oligonucleotides (**30** and the precursor to **32**, 1 μL, 2 μM in 0.1 M MES buffer, pH 6.3) were applied to the respective spots on the slide. Solutions of $NaBH_3CN$ (0.5 μL, 31 mM in PBS buffer, pH 7.4) were added to the droplets and the reaction was allowed to proceed in a humid chamber for 11 h at room temperature. The solutions were removed and the slide was immediately washed with 1×SSC/0.2% SDS (50 mL) and water, and then dried. Remaining aldehyde groups were treated with 3,6,9-trioxydecylamine by means of reductive amination of the slide surface as described above.

Hybridization and scanning: The 5′-Cy3-labeled DNA (**33**, 20 μL, 10 μM in 2×SSC/0.2% SDS buffer) was exposed to the slide surface under a cover slip for 17 h at room temperature. The cover slip was removed while dipping the slide into the first washing solution. The washing involved 1×SSC/0.2% SDS for 4 min, 0.1×SSC/0.2% SDS for 2 min, 0.1× SSC for 2 min, and water (twice) for 30 s. The washing steps with buffers were done with sonication for 5 s. For the washing steps, the appropriate solutions (50 mL) were used in separate beakers. The slides were dried with compressed air while being pulled out of the water. The fluorescence scans were performed with an Array WoRxe Biochip Reader (GeneScan, Freiburg, Germany). The integration of the fluorescence signals was obtained with the NIH Image/Scion Image program.[41]

UV melting experiments: UV melting experiments were performed with a Perkin–Elmer Lambda 10 spectrophotometer at 260 nm and with a path length of 1 cm, at heating or cooling rates of 1 °C min^{-1}. Buffer conditions are given in the respective tables. Prior to acquiring melting curves, duplexes were annealed by heating to 70 °C and cooling to 5 °C at a rate of 2 °C min^{-1}. Melting temperatures were determined with the program UV Winlab 2.0 (Perkin–Elmer) and are averages of the maxima of the first derivative of the 91-point smoothed curves from the heating and cooling experiments. Hyperchromicities were determined by calculating the difference in adsorption between high- and low-temperature baselines and dividing by the adsorption at the low-temperature baseline. Thermodynamic data were calculated by fitting to melting curves with the program Meltwin, which was provided by Dr. McDowell and Prof. Turner.[42]

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