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Twisted Intercalating Nucleic Acids – Intercalator Influence on Parallel Triplex Stabilities

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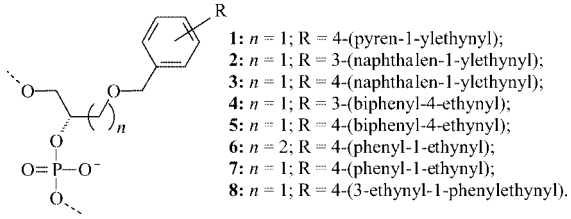
Phosphoramidites of several new twisted intercalating nucleic acid (TINA) monomers and the previously discovered (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**1**) were synthesized and used in DNA synthesis. Stabilization of Hoogsteen-type triplexes was observed in cases of insertion of the novel (*R*)-1-*O*-[3-(naphthalen-1-ylethynyl)phenylmethyl]glycerol (**2**) as a bulge into homopyrimidine oligodeoxynucleotides (ONs), whereas phenylethynyl and 4-(biphenylylethynyl) derivatives of TINAs resulted in destabilization of parallel triplexes relative to the wild-type triplex. It was concluded that TINA monomers should possess at least two fused phenyl rings attached through the triple bond at the 4-position of bulged (*R*)-1-*O*-(phenylmethyl)glycerol in homopyrimidine ONs in order to stabilize parallel triplexes. Slight destabilization of DNA/DNA Watson–Crick type duplexes (ΔT_m = 1.0–4.5 °C) was detected for **2** inserted as a bulge, while RNA/DNA duplexes and duplexes with other TINA analogues were considerably destabilized (ΔT_m > 6.0 °C). In cases of double insertion of **1** opposite to base inversions in dsDNA, the thermal stabilities of the triplexes were higher than that of the wild-type triplex, which is a new solution to overcome the problem of targeting homopurine stretches with single base pair inversions. A DNA three-way junction was considerably stabilized (ΔT_m in a range of 10.0–15.5 °C) upon insertion of TINA monomers in the junction point as a bulge.

Introduction

DNA triple helix formation is a subject of intensive research for gene targeting and alteration.[1–3] Triple helixes are formed when a single-stranded triplex-forming oligonucleotide (TFO) binds to a purine-containing strand of dsDNA through specific major groove interactions. Generally, the third-strand affinity of a TFO is low, due to the requirement for the formation of pH-sensitive C^+–G–C Hoogsteen base triplexes under physiological conditions in the parallel (pyrimidine) binding motif. Modification of TFOs has been attempted in order to improve their binding affinities to their targets and to alleviate restrictions in the dsDNA sequence with the design of new triplex nucleobases. Oligonucleotides containing modified nucleic acid components – such as peptide nucleic acids (PNAs),[4] locked nucleic acids (LNAs),[5,6] 2′-(aminoethyl)oligoribonucleotides (2′-AE-RNAs),[7] and N3′→P5′ phosphoramidates[8] – and inducing increased binding affinity are among the most successful chemically modified TFOs. Recently, we have found that bulge insertions of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**1**) into the middles of homopyrimidine oligodeoxynucleotides (twisted intercalating nucleic acids, TINAs) can give rise to extraordinarily high thermal stability in Hoogsteen-type triplexes and duplexes, whereas Watson–Crick-type duplexes of the same nucleotide content were destabilized.[9] Even at pH = 7.2, a TFO with two bulged insertions of **1** separated by three bases formed a stable triplex (T_m = 43.0 °C), whereas the native oligonucleotide was unable to bind to the target dsDNA. We believe that this technology, along with the development of modified nucleic bases designed for the recognition of inversion sites, should be applicable for sequence-selective dsDNA targeting (Scheme 1).



1: *n* = 1; R = 4-(pyren-1-ylethynyl);
2: *n* = 1; R = 3-(naphthalen-1-ylethynyl);
3: *n* = 1; R = 4-(naphthalen-1-ylethynyl);
4: *n* = 1; R = 3-(biphenyl-4-ethynyl);
5: *n* = 1; R = 4-(biphenyl-4-ethynyl);
6: *n* = 2; R = 4-(phenyl-1-ethynyl);
7: *n* = 1; R = 4-(phenyl-1-ethynyl);
8: *n* = 1; R = 4-(3-ethynyl-1-phenylethynyl).

Scheme 1. TINA monomers.

In order to evaluate how the structures of TINA monomers influence the thermal stabilities of parallel triplexes and antiparallel duplexes we synthesized several new TINA monomers. These analogues were incorporated into oligodeoxynucleotides (ONs) and the thermal stabilities of their

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complexes were compared with those of the previously investigated TINAs. The synthesis of the phosphoramidite of the monomer **1**, previously obtained by post-synthetic Sonogashira coupling,[9] is described and the compound was used in a DNA synthesis. It was found that positioning of the intercalator through a triple bond attached at the 4-position of the phenyl ring in (*R*)-1-*O*-(phenylmethyl)glycerol gave higher thermal stability than that through the 3-position in the case of Hoogsteen-type triplexes.

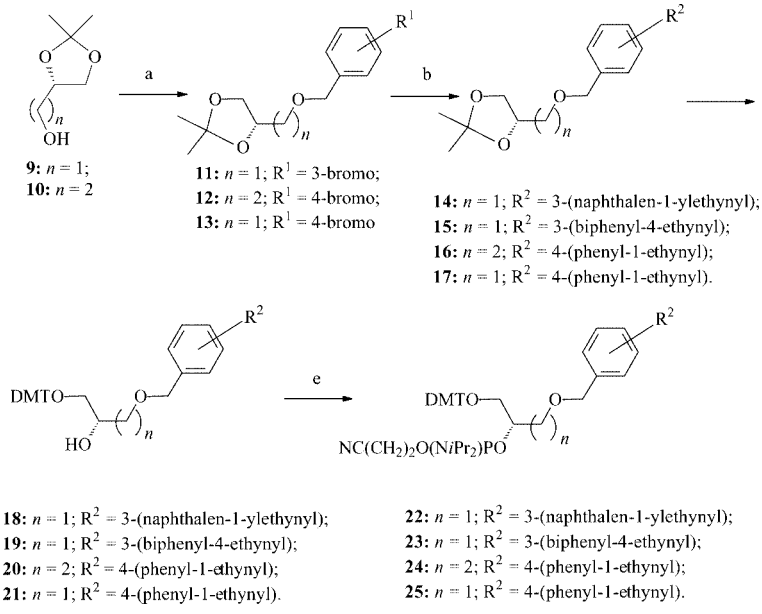
Results and Discussion

Synthesis of Phosphoramidites of TINA Monomers

As the planned synthetic route for phosphoramidites of TINA monomers was intended to be over only five steps, we decided to synthesize these phosphoramidites. In this way the previously developed post-synthetic Sonogashira reaction on the CPG support[9] can be avoided for repetitive synthesis of the same modification (although for screening of large numbers of different modifications we still prefer the post-synthetic approach). Compounds **9** and **10** were treated with 3- or 4-bromobenzyl bromide under Dean–Stark conditions (toluene, KOH), affording compounds **11**–**13** in quantitative yields (Scheme 2). The degree of conversion in the Sonogashira coupling reaction depended on the reactivity of the corresponding acetylenes: thus, under the same conditions [$Pd(PPh_3)_2Cl_2$, CuI, iPr_2NEt, THF, reflux under N_2], use of 1-ethynylnaphthalene resulted in the formation of compound **14** in 92% yield after 24 h, whereas with 4-ethynylbiphenyl only very low conversion of starting material was observed by TLC even after 4 d. In the latter case a yield of 28% of compound **15** was obtained in a dry DMF/1,4-dioxane (5:2) mixture as medium after heating at 80 °C for 3 d. The low reactivity of phenylacetylene in reactions with **12** and **13** [$Pd(PPh_3)_2Cl_2$, CuI, iPr_2NEt, THF, reflux under N_2] resulted in difficulties with isolation of the products on silica gel because of the similar mobilities of the products, byproducts and phenylacetylene, so compounds **16** and **17** were finally purified by semipreparative HPLC on a C_{18} column in 23% and 18% yields, respectively. It has been shown that application of microwave[10] or ultrasound[11] irradiation may result in increased rates and yields in Sonogashira coupling reactions. Attempts to obtain compound **17** with ultrasound irradiation using the above reagents were unsuccessful, but the yield of **17** was increased up to 33% through application of microwave irradiation (2×25 min, 120 °C) for the reaction mixture (also containing PPh_3 to improve the stability of the palladium catalyst) in a solution of Et_2NH/DMF.[11]

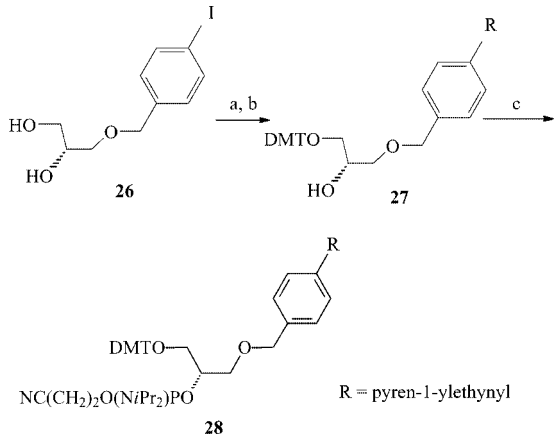
The isopropylidene protection groups of **14**–**17** were cleaved in 80% aq. AcOH and the primary alcohols were protected with DMTCl in CH_2Cl_2 in the presence of Et_3N, followed by silica gel purification to afford compounds **18**–**21** in moderate yields over two steps (65–77%). Phosphitylation of **18**–**21** by treatment with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite and diisopropylammonium tetrazolide in CH_2Cl_2 afforded the required phosphoramidites **22**–**25**, which were used in the synthesis of ONs.

The insertion of the monomer **1** into ONs had previously been achieved through post-synthetic Sonogashira modification of ONs immobilized on a CPG solid support comprising (*R*)-1-*O*-(4-iodobenzyl)glycerol through treatment with 1-ethynylpyrene.[9] Such post-synthetic derivatization



Scheme 2. Reagents and conditions: a) 4(3)-Bromobenzyl bromide, KOH, toluene, reflux. b) For compounds **14**, **16**, **17**: Ar-1-ylethynyl, $Pd(PPh_3)_2Cl_2$, CuI, iPr_2NEt, THF, reflux. For compound **15**: 4-ethynylbiphenyl, $Pd(PPh_3)_2Cl_2$, CuI, DMF/NEt_3, 80 °C. For compound **17**: phenylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, PPh_3, Et_2NH/DMF, microwave. c) 80% aq. CH_3COOH, room temp. d) DMTCl, NEt_3, CH_2Cl_2, room temp. e) $NC(CH_2)_2OP(NiPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, room temp.

of ONs is a good method for screening of different analogues, but it is a time-consuming process with already discovered compounds for routine DNA preparation. Moreover, we had observed that post-synthetic Sonogashira modification was not efficient with more than two insertions of (*R*)-1-*O*-(4-iodobenzyl)glycerol in the middle of an ON,[9] so the synthesis of the phosphoramidite of the monomer **1** was needed for routine incorporation into DNA. Because of the observed low reactivities of bromobenzyl derivatives **11**–**13** in Sonogashira reactions with arylethynyl compounds and problems with isolation of products we decided to synthesize the phosphoramidite **28** in three steps from (*R*)-1-*O*-(4-iodobenzyl)glycerol (**26**,[9] Scheme 3). This last compound was treated with a Sonogashira reaction mixture containing 1-ethynylpyrene and $Pd(PPh_3)_4$ as a catalyst to afford (*R*)-3-[4-(pyren-1-ylethynyl)benzyloxy]propane-1,2-diol, which was further used without chromatographic purification. Compound **27** was obtained in 57% yield (calculated from **26**) after protection of the primary alcohol with DMTCl in dry pyridine and silica gel purification. Overnight treatment of compound **27** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in the presence of diisopropylammonium tetrazolide in dry CH_2Cl_2, followed by silica gel column chromatography, gave the final phosphoramidite **28**, which was used in a DNA synthesis.



Scheme 3. Reagents and conditions: a) Pyren-1-ylethynyl, $Pd(PPh_3)_4$, CuI, DMF/NEt_3, room temp. b) DMTCl, pyridine, room temp. c) $NC(CH_2)_2OP(NiPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 0 °C to room temp.

Thermal Stability Studies

The thermal stabilities of parallel triplexes, DNA/DNA and DNA/RNA duplexes, and DNA three-way junctions with the synthesized ONs were assessed by thermal denaturation experiments. The melting temperatures (T_m [°C]), determined as the first derivatives of melting curves, are listed in Tables 1, 2, 3, and 4. For comparison we have included data for **ON2**, **ON10**, **ON12**, **ON15**, **ON22** and **ON30**, incorporating the previously published TINA monomers **1**, **3**, **5** and **8**.[9] The pH dependence of Hoogsteen-type base pairing in the sequences possessing different TINAs – both in a parallel triplex fashion toward the duplex **D1** and in a parallel dsDNA fashion toward **ON15** – was studied (Table 1). The thermal stabilities of single mismatched parallel triplexes (**D2**–**D4**) are listed in Table 2.

We also studied the hybridization affinities of TINAs containing **1**, **2**, **4**, and **6** toward mixed purine/pyrimidine sequences of ssDNA and ssRNA in Watson–Crick-type duplexes (Table 3) with sequences and conditions similar to those previously described.[9]

As can be seen from the T_m data in Table 1, stabilization of parallel triplexes and parallel duplexes [relative to the wild-type complexes (**ON1**)] was observed for the newly synthesized monomer **2** (**ON3**) and for the previously described TINA monomers **1** (**ON2**) and **3** (**ON10**) when inserted as bulges in the middle of the sequence at pH = 6.0. Incorporation of the monomer **2** at the 5′-end (**ON4**) gave better stabilization of Hoogsteen-type triplexes and duplexes than 3′-end incorporation did (**ON5**). Interestingly, at pH = 7.2 the TFO possessing intercalator **2** at the 5′-end (**ON4**) formed the most stable triplex among all the TFOs incorporating single insertion of **2**. Earlier we had observed the contrary effect for the monomer **1**.[9] On comparison of the triplex and parallel duplex thermal stabilities of **ON3** and **ON10** it can be concluded that attachment of a naphthalene-1-ylethynyl substituent at the 4-position of the phenyl ring in (*R*)-1-*O*-(phenylmethyl)glycerol inserted as a bulge in the middle of TFO is more efficient than attachment at the 3-position. This conclusion can also be applied to other aromatic structures, since the same tendency can be seen for **ON11** and **ON12**, with biphenyl-4-ylethynyl substituents at 3- and 4-positions, respectively. It is believed that the acetylene bond improves the intercalating properties,[12,13] as can also be seen on comparison of the T_m values for triplexes with **ON14** and **ON15**, with attachment of the acetylene bond to the phenyl ring resulting in a 2.5 °C increase in triplex thermal stability. Use of a longer linker, as in the case of monomer **6**, resulted in a slightly decreased thermal stability relative to the monomer **7**. These data support the general impression that aromatic structures containing more than two fused phenyl rings are best attached to the 4-positions in (*R*)-1-*O*-(phenylmethyl)glycerol components inserted as a bulge in the middle of a TFO. Ethynyl substitution also improves intercalating properties, and therefore thermal stability, for Hoogsteen-type helixes, and the terminal acetylene bond is also an attractive functional group for further post-synthetic chemical modifications through, for example, "click-chemistry".[14]

As previously described, a TFO with bulged insertion of **1** in the middle of the sequence significantly discriminated between matched and mismatched dsDNA (**ON2**, Table 2),[9] and the same effect was observed with the monomer **2** (**ON3**, Table 2). We decided to evaluate TINA monomers in their ability to behave in a universal base mode.[15] The triplexes formed with **ON17** and dsDNA (**D1**–**D4**) had very close values of T_m, although these were very low in comparison with the perfectly matched wild-type triplex (**ON1/D1**). We believe that this is a result of a

Table 1. T_m [°C] data for triplex and duplex melting, taken from UV melting curves (λ = 260 nm).

		Triplex[a] 3′-CTGCCCCTTTCTTTTTT 5′-GACGGGGAAAGAAAAAA (**D1**)		Parallel duplex[b] 5′-GACGGGGAAAGAAAAAA (**ON16**)
		pH = 6.0	pH = 7.2	pH = 6.0
ON1[c]	5′-CCCCTTTCTTTTTT	27.0	<5.0	19
ON2[c]	5′-CCCCTT**1**TCTTTTTT	46.0	28.0	33.5
ON3	5′-CCCCTT**2**TCTTTTTT	33.0	11.0	19.5
ON4	5′-**2**CCCCTTTCTTTTTT	36.5	11.5	26.0
ON5	5′-CCCCTTTCTTTTTT**2**	33.5	6.0	25.0
ON6	5′-2CCCCTT**2**TCTTTTTT	43.0	22.0	27.5
ON7	5′-CCCCTTTC**2**TTTTTT	29.0	<5.0	24.5
ON8	5′-CCCCTTT**2**CTTTTTT	33.5	10.5	22.5
ON9	5′-CCC**2**CTTTCTTTTTT	32.5	8.5	27.5
ON10[c]	5′-CCCCTT**3**TCTTTTTT	35.0	13.5	22.0
ON11	5′-CCCCTT**4**TCTTTTTT	23.5	<5.0	10.0
ON12[c]	5′-CCCCTT**5**TCTTTTTT	26.0	<5.0	17.0
ON13	5′-CCCCTT**6**TCTTTTTT	20.5	<5.0	9.5
ON14	5′-CCCCTT**7**TCTTTTTT	23.5	<5.0	11.0
ON15[c]	5′-CCCCTT**8**TCTTTTTT	26.0	<5.0	<5.0

[a] c = 1.5 μM of **ON1**–**15** and 1.0 μM of each strand of dsDNA (**D1**) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH = 6.0 and 7.2; duplex T_m = 58.5 °C (pH = 6.0) and 57.0 °C (pH = 7.2). [b] c = 1.0 μM of each strand in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH = 6.0. [c] Oligonucleotides and T_m data are from ref.[9]

Table 2. T_m [°C] data for mismatched parallel triplex,[a] taken from UV melting curves (λ = 260 nm).

Sequence 3′-CTGCCCCTT**X**CTTTTTT 5′-GACGGGGAA**Y**GAAAAAA		**D1**: **X**·**Y** = T·A	**D2**: A·T	**D3**: C·G	**D4**: G·C
ON1[b]	5′-CCCCTTTCTTTTTT	27.0	<5.0	<5.0	<5.0
ON2[b]	5′-CCCCTT**1**TCTTTTTT	46.0	27.0	34.5	28.5
ON3	5′-CCCCTT**2**TCTTTTTT	33.0	15.0	19.5	17.5
ON17	5′-CCCCTT**2**CTTTTTT	10.0	11.0	8.5	11.0
ON18	5′-CCCCTT**11**CTTTTTT	32.0[c]	30.5[c]	30.0[c]	30.0[c]
ON19	5′-CCCCTT**11**TCTTTTTT	35.0	28.0	28.5	26.0
ON20	5′-CCCCTT**111**CTTTTTT	<5.0	<5.0	<5.0	<5.0

	3′-CTGCC**X**^{1}CTTTCTT**X**^{2}TTT 5′-GACGG**Y**^{1}GAAAGAA**Y**^{2}AAA	**D5**: **X**^{1}·**Y**^{1} = G·C; **X**^{2}·**Y**^{2} = T·A	**D6**: **X**^{1}·**Y**^{1} = C·G; **X**^{2}·**Y**^{2} = A·T
ON18	5′-CCCCTT**11**CTTTTTT	14.0	<5.0
ON19	5′-CCCCTT**11**TCTTTTTT	15.0	12.5

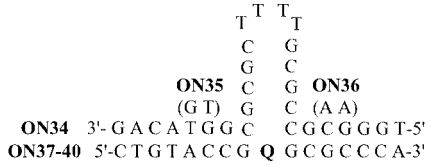
[a] c = 1.5 μM of **ON1**–**3**, **ON17**–**20** and 1.0 μM of each strand of dsDNA (**D1**–**6**) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH = 6.0. [b] Oligonucleotides and T_m data are from ref.[9] [c] Reduced hyperchromicity for triplex transition relative to **ON2**.

Table 3. T_m [°C] data for Watson–Crick duplexes,[a] taken from UV melting curves (λ = 260 nm).

		DNA 5′-AGCTTG**Z**CTTGAG		RNA 5′-AGCUUGCUUGAG
		ON29: **Z** = 0		**ON33**
ON21	3′-TCGAACGAACTC	47.5[b]	32.0 (**ON30**: **Z** = **1**)[b] 43.0 (**ON31**: **Z** = **2**) 38.0 (**ON32**: **Z** = **6**)	40.5[b]
ON22[b]	3′-TCGAAC**1**GAACTC	39.5	36.0 (**ON30**)[b]	30.5
ON23	3′-TCGAACGAACTC**1**	54.0		45.5
ON24	3′-TCGAAC**11**GAACTC	31.5	36.5 (**ON30**)	24.0
ON25	3′-TCGAAC**2**GAACTC	46.5	40.0 (**ON31**)	34.5
ON26	3′-TCGAAC**4**GAACTC	36.0		28.5
ON27	3′-TCGAAC**6**GAACTC	41.0	41.0 (**ON32**)	35.0
ON28	3′-TCGAAC**7**GAACTC	40.5		33.0

[a] c = 1.0 μM of each oligonucleotide in 140 mM NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH = 7.0. [b] Oligonucleotides and T_m data are from ref.[9]

Table 4. T_m [°C] data for DNA three-way junction,[a] taken from UV melting curves (λ = 260 nm).



	ON37 (Q = A)	ON38 (Q = 1)	ON39 (Q = 6)	ON40 (Q = 7)
ON34	36.5	52.0	48.5	46.5
ON35	16.5	31.5	28.5	34.0
ON36	16.5	29.5	30.0	24.0

[a] c = 1.0 μM of each oligonucleotide in 140 mM NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH = 7.0. T_m of the hairpin (**ON34**) is 70.0 °C.

helix distortion by the 1-*O*-methylglycerol linker, which is too short to mimic the 2′-deoxyribofuranose of nucleotides. On the other hand, the linker has an optimum shape to be adopted by the helix when inserted as a bulge. In order to stabilize parallel triplexes with single base inversion in dsDNA, we synthesized **ON18**, possessing two monomers **1** instead of thymidine in the middle of the TFO. Two 1-*O*-methylglycerol linkers can mimic the 2′-deoxyribofuranose backbone better than the single insertion. As a result, slightly increased triplex thermal stability, but decreased hyperchromicity for triplex melting were detected for **ON18** with all possible combinations of base pairs in dsDNA relative to the native triplex (**ON1/D1**). The observed thermal stability difference, in a range of 1.5–2.0 °C, is a good indication that double insertion of **1** in the middle of TFO can be used to bind to dsDNA with single purine/pyrimidine inversion. Since reduced hyperchromicity could be an indication of missing base-pairing in some part of the sequence, we checked the thermal stability of **ON18** toward dsDNAs with base inversions three bases away from the incorporation of intercalators (**D5** and **D6**, Table 2). In both cases a lowering of T_m for triplex ($\Delta T_m > 18.0$ °C) was observed. That confirms that the whole sequence binds to the dsDNA and represents discrimination of binding to matched dsDNA over dsDNA containing the mismatch several bases away from the intercalator. The ability to use double insertion of **1** in TFO as a universal base should be especially applicable for targeting of dsDNA with TA inversion.[16] Success in the synthesis of compounds for recognition of TA is still limited because such modified bases must avoid the steric clash with the 5-methyl group of thymidine.[17]

Unlike in the case of TA, there are several analogues available for recognition of CG,[18–20] and the thermal stability of this type of triplexes can be further increased by incorporation of TINA monomer **1** in another part of the sequence as a bulge. Double bulged insertion of **1** in the TFO in the sequence **ON19** also resulted in a decreased T_m of the parallel triplex relative to the **ON2/D1**, although the triplex was more stable than the wild-type triplex (**ON1/D1**). In this case, however, a drop in T_m in the range of 6.5–9.0 °C was observed when single mismatched dsDNAs (**D2**–**D4**) with **ON19** were targeted. To determine the limits of bulged insertions of **1** in the sequence we synthesized oligonucleotide **ON20**, with triple insertions of **1**, and no triplex formation was detected. From our experience we can recommend not synthesizing TFOs with the content of TINA monomer exceeding 30% in the sequence: the high content of polyaromatic structures results in self aggregation of the oligonucleotide and inability to target dsDNA. Bulged insertions of **1** as next-nearest neighbors are also inefficient for binding to dsDNA at neutral conditions.[9]

We also studied the hybridization affinities of TINAs possessing the new bulged intercalating monomers **2**, **4**, **6**, and **7** in mixed purine/pyrimidine sequences toward ssDNA and ssRNA in Watson–Crick-type duplexes (Table 3). Destabilization of TINA/DNA and TINA/RNA duplexes relative to the wild-type duplexes was observed for all TINA monomers as a bulge in the middle of the sequence. Incorporation of **1** at the 5′-end of the sequence **ON23** resulted in stabilization of duplexes toward DNA (ΔT_m = +6.5 °C) and RNA (ΔT_m = +5.0 °C), which can be ascribed to the stacking of an aromatic structure on the adjacent nucleobase.[21,22] Incorporation of two bulged TINA monomers **1** in the middle of the Watson–Crick duplex produced further destabilization of the duplex relative to the single bulged **1** (**ON22/ON29**). The placement of the monomer **1** opposite the double bulged **1** resulted in a formation of a duplex (**ON24/ON30**) with increased thermal stability (ΔT_m = +5.0 °C) relative to the duplex **ON24/ON29**. When 3-(naphthalen-1-ylethynyl)phenyl residue **2** was positioned in the middle of the sequence, it gave the least destabilized duplex among the TINA monomers tested. Interestingly, the placement of two intercalators **2** opposite each other in the Watson–Crick helix gave a duplex (**ON25/ON31**) with a T_m value indicating a thermal stability lower than those of the corresponding duplexes with unmodified DNAs (**ON25/ON29** and **ON21/ON31**). This contrasts with the results observed with TINA monomers **1** and **6** and is believed to be a consequence of the attachment of the naphthalen-1-ylethynyl moiety to the 3-position of the phenyl substituent in (*R*)-1-*O*-(phenylmethyl)glycerol.

As another target in our investigation we chose a DNA three-way junction (TWJ) composed of two arms linked to a stem (**ON34**, Table 4).[23] Here we observed considerable stabilization with all TINA monomers **1** (**ON38**), **6** (**ON39**), and **7** (**ON40**) upon insertion at the junction point relative to the oligonucleotide with dA at the same position (**ON37**). Interestingly, despite a huge difference (ΔT_m = 22.5–25.5 °C) in the abilities of bulged monomers **1**, **6**, and **7** to stabilize parallel triplexes, the difference in T_m for TWJ stabilization with the same monomers was quite small (ΔT_m = 3.5–5.5 °C). Unlike in the case of the parallel triplex, the use of a longer linker in the monomer **6** (**ON39**) resulted in stronger stabilization of TWJ in relation to the structure **7** (**ON40**). To be sure that hybridization in the arms is important for the stability of the complex, we prepared oligonucleotides with mismatches in either arm of the TWJ (**ON35** and **ON36**). In both cases this resulted in a large lowering of the hybridization affinity (ΔT_m in a range of 18.5–

22.5 °C). This finding confirms the applicability of TINAs for targeting of DNA hairpins, which may also be useful in the design of oligonucleotides targeting partially melted dsDNA, as can occur during DNA replication or transcription, such as, for example, in the formation of the "open complex" initiated by RNA polymerase on dsDNA at the transcription start.

Conclusions

Several phosphoramidites of twisted intercalating nucleic acid monomers were synthesized and used in DNA synthesis. In thermal denaturation studies of parallel triplexes stabilized by bulged TINA insertion it was found to be preferable to have more than two fused phenyl rings attached through a triple bond at the 4-position (rather than connection at the 3-position) of the (*R*)-1-*O*-(phenylmethyl)glycerol component in homopyrimidine ONs. Double insertion of (*R*)-1-*O*-[4-(pyren-1-ylethynyl)phenylmethyl]glycerol (**1**) instead of the single nucleotide in the middle of the triplex-forming oligonucleotide was found to be useful to provide a universal base with similar thermal stabilities for all possible matched base-pairing in the dsDNA. Extraordinary stabilization of a DNA three-way junction was observed upon insertion of the TINA monomers in the junction region as a bulge.

Experimental Section

General Remarks: NMR spectra were recorded with a Varian Gemini 2000 spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The internal standard used in the ^{1}H NMR spectra was TMS (δ = 0.00 ppm) in $CDCl_3$, in ^{13}C NMR it was $CDCl_3$ (δ = 77.0 ppm), and in ^{31}P NMR it was external H_3PO_4 (δ = 0.00 ppm). Accurate ion mass determinations were performed with a 4.7 Tesla Ultima Fourier Transform (FT) mass spectrometer (Ion Spec, Irvine, CA). The $[M + Na]^+$ ions were peak-matched by use of ions derived from the 2,5-dihydroxybenzoic acid matrix. EI-MS was performed with a Finnigan SSQ 710 instrument. Thin layer chromatography (TLC) analyses were carried out with use of 60 F_{254} TLC plates purchased from Merck and were visualized under UV light (254 nm). The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck. Solvents used for column chromatography were distilled prior to use, while reagents were used as purchased. Unmodified ONs were purchased from DNA Technology A/S (Århus, Denmark) and from TAG Copenhagen A/S (Copenhagen, Denmark).

(*S*)-4-(3-Bromobenzyloxymethyl)-2,2-dimethyl-1,3-dioxolane (11): Pulverized KOH (12.5 g, 220 mmol) and 3-bromobenzyl bromide (2.5 g, 10 mmol) were added to a solution of [(*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]methanol (**9**, 1.3 g, 10 mmol) in dry toluene (125 mL). The mixture was heated at reflux under Dean–Stark conditions for 16 h and cooled, and H_2O (100 mL) was added. After separation of the phases, the organic layer was washed with H_2O (3 × 100 mL), dried ($MgSO_4$), and concentrated under reduced pressure to give viscous oil **11** in a quantitative yield (3 g). ^{1}H NMR ($CDCl_3$): δ = 1.37 (s, 3 H, CH_3), 1.42 (s, 3 H, CH_3), 3.50 (m, 2 H, CHCH_2OCH$_2$), 3.71–3.78 (dd, *J* = 6.5, 7.6 Hz, 1 H, [CH_2OC(CH$_3$)$_2$]), 4.06 (m, 1 H, [CH_2OC(CH$_3$)$_2$]), 4.31 (m, 1 H, CH_2CHCH_2), 4.54 (s, 2 H, OCH_2Ar), 7.21 (m, 2 H, Ar), 7.40 (d, *J* = 7.5 Hz, 1 H, Ar), 7.49 (s, 1 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.3, 26.7 (2 × CH_3), 66.7 [CH_2OC(CH$_3$)$_2$], 71.3 (CH_2CHCH_2), 72.6 (CH_2Ar), 74.6 (CH_2OCH_2Ar), 109.4 [C(CH$_3$)$_2$], 122.5, 126.0, 129.9, 130.5, 130.7, 140.3 (Ar) ppm. MS (EI): *m*/*z* = 302 [$C_{13}H_{17}O_3{}^{81}Br]^+$, 300 [$C_{13}H_{17}O_3{}^{79}Br]^+$.

(*S*)-4-[2-(4-Bromobenzyloxy)ethyl]-2,2-dimethyl-1,3-dioxolane (12): This compound was synthesized in a quantitative yield (2.7 g) by the same procedure as applied for compound **11**, from 4-bromobenzyl bromide and 2-[(*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]ethanol (**10**). ^{1}H NMR ($CDCl_3$): δ = 1.35 (s, 3 H, CH_3), 1.40 (s, 3 H, CH_3), 1.88 (m, 2 H, CHCH_2CH$_2$), 3.57 (m, 3 H, OCH_2CH, CH_2CH$_2$O), 4.06 (dd, *J* = 6.1, 8.1 Hz, 1 H, OCH_2CH), 4.21 (m, 1 H, CH_2CHCH_2), 4.45 (s, 2 H, OCH_2Ar), 7.15–7.50 (m, 4 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.9, 27.0 (2 × CH_3), 34.0 (CHCH_2CH$_2$), 67.3 (CHCH_2O), 69.7 (CH_2CHCH_2), 72.4 (CH_2Ar), 73.9 (CH_2OCH_2Ar), 108.7 [C(CH$_3$)$_2$], 121.5, 129.3, 131.6, 137.5 (Ar) ppm. MS (EI): *m*/*z* = 316 [$C_{14}H_{19}O_3{}^{81}Br]^+$, 314 [$C_{14}H_{19}O_3{}^{79}Br]^+$.

(*S*)-4-(4-Bromobenzyloxymethyl)-2,2-dimethyl-1,3-dioxolane (13): This compound was synthesized in a quantitative yield (3.0 g) by the same procedure as applied for compound **11**, from 4-bromobenzyl bromide and [(*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]methanol (**9**). ^{1}H NMR ($CDCl_3$): δ = 1.36 (s, 3 H, CH_3), 1.42 (s, 3 H, CH_3), 3.51 (m, 2 H, CHCH_2OCH$_2$), 3.70 (dd, *J* = 6.3, 8.0 Hz, 1 H, [CH_2OC(CH$_3$)$_2$]), 4.04 (dd, *J* = 6.3, 8.0 Hz, 1 H, [CH_2OC(CH$_3$)$_2$]), 4.29 (m, 1 H, CH_2CHCH_2), 4.51 (s, 2 H, OCH_2Ar), 7.23 (d, *J* = 6.4 Hz, 2 H, Ar), 7.46 (d, *J* = 6.4 Hz, 2 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.4, 26.7 (2 × CH_3), 66.7 [CH_2OC(CH$_3$)$_2$], 71.2 (CH_2CHCH_2), 72.7 (CH_2Ar), 74.7 (CH_2OCH_2Ar), 109.4 [C(CH$_3$)$_2$], 121.5, 129.0, 129.2, 131.4, 131.5, 137.0 (Ar) ppm. MS (EI): *m*/*z* = 302 [$C_{13}H_{17}O_3{}^{81}Br]^+$, 300 [$C_{13}H_{17}O_3{}^{79}Br]^+$.

General Procedure for Sonogashira Coupling: A mixture of aryl bromide (1.42 mmol), $Pd(PPh_3)_2Cl_2$ (20 mg, 0.03 mmol), CuI (20 mg, 0.11 mmol), *i*Pr$_2$NEt (1.0 mL, 5.7 mmol), and the aromatic structure containing the terminal acetylene moiety (3.00 mmol) was heated at reflux under N_2 in dry THF (10 mL) for 24 h. The reaction mixture was poured into H_2O (50 mL) and extracted with CH_2Cl_2 (3 × 25 mL), and the combined organic layers were washed with H_2O (2 × 25 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography with EtOAc/petroleum ether (1:4, v/v) as an eluent, affording the target compound. In the cases of compounds **16** and **17** the final purification was carried out by semipreparative HPLC on a Delta Pak C_{18} column (19 × 300 mm). Buffer A was 100% CH_3CN, buffer B was 10% CH_3CN in H_2O, the crude product was dissolved in CH_3CN (2 mL) and injected onto the column, and the elution program was: 0–25 min linear gradient from 50% to 85% of A in B; 25–30 min with 85% of A in B; 30–35 min linear gradient from 85% to 100% of A in B; flow was 5 mL min^{-1}, monitoring at 260 nm. The fractions at 36–40 min were concentrated to half the volume and the product was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with H_2O (2 × 25 mL), dried ($MgSO_4$), and filtered, and the solvents were evaporated in vacuo to give the pure products **14**, **16**, or **17**.

(*S*)-2,2-Dimethyl-4-[3-(naphthalen-1-ylethynyl)benzyloxymethyl]-1,3-dioxolane (14): Yield 480 mg, 92%. ^{1}H NMR ($CDCl_3$): δ = 1.37 (s, 3 H, CH_3), 1.44 (s, 3 H, CH_3), 3.52 (m, 2 H, CHCH_2OCH$_2$), 3.76 [dd, *J* = 6.3, 8.2 Hz, 1 H, CH_2OC(CH$_3$)$_2$], 4.07 [dd, *J* = 6.3, 8.2 Hz, 1 H, CH_2OC(CH$_3$)$_2$], 4.34 (m, 1 H, CH_2CHCH_2), 4.61 (s, 2 H, OCH_2Ar), 7.20–8.43 (m, 11 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.4, 26.8 (2 × CH_3), 66.8 [CH_2OC(CH$_3$)$_2$], 71.2 (CH_2CHCH_2),

73.0 (CH_2Ar), 74.7 (CH_2OCH_2Ar), 87.6, 94.1 (C≡C), 109.5 [$C(CH_3)_2$], 120.8, 123.5, 125.2, 126.2–133.2, 138.3 (Ar) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{25}H_{24}NaO_3^+$ $[M+Na]^+$ 395.1617; found 395.1614.

(*S*)-2,2-Dimethyl-4-[2-(4-phenylethynylbenzyloxy)ethyl]-1,3-dioxolane (16): Yield 321 mg, 23% by the general procedure for Sonogashira coupling described above. ^1H NMR ($CDCl_3$): δ = 1.36 (s, 3 H, CH_3), 1.41 (s, 3 H, CH_3), 1.90 (m, 2 H, $CHCH_2CH_2$), 3.59 (m, 3 H, OCH_2CH, CH_2CH_2O), 4.06 [m, 1 H, $CH_2OC(CH_3)_2$], 4.22 (m, 1 H, CH_2CHCH_2), 4.51 (s, 2 H, OCH_2Ar), 7.20–7.60 (m, 9 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.7, 26.9 (2×CH_3), 33.8 ($CHCH_2CH_2$), 67.2 ($CHCH_2O$), 69.5 (CH_2CHCH_2), 72.3 (CH_2Ar), 73.7 (CH_2OCH_2Ar), 89.2, 89.3 (C≡C), 108.7 [$C(CH_3)_2$], 122.4, 123.2, 128.2, 127.4–131.6, 137.3, 138.6 (Ar) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{22}H_{24}NaO_3^+$ $[M+Na]^+$ 359.1617; found 359.1622.

(*S*)-4-[3-(Biphenyl-4-ylethynyl)benzyloxymethyl]-2,2-dimethyl-1,3-dioxolane (15): 4-Ethynylbiphenyl (0.51 g, 2.8 mmol), $Pd(PPh_3)_2Cl_2$ (40 mg, 0.057 mmol), CuI (27 mg, 0.14 mmol), and Et_3N (0.594 mL, 4.26 mmol) were added to a solution of compound **11** (0.43 g, 1.42 mmol) in dry DMF (50 mL) and 1,4-dioxane (20 mL). The reaction mixture was heated at 80 °C under nitrogen for 3 d, and was then allowed to cool to room temp., diluted with CH_2Cl_2 (150 mL), and washed with a aq. solution of EDTA ammonium salt (0.3 M, 100 mL) and H_2O (5×100 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo, and the residue was purified by silica gel column chromatography with EtOAc/petroleum ether (1:8, v/v), affording compound **15** (0.156 g, 28%). ^1H NMR ($CDCl_3$): δ = 1.38 (s, 3 H, CH_3), 1.49 (s, 3 H, CH_3), 3.53 (m, 2 H, $CHCH_2OCH_2$), 3.76 [t, *J* = 7.2 Hz, 1 H, $CH_2OC(CH_3)_2$], 4.08 [t, *J* = 7.2 Hz, 1 H, $CH_2OC(CH_3)_2$], 4.31 (m, 1 H, CH_2CHCH_2), 4.58 (s, 2 H, OCH_2Ar), 7.30–7.60 (m, 13 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.4, 26.8 (2×CH_3), 66.8 [$CH_2OC(CH_3)_2$], 71.3 (CH_2CHCH_2), 73.0 (CH_2Ar), 74.7 (CH_2OCH_2Ar), 89.4, 89.9 (C≡C), 109.5 [$C(CH_3)_2$], 122.1, 123.4, 127.0–132.0, 138.3, 140.3, 141.0 (Ar) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{27}H_{26}NaO_3^+$ $[M+Na]^+$ 421.1774; found 421.1761.

(*S*)-2,2-Dimethyl-4-(3-phenylethynylbenzyloxy)methyl-1,3-dioxolane (17). Method A: Yield 105 mg, 18% by the general procedure for Sonogashira coupling described above. ^1H NMR ($CDCl_3$): δ = 1.39 (s, 3 H, CH_3), 1.45 (s, 3 H, CH_3), 3.50 (m, 2 H, $CHCH_2OCH_2$), 3.73 [dd, *J* = 6.5, 8.0 Hz, 1 H, $CH_2OC(CH_3)_2$], 4.05 [t, *J* = 6.5, 8.0 Hz, 1 H, $CH_2OC(CH_3)_2$], 4.30 (m, 1 H, CH_2CHCH_2), 4.58 (s, 2 H, OCH_2Ar), 7.29–7.54 (m, 9 H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ = 25.3, 26.7 (2×CH_3), 66.7 [$CH_2OC(CH_3)_2$], 71.2 (CH_2CHCH_2), 73.0 (CH_2Ar), 74.7 (CH_2OCH_2Ar), 89.1, 89.4 (C≡C), 109.4 [$C(CH_3)_2$], 122.5, 123.2, 127.5, 128.2, 128.3, 131.6, 138.2 (Ar) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{21}H_{22}NaO_3^+$ $[M+Na]^+$ 345.1461; found 345.1447. **Method B:** Compound **13** (270 mg, 0.90 mmol), $Pd(PPh_3)_2Cl_2$ (35 mg, 0.05 mmol), CuI (12 mg, 0.06 mmol), PPh_3 (50 mg, 0.20 mmol) and phenylacetylene (115 mg, 1.13 mmol) were dissolved in diethylamine (1.5 mL, 13.60 mmol) and DMF (0.5 mL), and the reaction mixture was stirred at 120 °C under nitrogen in the microwave cavity for 25 min. The reaction was checked by analytical HPLC on a Delta Pak C_{18} column (3.9×300 mm) with the following program: 0–5 min elution with 30% buffer A in B, 5–10 min linear gradient from 30% to 15% of A in B, 10–15 min of 15% of A in B, 15–20 min of linear gradient from 15% to 0% of A in B, 20–25 min of the linear gradient from 0% to 30% of A in B, where buffer A was 20% of CH_3CN in H_2O and buffer B was 80% of CH_3CN in H_2O, flow was 1 mL min^{-1}, monitoring at 260 nm. Since HPLC analysis showed all the phenylacetylene to have been consumed, an additional quantity of phenylacetylene (93 mg, 0.91 mmol) was injected into the vial with the reaction mixture, followed by further stirring at 120 °C under nitrogen in the microwave cavity for 25 min. The reaction mixture was cooled and poured into aq. HCl (0.1 M, 10 mL) and extracted with diethyl ether (3×10 mL), and the combined organic layer was washed with saturated aq. $NaHCO_3$ (10 mL) and H_2O (10 mL). The combined water phase was re-extracted with diethyl ether (2×10 mL), the combined organic layers were concentrated under diminished pressure, and the residue was purified by silica gel column chromatography with EtOAc/petroleum ether (1:4, v/v), affording compound **17** together with impurities. The final purification of **17** was carried out by semipreparative HPLC as described in the general procedure for Sonogashira coupling. Yield 98 mg, 33%.

General Procedure for Isopropylidene Group Cleavage and DMT Protection: The 2,2-dimethyl-4-substituted-1,3-dioxolane was stirred in aq. AcOH (80%, 10–20 mL) at room temp. for 24 h and the mixture was then concentrated under reduced pressure. The residue was co-evaporated twice with toluene/EtOH (25 mL, 1:1, v/v), giving an oily product which was dried in vacuo overnight and used in the next step without further purification. 4,4′-Dimethoxytrityl chloride (1.1 equiv.) was added to a solution of diol (1 equiv.) and dry Et_3N (2 equiv.) in dry CH_2Cl_2 (10 mL). The reaction mixture was stirred at room temp. under nitrogen overnight, quenched with MeOH (2 mL) and concentrated in vacuo. The residue was purified by silica gel column chromatography with NEt_3 (2%, v/v)/EtOAc (30%)/petroleum ether. UV-active fractions were collected and concentrated under diminished pressure, affording the DMT-protected diols **18**–**21**.

(*S*)-1-*O*-(4,4′-Dimethoxytrityl)-3-*O*-[3-(naphthalen-1-ylethynyl)phenylmethyl]glycerol (18): Yield 350 mg, 65%. ^1H NMR ($CDCl_3$): δ = 2.44 (d, *J* = 4.7 Hz, 1 H, OH), 3.23 (m, 2 H, $CHCH_2OCH_2$), 3.53 (m, 2 H, CH_2ODMT), 3.74 (s, 6 H, 2×OCH_3), 4.02 (m, 1 H, *CH*OH), 4.56 (s, 2 H, OCH_2Ph), 6.78 (d, *J* = 8.5 Hz, 4 H, DMT), 7.20–8.43 (m, 20 H, DMT + Ph + naphthalene) ppm. ^{13}C NMR ($CDCl_3$): δ = 55.1 (OCH_3), 64.2 (CH_2ODMT), 70.0 (CH_2CHOH), 71.7 (CHOH), 72.9 (CH_2Ph), 86.1 (CAr_3), 87.6, 94.1 (C≡C), 113.1, 120.8, 123.5, 125.2–133.2, 135.9, 138.4, 144.8, 158.4 (Ph, naphthalene, DMT) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{43}H_{38}NaO_5^+$ $[M+Na]^+$ 657.2611; found 657.2601.

(*S*)-3-*O*-[3-(Biphenyl-4-ylethynyl)phenylmethyl]-1-*O*-(4,4′-dimethoxytrityl)glycerol (19): Yield 240 mg, 71%. ^1H NMR ($CDCl_3$): δ = 2.50 (br s, 1 H, OH), 3.24 (m, 2 H, $CHCH_2OCH_2$), 3.63 (m, 2 H, CH_2ODMT), 3.76 (s, 6 H, 2×OCH_3), 4.01 (m, 1 H, *CH*OH), 4.53 (s, 2 H, OCH_2Ph), 6.81 (d, *J* = 8.5 Hz, 4 H, DMT), 7.17–7.62 (m, 22 H, DMT + Ph + biphenyl) ppm. ^{13}C NMR ($CDCl_3$): δ = 55.1 (OCH_3), 64.3 (CH_2ODMT), 69.9 (CH_2CHOH), 71.7 (CHOH), 72.9 (CH_2Ph), 86.1 (CAr_3), 89.4, 89.9 (C≡C), 113.1, 122.1, 123.4, 126.8–133.0, 135.9, 138.3, 140.2, 144.8, 158.4 (Ph, biphenyl, DMT) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{45}H_{40}NaO_5^+$ $[M+Na]^+$ 683.2768; found 683.2740.

(*S*)-1-(4,4′-Dimethoxytrityloxy)-4-[4-(phenylethynyl)phenylmethoxy]butan-2-ol (20): Yield 230 mg, 77%. ^1H NMR ($CDCl_3$): δ = 1.79 (m, 2 H, CH_2CH_2O), 2.70 (br s, 1 H, OH), 3.13 (m, 2 H, CH_2CH_2O), 3.59 (m, 2 H, CH_2ODMT), 3.77 (s, 6 H, 2×OCH_3), 3.90 (m, 1 H, *CH*OH), 4.46 (s, 2 H, OCH_2Ph), 6.81 (d, *J* = 8.5 Hz, 4 H, DMT), 7.10–7.60 (m, 18 H, DMT + Ph) ppm. ^{13}C NMR ($CDCl_3$): δ = 33.5 (CH_2CH_2O), 55.2 (OCH_3), 67.3 (CH_2OCH_2Ph), 68.0 (CH_2ODMT), 69.5 (CH_2Ph), 72.8 (CHOH), 86.0 (CAr_3), 89.2, 89.3 (C≡C), 113.1, 122.5, 123.3, 126.7–131.6, 136.1, 138.4, 144.9,

158.5 (Ph, DMT) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{40}H_{38}NaO_5^+$ $[M+Na]^+$ 621.2611; found 621.2606.

(*S*)-1-*O*-(4,4′-Dimethoxytrityl)-3-*O*-[4-(phenylethynyl)phenylmethyl]glycerol (21): Yield 126 mg, 61%. ^{1}H NMR ($CDCl_3$): δ = 2.46 (br s, 1 H, OH), 3.22 (m, 2 H, CHCH_2OCH$_2$), 3.58 (m, 2 H, CH_2ODMT), 3.75 (s, 6 H, 2×OCH_3), 3.95 (m, 1 H, *CH*OH), 4.53 (s, 2 H, OCH_2Ph), 6.81 (d, J = 8.5 Hz, 4 H, DMT), 7.14–7.54 (m, 18 H, DMT + Ph) ppm. ^{13}C NMR ($CDCl_3$): δ = 55.2 (OCH_3), 64.3 (CH_2ODMT), 69.9 (CH_2CHOH), 71.6 (CHOH), 72.9 (CH_2Ph), 86.1 (CAr_3), 89.2, 89.4 (C≡C), 113.1, 122.5, 123.2, 126.7–131.6, 135.9, 138.2, 144.8, 158.4 (Ph, naphthalene, DMT) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{39}H_{36}NaO_5^+$ $[M+Na]^+$ 607.2455; found 607.2456.

General Procedure for Phosphitylation: DMT-protected diol (1 equiv.) was dissolved under nitrogen in anh. CH_2Cl_2 (10–50 mL). Diisopropylammonium tetrazolide (1.6 equiv.) was added, followed by dropwise addition of 2-cyanoethyl tetraisopropylphosphordiamidite (2 equiv.) with external cooling with an ice/water bath. After 24 h, analytical TLC showed no more starting material and the reaction was quenched with H_2O (10–30 mL). The layers were separated and the organic phase was washed with H_2O (10–30 mL), the combined water layers were washed with CH_2Cl_2 (25 mL), the organic phase was dried (Na_2SO_4) and filtered, and the solvents were evaporated in vacuo. The residue was purified by silica gel column chromatography with NEt_3 (0.5–2%, v/v)/EtOAc (0–25%)/cyclohexane and the combined UV-active fractions were concentrated in vacuo to afford the final compound, which was used in DNA synthesis after drying under diminished pressure.

(*S*)-2-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphanyl]-1-*O*-(4,4′-dimethoxytrityl)-3-*O*-[3-(naphthalen-1-ylethynyl)phenylmethyl]glycerol (22): Yield 290 mg, 83%. ^{32}P NMR ($CDCl_3$) δ = 150.2, 150.4 ppm in a 5:4 ratio. HR-MALDI-MS: *m*/*z* calcd. for $C_{52}H_{55}N_2NaO_6P^+$ $[M+Na]^+$ 857.3690; found 857.3684.

(*S*)-3-*O*-[3-(Biphenyl-4-ylethynyl)phenylmethyl]-2-*O*-[(2-cyanoethoxy)(diisopropylamino)phosphanyl]-1-*O*-(4,4′-dimethoxytrityl)-glycerol (23): Yield 115 mg, 57%. ^{32}P NMR ($CDCl_3$) δ = 150.3, 150.4 ppm in a ratio 3:2. HR-MALDI-MS: *m*/*z* calcd. for $C_{54}H_{57}N_2NaO_6P^+$ $[M+Na]^+$ 883.3846; found 883.3865.

(*S*)-2-[(2-Cyanoethoxy)(diisopropylamino)phosphanyloxy]-1-(4,4′-dimethoxytrityloxy)-4-[4-(phenylethynyl)phenylmethoxy]butane (24): Yield 260 mg, 90%. ^{32}P NMR ($CDCl_3$): δ = 149.3, 149.7 ppm in a 3:2 ratio. HR-MALDI-MS: *m*/*z* calcd. for $C_{49}H_{55}N_2NaO_6P^+$ $[M+Na]^+$ 821.3690; found 821.3721.

(*S*)-2-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphanyl]-1-*O*-(4,4′-dimethoxytrityl)-3-*O*-[4-(phenylethynyl)phenylmethyl]glycerol (25): Yield 139 mg, 82%. ^{32}P NMR ($CDCl_3$): δ = 150.3, 150.4 ppm in a 5:4 ratio. HR-MALDI-MS: *m*/*z* calcd. for $C_{48}H_{53}N_2NaO_6P^+$ $[M+Na]^+$ 807.3533; found 807.3498.

(*S*)-1-*O*-(4,4′-Dimethoxytrityl)-3-*O*-[4-(pyren-1-ylethynyl)phenylmethyl]glycerol (27): A solution of (*R*)-3-(4-iodobenzyloxy)propane-1,2-diol (**26**,[9] 1.18 g, 4.2 mmol) in DMF (40 mL) and Et_3N (5.8 mL) was bubbled with argon for 30 min. Afterwards, 1-ethynylpyrene (1.05 g, 4.65 mmol) was dissolved under argon, and CuI (56 mg, 0.3 mmol) and $Pd(PPh_3)_4$ (125 mg, 0.11 mmol) were added to the solution. The reaction mixture was stirred at room temp. under argon overnight, followed by the addition of CH_2Cl_2 (150 mL) and extraction with an aq. solution of EDTA ammonium salt (0.3 M, 2×75 mL). The organic layer was washed with H_2O (3×75 mL), dried (Na_2SO_4), and filtered, and the solvents were evaporated to dryness in vacuo. The residue was co-evaporated twice with toluene/EtOH (30 mL, 1:1, v/v), affording (*R*)-1-*O*-[4-(pyren-1-ylethynyl)phenylmethyl]glycerol as an oil (3.1 g). The oil was co-evaporated with pyridine (20 mL) and then dissolved in anh. pyridine (50 mL), cooled in an ice/water bath, and 4,4′-dimethoxytrityl chloride (1.45 g, 4.41 mmol) was added under argon. The reaction mixture was stirred at room temp. for 16 h and an extra portion of 4,4′-dimethoxytrityl chloride (0.5 g, 1.5 mmol) was then added. After 24 h, TLC showed no more starting material and the reaction mixture was quenched with MeOH (2 mL), diluted with EtOAc (150 mL), and extracted with satd. aq. $NaHCO_3$ (2×100 mL). The water phase was extracted with EtOAc (2×50 mL) and the combined organic layers were dried (Na_2SO_4), filtered, and concentrated under diminished pressure. The residue was co-evaporated twice with toluene/EtOH (25 mL, 1:1, v/v). The residue was adsorbed from EtOAc (50 mL) on silica gel (2.0 g) and purified by silica gel dry column vacuum chromatography with EtOAc (0–100%, v/v) in cyclohexane to afford **27** (1.75 g, 60%) as a yellow foam. ^{1}H NMR ($CDCl_3$): δ = 2.48 (d, 1 H, J = 5.0 Hz, OH), 3.24 [m, 2 H, CH(OH)CH_2OCH$_2$], 3.31 (m, 2 H, CH_2ODMT), 3.78 (s, 6 H, 2×OCH_3), 4.00 (m, 1 H, *CH*OH), 4.58 (s, 2 H, CH_2Ar), 6.80 (d, 4 H, J = 8.5 Hz, DMT), 7.10–7.45 (m, 11 H, DMT), 7.72 (d, 2 H, J = 8.0 Hz, phenyl), 8.00–8.30 (m, 9 H, pyren-1-yl) ppm. ^{13}C NMR ($CDCl_3$): δ = 55.2 (OCH_3), 64.3 (CH_2ODMT), 70.0 [CH(OH)CH_2OCH$_2$], 71.7 (*C*HOH), 72.9 (CH_2-phenyl), 86.1 [$C(Ar)_3$], 88.7, 94.9 (C≡C), 117.7, 127.7, 138.5, 139.4 (phenyl), 113.1, 124.5–131.8, 136.0, 144.8, 158.5 (DMT, pyren-1-yl) ppm. HR-MALDI-MS: *m*/*z* calcd. for $C_{49}H_{40}NaO_5^+$ $[M+Na]^+$ 731.2768; found 731.2739.

(*S*)-2-*O*-[(2-Cyanoethoxy)(diisopropylamino)phosphanyl]-1-*O*-(4,4′-dimethoxytrityl)-3-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (28): (*S*)-1-*O*-(4,4′-Dimethoxytrityl)-3-*O*-[4-(1-pyrenylethynyl)-phenylmethyl]glycerol (**27**, 1.7 g, 2.4 mmol) was dissolved under argon in anh. CH_2Cl_2 (50 mL). Diisopropylammonium tetrazolide (0.620 g, 3.6 mmol) was added, followed by dropwise addition of 2-cyanoethyl tetraisopropylphosphordiamidite (1.150 g, 3.8 mmol) with external cooling with an ice/water bath. After 24 h, analytical TLC showed no more starting material and the reaction was quenched with H_2O (30 mL). The layers were separated, the organic phase was washed with H_2O (30 mL), and the combined water layers were washed with CH_2Cl_2 (25 mL). The organic phase was dried (Na_2SO_4) and filtered, silica gel (1.5 g) and pyridine (0.5 mL) were added, and the solvents were removed under reduced pressure. The residue was purified by silica gel dry column vacuum chromatography with NEt_3 (0.5%, v/v)/EtOAc (0–25%)/cyclohexane and the combined UV-active fractions were concentrated in vacuo to afford the final compound **28** (1.8 g, 83%) as a foam that was used in DNA synthesis. ^{32}P NMR ($CDCl_3$): δ = 150.3, 150.5 ppm in a 3:2 ratio. HR-MALDI-MS: *m*/*z* calcd. for $C_{58}H_{57}N_2NaO_6P^+$ $[M+Na]^+$ 931.3846; found 931.3814.

Synthesis and Purification of TINAs: ONs were synthesized with an Expedite™ Nucleic Acid Synthesis System Model 8909 from Applied Biosystems with 4,5-dicyanoimidazole as an activator. An increased deprotection time (100 s) and coupling time (2 min) for 0.075 M solutions of the phosphoramidites **22**–**25** and **28** in 1:1 mixtures of dry MeCN/CH_2Cl_2 were applied. The 5′-DMT-on ONs were cleaved from the solid support (room temp., 2 h) and deprotected (55 °C, overnight) by treatment with 32% aqueous ammonia. Purification of 5′-*O*-DMT-on TINAs was accomplished by reversed-phase semipreparative HPLC on a Waters Xterra™ MS C_{18} column. Buffer A [0.05 M triethylammonium acetate in H_2O (pH = 7.0)] and buffer B (75% CH_3CN in H_2O). Flow 2.5 mL min^{-1}. Gradients: 2 min 100% A, linear gradient to 70% B in 38 min, linear gradient to 100% B in 7 min, 100% B in 3 min and then 100% A in 10 min. The fractions containing the ONs eluted at 25–

30 min and were concentrated, and the DMT group was cleaved by treatment with aq. AcOH (80%, 100 μL) over 20 min, followed by dilution with aq. NaOAc (1 M, 150 μL) and precipitation of the ONs from chilled EtOH (550 μL). The modified ONs were confirmed by MALDI-TOF analysis with a PerSeptive Biosystems Voyager Elite Biospectrometry Research Station (see Supporting Information). The purities of the final ONs were found to be > 85% by analytical ion-exchange chromatography with a Merck Hitachi LaChrom system on a GenPak-Fax column (Waters).

Melting Temperature Measurements: Melting temperature measurements were performed with a Perkin–Elmer Lambda 35 UV/Vis spectrometer with a PTP-6 temperature programmer. The triplexes were formed by first mixing the two strands of the Watson–Crick duplex, each at a concentration of 1.0 μM in the corresponding buffer solution. The solution was heated to 80 °C for 5 min and then cooled to room temp., and the third (TFO) strand was added and then kept at 15 °C for 30 min. The duplexes were formed by mixing the two strands, each at a concentration of 1.0 μM, in the corresponding buffer solution, followed by heating to 70 °C for 5 min and then cooling to room temp. The melting temperatures (T_m [°C]) were determined as the maxima of the first derivative plots of the melting curves obtained by measuring absorbance at 260 nm against increasing temperature (1.0 °C per 1 min). Use of a lower rate of temperature increase (0.5 °C per 1 min) resulted in the same curves. Experiments were also performed at 373 nm for ONs possessing monomer **1**. All melting temperatures are within the uncertainty of ± 0.5 °C as determined by repetitive experiments.

Supporting Information (see footnote on the first page of this article): MALDI-TOF-MS of synthesized ONs (Table S1).

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