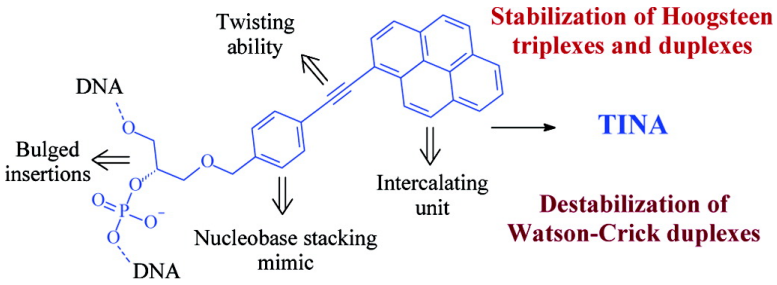
Article

Stable and Selective Formation of Hoogsteen-Type Triplexes and Duplexes Using Twisted Intercalating Nucleic Acids (TINA) Prepared via Postsynthetic Sonogashira Solid-Phase Coupling Reactions

Vyacheslav V. Filichev, and Erik B. Pedersen

J. Am. Chem. Soc., **2005**, 127 (42), 14849-14858• DOI: 10.1021/ja053645d • Publication Date (Web): 01 October 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 8 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 10/01/2005

Stable and Selective Formation of Hoogsteen-Type Triplexes and Duplexes Using Twisted Intercalating Nucleic Acids (TINA) Prepared via Postsynthetic Sonogashira Solid-Phase Coupling Reactions

Vyacheslav V. Filichev and Erik B. Pedersen*

Contribution from the Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

Received June 3, 2005; E-mail: EBP@chem.sdu.dk

Abstract: Bulge insertions of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**5**) into the middle of homopyrimidine oligodeoxynucleotides (twisted intercalating nucleic acids, TINA) obtained via postsynthetic Sonogashira coupling reaction led to extraordinary high thermal stability of Hoogsteen-type triplexes and duplexes, whereas Watson−Crick-type duplexes of the same nucleotide content were destabilized. Modified oligonucleotides were synthesized using the phosphoramidite of (*S*)-1-(4,4′-dimethoxytriphenylmethyloxy)-3-(4-iodo-benzyloxy)-propan-2-ol followed by treatment of the oligonucleotide on a CPG-support with the Sonogashira-coupling reaction mixture containing different ethynylaryls. Bulged insertion of the pyrene derivative **5** into oligonucleotides was found to be the best among the tested modifications for binding to the Hoogsteen-type triplexes and duplexes. Thus, at pH 7.2 an oligonucleotide with cytidine content of 36% possessing two bulged insertions of **5** separated by three bases formed a stable triplex (T_m = 43.0 °C), whereas the native oligonucleotide was unable to bind to the target duplex. The corresponding Watson−Crick-type duplex with the same oligonucleotide had T_m of 38.0 °C at pH 7.2, while the T_m of unmodified dsDNA was 47.0 °C. Experiments with mismatched oligonucleotides, luminescent properties, and potential applications of TINA technology is discussed.

Introduction

The sequence-specific recognition of double-stranded DNA (dsDNA) is a topic of considerable interest in the development of oligonucleotide-based tools in molecular biology, therapeutics, and bionanotechnology. Triple helixes are formed when a single-stranded triplex-forming oligonucleotide (TFO) binds to dsDNA through specific major groove interactions, and this has been the subject of intense research for gene targeting. This approach allows transcriptional control, gene knock-out, and sequence-selective treatment of genomic DNA aiming for mutated or recombined genes.[1]

The third strand affinity of TFOs to their targets is generally problematic due to their required recognition to homopurine sequences of dsDNA and the disfavored formation of pH sensitive C^+·G−C Hoogsteen base triples at physiological conditions in the parallel (pyrimidine) binding motif. During the past decade, many efforts have been devoted to modifying TFOs to improve binding affinity to their targets along with designing triplex nucleobases which could alleviate restriction of the dsDNA sequence. Oligonucleotides possessing modified nucleic acids such as peptide nucleic acids (PNAs),[2] locked nucleic acids (LNAs),[3] 2′-aminoethyl-oligoribonucleotides (2′-AE-RNAs),[4] and N3′→P5′ phosphoramidates[5] inducing increased binding affinity are among the most successful chemically modified TFOs. The stabilization of the triplex structures has been also observed upon addition of heterocyclic compounds (intercalators) sometimes possessing a positively charged side chain to the aqueous solution containing all three oligonucleotide sequences.[6] It has been also shown that an intercalator covalently linked to the 3′-end or the 5′-end of a TFO led to thermal stabilization of parallel triplexes in the range +3.0 to +16.1 °C depending on linker length and type of intercalator.[7] However, there has been limited attention paid to the covalently attached intercalators inserted as a bulge in the middle of TFO.[8] This design could have several advantages. First, in comparison

(1) Reviews about application and development of triplex forming oligonucleotides: (a) Uil, T. G.; Haisma, H. J.; Rots, M. G. *Nucleic Acids Res.* **2003**, *31*, 6064−6078. (b) Knauert, M. P.; Glazer, P. *Hum. Mol. Genet.* **2001**, *10*, 2243−2251. (c) Guntaka, R. V.; Varma, B. R.; Weber, K. T. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 22−31. (d) Buchini, S.; Leumann, C. J. *Curr. Opin. Chem. Biol.* **2003**, *7*, 717−726. (e) Faria, M.; Giovannageli, C. *J. Gene Med.* **2001**, *3*, 299−310. (f) Gowers, D. M.; Fox, K. R. *Nucleic Acids Res.* **1999**, *27*, 1569−1577.

(2) (a) Nielsen, P. E.; Egholm, M.; Berg, R.; Buchardt, O. *Science* **1991**, *254*, 1497−1500. (b) Larsen, H. J.; Bentin, T.; Nielsen, P. E. *Biochim. Biophys. Acta* **1999**, *1489*, 159−166. (c) Demidov, V.; Frank-Kamenetskii, M. D. *Methods* **2001**, *23*, 108−122.

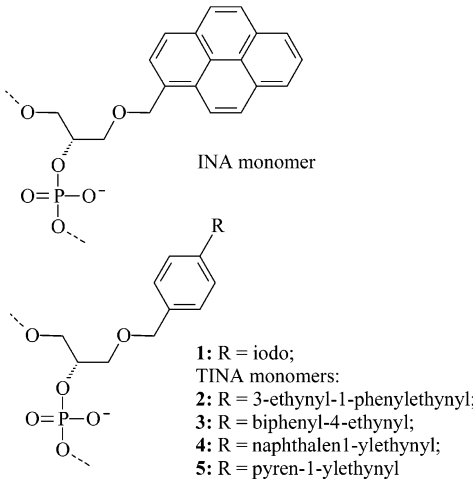
(3) (a) Obika, S.; Hari, Y.; Sugimoto, T.; Sekiguchi, M.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 8923−8927. (b) Sun, B.-W.; Babu, B. R.; Sørensen, M. D.; Zakrzewska, K.; Wengel, J.; Sun, J.-S. *Biochemistry* **2004**, *43*, 4160−4169.

(4) (a) Blommers, M. J. J.; Natt, F.; Jahnke, W.; Cuenoud, B. *Biochemistry* **1998**, *37*, 17714−17725. (b) Cuenoud, B.; Casset, F.; Hüsken, D.; Natt, F.; Wolf, R. M.; Altmann, K.-H.; Martin, P.; Moser, H. E. *Angew. Chem.* **1998**, *110*, 1350−1353; *Angew. Chem., Int. Ed.* **1998**, *37*, 1288−1291.

(5) (a) Gryaznov, S. M.; Lloyd, D. H.; Chen, J. K.; Schulz, R. G.; Dedionisio, L. A.; Ratmeyer, L.; Wilson, W. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5798−5802. (b) Escude, C.; Giovannageli, C. Sun, J.-S.; Lloyd, D. H.; Chen, J.-K.; Gryaznov, S. M.; Garestier, T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4365−4369.

10.1021/ja053645d CCC: $30.25

Scheme 1



with the synthesis of at least four nucleotide monomers needed for sugar-modified nucleic acids, the synthesis of one phosphoramidite of intercalating pseudonucleotides is required. Second, several bulged insertions of an intercalator monomer into the sequence could considerably increase duplex and triplex stabilities compared to the single insertion. Moreover, the structural difference between Watson−Crick and Hoogsteen binding modes along with the absence or presence of 2′-OH in DNA and RNA gives rise to different properties for the various types of helixes. As a result, bulged insertions of a linker and the breaking up of the helix by intercalators are expected to give unique properties for appropriately chosen helixes. This has led to chemically modified oligonucleotides which could discriminate between different types of single-stranded nucleic acids. Recently, we have reported the synthesis and properties of several intercalating nucleic acids designed for Watson−Crick-type duplexes (Scheme 1).[9] Bulged insertions of (*R*)-1-*O*-(1-pyrenylmethyl)glycerol in the middle of the oligodeoxynucleotides (INA) resulted in significantly increased affinities toward complementary ssDNA, whereas INA/RNA duplexes and the Hoogsteen-type triplex and duplex were destabilized.[9a,e] It has to also be mentioned that mismatch sensitivity on duplex formation was maintained upon bulged insertions of intercalators into the oligodeoxynucleotides.[9b] The unique combination of the flexible, short glycerol linker which distorted the phosphate backbone and the appropriate intercalator which stabilized INA/DNA duplex by desolvation and by stacking with nucleobases led to a valuable molecule which is now used in nucleic acid chemical biology.[10]

We decided to explore this type of intercalator for the design of TFO. To enhance the stability of the TFO using a short and flexible linker, the aromatic structure of intercalators should be long enough to place an intercalator into the dsDNA part of the triple helix. Therefore, (*R*)-1-*O*-(4-polyaryl-phenyl)methylglycerol could be a good choice because phenyl could also mimic a nucleobase in the TFO part of the triple helix. The polyaryl intercalator can also be attached to this phenyl via an acetylene bridge which provides the necessary structural rigidity and twisting ability and still unites the aromatic structures. The acetylene bond itself is believed to improve the intercalating properties.[11] According to the molecular modeling of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol by MacroModel 8.0, there is a twisting of 1-pyrenyl and phenyl residues around the triple bond with a torsion angle of 15.3°. It is believed that this twisting ability can help the intercalator to adjust itself to an appropriate position inside the dsDNA. Therefore, we refer to this type of nucleic acid as a twisted intercalating nucleic acid (TINA, Scheme 1). In a recent work, we deduced from molecular modeling that twisting of aromatics around triple bonds was a contributing factor to stabilize the intercalating moiety in 5′-5′ alternate strand triplexes.[7e] Here, we report the postsynthetic Sonogashira-type on-column derivatization of oligodeoxynucleotides leading to different TINAs, which were found to have extraordinarily high affinities in Hoogsteen-type duplexes and triplexes. Thermal stability and fluorescence studies of nucleic acid helixes with insertion of TINA monomers as a bulge formed according to either the Watson−Crick or Hoogsteen binding mode are also discussed.

Results and Discussion

The postsynthetic oligonucleotide modification is a better alternative to the routine and time-consuming preparation of several pseudonucleoside phosphoramidites, which are required for the selection of the right candidate for TINA. There have been several reports devoted to the palladium(0)-catalyzed modification of oligonucleotides during solid-phase synthesis.[12] Sonogashira coupling conditions were found to be compatible

(6) (a) Marchand, C.; Bailly, C.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Helene, C.; Waring, M. J. *Biochemistry* **1996**, *35*, 5022−5032. (b) Kukreti, S.; Sun, J.-S.; Loakes, D.; Brown, D. M.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Helene, C. *Nucleic Acids Res.* **1998**, *26*, 2179−2183. (c) Cassidy, S. A.; Strekowski, L.; Fox, K. R. *Nucleic Acids Res.* **1996**, *24*, 4133−4138. (d) Escude, C.; Sun, J.-S.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Helene, C. *Biochemistry* **1996**, *35*, 5735−5740. (e) Strekowski, L.; Say, M.; Zegrocka, O.; Tanious, F. A.; Wilson, W. D.; Manzel, L.; Macfarlane, D. E. *Bioorg. Med. Chem.* **2003**, *11*, 1079−1085. (f) Strekowski, L.; Hojjat, M.; Wolinska, E.; Parker, A. N.; Paliakov, E.; Gorecki, T.; Tanious, F. A.; Wilson, W. D. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1097−1100. (g) Sandstrom, K.; Warmlander, S.; Bergman, J.; Engqvist, R.; Leijon, M.; Graslund, A. *J. Mol. Recognit.* **2004**, *17*, 277−285.

(7) (a) Puri, N.; Zamaratski, E.; Sund, C.; Chattopadhyaya, J. *Tetrahedron* **1997**, *53*, 10409−10432. (b) Mouscadet, J.-F.; Ketterle, C.; Goulaouic, H.; Carteau, S.; Subra, F.; Le Bret, M.; Auclair, C. *Biochemistry* **1994**, *33*, 4187−4196. (c) Mohammadi, S.; Slama-Schwok, A.; Leger, A.; El Manouni, D.; Shchyolkina, A.; Leroux, Y.; Taillandier, E. *Biochemistry* **1997**, *36*, 14836−14844. (d) Cheng, E.; Asseline, U. *Tetrahedron Lett.* **2001**, *42*, 9005−9010. (e) Jessen, C. H.; Pedersen, E. B. *Helv. Chim. Acta* **2004**, *87*, 2465−2471.

(8) (a) Zhou, B.-W.; Puga, E.; Sun, J.-S.; Garestier, T.; Helene, C. *J. Am. Chem. Soc.* **1995**, *117*, 10425−10428. (b) Kukreti, S.; Sun, J.-S.; Garestier, T.; Helene, C. *Nucleic Acids Res.* **1997**, *25*, 4264−4270. (c) Ossipov, D.; Zamaratski, E.; Chattopadhyaya, J. *Helv. Chim. Acta* **1999**, *82*, 2186−2200. (d) Abdel-Rahman, A. A.-H.; Ali, O. M.; Pedersen, E. B. *Tetrahedron* **1996**, *52*, 15311−15324. (e) Aubert, Y.; Asseline, U. *Org. Biomol. Chem.* **2004**, *2*, 3496−3503.

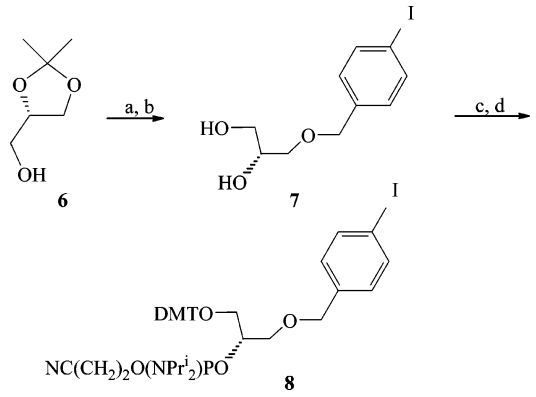
(9) (a) Christensen, U. B.; Pedersen, E. B. *Nucl. Acids Res.* **2002**, *30*, 4918−4925. (b) Christensen, U. B.; Pedersen, E. B. *Helv. Chim. Acta* **2003**, *86*, 2090−2097. (c) Filichev, V. V.; Pedersen, E. B. *Org. Biomol. Chem.* **2003**, *1*, 100−103. (d) Filichev, V. V.; Hilmy, K. M. H.; Christensen, U. B.; Pedersen, E. B. *Tetrahedron Lett.* **2004**, *45*, 4907−4910. (e) Christensen, U. B.; Wamberg, M.; El-Essawy, F. A. G.; Ismail, A. E.-H.; Nielsen, C. B.; Filichev, V. V.; Jessen, C. H.; Petersen, M.; Pedersen, E. B. *Nucleosides, Nucleotides Nucleic Acids* **2004**, *23*, 207−225. (f) Nielsen, C. B.; Petersen, M.; Pedersen, E. B.; Hansen, P. E.; Christensen, U. B. *Bioconjugate Chem.* **2004**, *15*, 260−269.

(10) (a) Filichev, V. V.; Vester, B.; Hansen, L. K.; Abdel Aal, M. T.; Babu, B. R.; Wengel, J.; Pedersen, E. B. *ChemBioChem* **2005**, *6*, 1181−1184. (b) Filichev, V. V.; Christensen, U. B.; Pedersen, E. B.; Babu, B. R.; Wengel, J. *ChemBioChem* **2004**, *5*, 1673−1679. (c) Louët, S. *Bioentrepreneur* 23 June 2003, http://dx.doi.org/10.1038/bioent748. INA is commercially available from Human Genetic Signatures Pty Ltd., Sydney, Australia.

(11) (a) Froehler, B. C.; Wadwani, S.; Terhorst, T. J.; Gerrard, S. R. *Tetrahedron Lett.* **1992**, *33*, 5307−5310. (b) Sági, J.; Szemzö, A.; Ebinger, K.; Szabolcs, A.; Sági, G.; Ruff, E.; Ötvös, L. *Tetrahedron Lett.* **1993**, *34*, 2191−2194. (c) Colocci, N.; Dervan, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 785−786.

(12) (a) Khan, S. I.; Grinstaff, M. W. *J. Am. Chem. Soc.* **1999**, *121*, 4704−4705. (b) Beilstein, A. E.; Grinstaff, M. W. *Chem. Commun.* **2000**, 509−510. (c) Rist, M.; Amann, N.; Wagenknecht, H.-A. *Eur. J. Org. Chem.* **2003**, 2498−2504. (d) Mayer, E.; Valis, L.; Wagner, C.; Rist, M.; Amann, N.; Wagenknecht, H.-A. *ChemBioChem* **2004**, *5*, 865−868.

Scheme 2 [a]



[a] Reagents and conditions: (a) 4-iodobenzylbromide, KOH, toluene; (b) 80% aq CF_3COOH, room temperature, 100% over two steps; (c) DMTCl, pyridine, room temperature, 70%; (d) $NC(CH_2)_2OP(NPr^i_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 0 °C to room temperature, overnight, 67%.

with the DNA synthesis, and no side reactions were observed for nucleobases possessing protective groups.[12a] According to the known protocols,[12] the DNA synthesis is stopped after the incorporation of 5′-*O*-DMT-2′-deoxy-5-iodouridine at the 5′-end of the sequence followed by treatment of the oligonucleotide support under Sonogashira conditions. Afterward, the oligonucleotide support is attached to the DNA synthesizer, and oligo synthesis is continued automatically. There is a risk that the coupling efficiency for the standard phosphoramidites drops after on-column derivatization, which we have also observed in our experiments described below. Despite the fact that some organometallic couplings were applied for postsynthetic oligonucleotide modification,[13] the postsynthetic Sonogoshira-type reactions on the convertible nucleoside 2′-deoxy-5-iodouridine located in the middle of the sequence were reported to be unsuccessful.[12b] Instead, we took a chance to use (*R*)-1-*O*-(4-iodobenzyl)glycerol in Sonogoshira-type reactions after its incorporation into the middle of the oligos. A number of aromatic structures with the terminal triple bond (**2**−**5**) were used in this context (Scheme 1).

The required phosphoramidite **8** was synthesized in four steps from 4-iodobenzylbromide and (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol in 47% overall yield (Scheme 2). The coupling efficiency of compound **8** during DNA synthesis on a 0.2 μmol scale using standard nucleotide coupling conditions (2 min coupling, 4,5-dicyanoimidazole as an activator) and increased deprotection time (100 s) was estimated to be more than 99%. After the DNA synthesis, the CPG-supports with DMT-on oligonucleotides possessing 4-iodophenyl moieties were treated with a Sonogashira-coupling reagent mixture containing $Pd(PPh_3)_4$ or $Pd(PPh_3)_2Cl_2$ (7.5 mM), an aromatic structure possessing a terminal acetylene (22.5 mM), and CuI (7.5 mM) in dry DMF/Et_3N (3.5/1.5, 500 μL) in 1 mL syringes under dry conditions at room temperature. It was important to flush supports and syringes with argon instead of nitrogen prior to the coupling reaction in order to avoid Glazer oxidative dimerization. The conversion was found to be better when the Sonogashira reaction mixture was prepared directly in the plastic syringe for each individual oligo instead of preparing the Sonogashira reaction mixture as a large portion for several coupling reactions. After the coupling reaction (3−4 h), the CPGs were flushed with DMF (2 × 0.5 mL) and CH_3CN (2 × 1 mL), and dried. Then, the oligonucleotides were cleaved off from the CPG-support with 32% NH_4OH (2 h), and deprotected at 55 °C (overnight). Due to the different lypophilic ability, the unreacted oligomer and the target TINA were separated by semipreparative HPLC on a C_{18} column. In the case of the overlapping peaks (structure **2**), a longer HPLC program was applied (see Supporting Information). After the first separation, DMT-on oligonucleotides were treated with 10% AcOH, purified again on HPLC, and precipitated from ethanol. The purity of the final TFOs was found to be over 90% for pure pyrimidine-containing oligodeoxynucleotides and 85−88% for oligodeoxynucleotides with purines as judged by ion-exchange HPLC. The composition was verified by MALDI-TOF.

The conversion during the Sonogashira coupling depended on the reactivity of acetylenes and on the oligo sequence. As can be judged from a number of experiments with 1-ethynylpyrene, one more treatment with the fresh reaction mixture was more efficient than the prolonged reaction time (4−16 h). Smaller amounts of sparingly soluble Glazer byproducts were formed and better oligo derivatization was observed for $Pd(PPh_3)_4$ than for $Pd(PPh_3)_2Cl_2$ as the catalyst in the case of 1-ethynylpyrene. The presence of purines in the sequence resulted in lower conversion (50−60%) to the target TINA even after double treatment of the support with the oligonucleotide by the Sonogashira-coupling reagent mixture containing 1-ethynylpyrene compared to the homopyrimidine sequences (80−85%) after a single treatment. This also seems true for other aromatic acetylenes, because in a purine-containing sequence no target oligonucleotides were obtained using 4-ethynylbiphenyl, which was found to be the least reactive compound among the tested acetylenes. In the synthesis of **ON14**, we experienced that the treatment of a complete oligonucleotide with a Sonogashira reaction mixture with 1-ethynylpyrene gave a more pure oligomer than interruption of the DNA synthesis after the second insertion of **8** followed by Sonogashira reaction and continued DNA synthesis. In the latter case, short oligomers possessing pyrenes contaminated the final TINA as judged by ion-exchange HPLC.

Very recently, B. Liang et al.[14] have reported the copper-free Sonogashira coupling reaction with $PdCl_2$ in water in the presence of pyrrolidine. The compatibility with water, aerobic conditions, and traces of homocoupling products are the very big advantages of this method. We applied the analogous conditions on the fully deprotected **ON2**. However, after treatment of **ON2** with 1-ethynylnaphthalene and $PdCl_2$ in water/pyrrolidine (1:1) at 50 or 20 °C overnight, no trace of the desired nucleic acids was observed after HPLC purification.

The thermal stability of triplexes and DNA/DNA and DNA/RNA duplexes with the synthesized oligonucleotides was assessed by thermal denaturation experiments. The melting temperatures (T_m's, °C) determined as first derivatives of melting curves are listed in Tables 1−4. The sequences possessing different TINAs were studied in pH-dependent Hoogsteen-type base pairing, both in the parallel triplex[15a] toward the duplex

(13) (a) Gartner, Z. J.; Kanan, M. W.; Liu, D. R. *Angew. Chem.* **2002**, *114*, 1874−1878; *Angew. Chem., Int. Ed.* **2002**, *41*, 1796−1800. (b) Gartner, Z. J.; Grubina, R.; Calderone, C. T.; Liu, D. R. *Angew. Chem.* **2003**, *115*, 1408−1413; *Angew. Chem., Int. Ed.* **2003**, *42*, 1370−1375.

(14) Liang, B.; Dai, M.; Chen, J.; Yang, Z. *J. Org. Chem.* **2005**, *70*, 391−393.

(15) (a) Felsenfeld, G.; Miles, H. T. *Annu. Rev. Biochem.* **1967**, *36*, 407−449. (b) Liu, L.; Miles, H. T.; Frazier, J.; Sasisekharan, V. *Biochemistry* **1993**, *32*, 11802−11809.

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 (**ON15**)		antiparallel duplex[c] 3′-GGGGAAAGAAAAAA (**ON16**)		
		pH 5.0	pH 6.0	pH 7.2	pH 5.0	pH 6.0	pH 5.0	pH 6.0	pH 7.2
ON1	5′-CCCCTTTCTTTTTT	55.0[e]	27.0	<5.0	29.5	19	47.0	48.0	47.0
ON2	5′-CCCCTT**1**TCTTTTTT	*i*	15.0	<5.0	*i*	<5.0	*i*	40.5	*i*
ON3[f]	5′-CCCCTT**2**TCTTTTTT	*i*	26.0	<5.0	*i*	<5.0	*i*	42.0	*i*
ON4[g]	5′-CCCCTT**3**TCTTTTTT	*i*	26.0	<5.0	*i*	17.0	*i*	40.0	*i*
ON5[f]	5′-CCCCTT**4**TCTTTTTT	57.0	35.0	13.5	33.5	22.0	44.5	45.0	46.0
ON6[f,g]	5′-CCCCTT**5**TCTTTTTT	59.0[e]	46.0	28.0	42.0	33.5	44.0	46.5	45.5
ON7[f]	5′-CCCCTTTC**5**TTTTTT	*i*	39.5	21.5	*i*	30.0	*i*	44.5	*i*
ON8[f]	5′-CCCCTTT**5**CTTTTTT	*i*	42.5	26.0	*i*	28.0	*i*	45.0	*i*
ON9[g]	5′-CCC**5**CTTTCTTTTTT	*i*	41.0	24.0	*i*	31.5	*i*	45.5	*i*
ON10[f]	5′-**5**CCCCTTTCTTTTTT	61.0	44.5	20.5	46.0	36.0	49.5	53.0	52.0
ON11[g]	5′-**5**CCCCTT**5**TCTTTTTT	65.5[e]	57.0[d]	35.5	53.5	45.5	46.5	47.0	46.5
ON12[h]	5′-CCCCTT**5**T**5**CTTTTTT	55.5[e]	40.0	<5.0	37.0	26.5	37.5	41.0	41.0
ON13[h]	5′-CCCCT**5**TT**5**CTTTTTT	59.5[e]	56.5[e]	40.0	41.0	38.0	44.5	45.0	42.0
ON14[h]	5′-CCCCTT**5**TCT**5**TTTTT	63.0[e]	56.5[e]	43.0	45.5	38.0	42.5	41.0	38.0

[a] C = 1.5 μM of **ON1**-**14** and 1.0 μM of each strand of dsDNA (**D1**) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 5.0, 6.0 and 7.2; duplex T_m = 56.5 °C (pH 5.0), 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 or pH 5.0. [c] C = 1.0 μM of each strand in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 5.0, 6.0 and pH 7.2. [d] Third strand and duplex melting overlaid. Transition with T_m = 54.5 °C was determined at 373 nm. [e] Third strand and duplex melting overlaid. [f] Prepared by Sonogashira reaction mixture: $Pd(PPh_3)_2Cl_2$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [g] Prepared by Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [h] Prepared by double treatment with Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), 1-ethynylpyrene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [i] Not determined.

Table 2. T_m [°C] Data for Mismatched Parallel Triplex[a] and Parallel Duplex[b] Melting Taken from UV Melting Curves (λ = 260 nm) at pH 6.0

sequence
3′-CTGCCCCTT**X**CTTTTTT
5′-GACGGGGAA**Y**GAAAAAA

		D1, **X**·**Y**= T·A	**D2**, **X**·**Y**= A·T	**D3**, **X**·**Y**= C·G	**D4**, **X**·**Y**= G·C
ON1	5′-CCCCTTTCTTTTTT	27.0	<5.0	<5.0	<5.0
ON6	5′-CCCCTT**5**TCTTTTTT	46.0	27.0	34.5	28.5
ON8	5′-CCCCTTT**5**CTTTTTT	42.5	28.5	26.5	26.5
ON10	5′-**5**CCCCTTTCTTTTTT	44.5	22.5	27.0	28.0
ON11	5′-**5**CCCCTT**5**TCTTTTTT	57.0	40.5	45.5	42.0

5′-GACGGGGAA**Y**GAAAAAA

		ON15, **Y**=A	**ON17**, **Y**= T	**ON18**, **Y**= G	**ON19**, **Y**= C
ON1	5′-CCCCTTTCTTTTTT	19.0	<5.0	10.0	<5.0
ON6	5′-CCCCTT**5**TCTTTTTT	33.5	21.5	20.5	20.5
ON8	5′-CCCCTTT**5**CTTTTTT	28.0	20.0	18.5	20.0

[a] C = 1.5 μM of **ON1**−**14** and 1.0 μM of each strand of dsDNA in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0. [b] C = 1.0 μM of **ON1**−**14** and 1.0 μM of purine strand.

D1 and in parallel dsDNA[15b] toward **ON15** (Table 1). The same sequences (**ON1**−**14**) were used for Watson−Crick DNA/DNA antiparallel duplexes toward **ON16**. For the latter type of duplexes, mixed pyrimidine/purine sequences similar to those described earlier for INA[9a,d] were also used for TINA oligonucleotides for hybridization with ssDNA and ssRNA (Table 4).

As can be seen from the T_m data in Table 1, considerable destabilization of the Hoogsteen-type triplex and duplex was observed for **ON2** with (*R*)-1-*O*-(4-iodophenylmethyl)glycerol as a bulge in the middle of the sequence compared to the wild-type complexes at pH 6.0 (**ON1** toward **D1** and **ON15**). Substitution of the iodine with aryl substituents gave more stable triplexes (**ON3**−**6** toward **D1**, pH 6.0). The highest T_m value 46.0 °C was observed for the 1-pyrenylethynyl substituent at pH 6.0 which corresponds to ΔT_m = 19.0 °C when compared to the wild-type triplex. Even at pH 7.2 a single incorporation of **5** led to a considerable stabilization of the triplex (**ON6**/**D1**), despite a high cytosine content (36%). At this pH, no hybridization could be detected for the wild-type triplex (Figure 1). For the parallel duplexes at pH 6.0, the stabilization of 3.0 and 14.5 °C per modification was detected for 1-naphthalenylethynyl (**ON5**) and 1-pyrenylethynyl (**ON6**), respectively. As expected, at lower pH (pH = 5.0) parallel duplexes were found to be more stable due to protonation of cytosine. It could be concluded that attaching the aromatic structures at the 4-position of the phenyl ring in (*R*)-1-*O*-(phenylmethyl)glycerol resulted in increasing hybridization affinity in Hoogsteen-type helixes. Interestingly, naphthalene and pyrene rings gave considerably better stabilization than 4-biphenyl and benzene. This supports the idea that aromatic structures with a large surface such as pyrene are preferred for attachment to (*R*)-1-*O*-(4-substituted phenylmethyl)glycerol over small aromatic structures to achieve good binding in Hoogsteen-type helixes.

Destabilization of antiparallel dsDNA was observed for all studied modified oligodeoxynucleotides except when the intercalator **5** was placed at the 5′-end (**ON10**/**ON16**) as compared with the wild-type dsDNA (**ON1**/**ON16**, Table 1). The stabilizing effect in the latter case could be ascribed to stacking of an aromatic polycyclic system on the adjacent nucleobase (the effect as a lid), while the effect of the acyclic linker is marginal.[16] Hybridization affinity was also dependent on the structure of TINA. The least destabilized duplexes were formed with **4** and **5**, whereas the destabilization of dsDNA was larger for structures **1**−**3** incorporated as a bulge in the middle of the sequence. Already at this stage it can be concluded that TINA incorporated as a bulge into helixes is improving the stability of Hoogsteen-type helixes and not Watson−Crick type duplexes.

(16) (a) Ren, R. X.-F.; Chaudhuri, N. C.; Paris, P. L.; Rumney, I. S.; Kool, E. T. *J. Am. Chem. Soc.* **1996**, *118*, 7671−7678. (b) Guckian, K. M.; Schweitzer, B. A.; Ren, R. X.-F.; Sheilis, C. J.; Paris, P. L.; Tahmassebi, D. C.; Kool, E. T. *J. Am. Chem. Soc.* **1996**, *118*, 8182−8183.

Table 3. T_m [°C] Data for Parallel Triplex Meltings[a] for Insertions of **5** in the Sequence of the Watson−Crick Duplex Taken from UV Melting Curves (λ = 260 nm) at pH 6.0[b]

		triplex				parallel duplex	
			D5, 3′-CTGCCCCTT**5**TCTTTTTT (**ON20**)[c]	**D6**, 3′-CTGCCCCTTTCTTTTTT			
sequence		**D1**	5′-GACGGGGAAAGAAAAAA	5′-GACGGGGAA**5**AGAAAAAA (**ON21**)[d]	**D7**, **ON20**/**ON21**	**ON15**	**ON21**
ON1	5′-CCCCTTTCTTTTTT	27.0	38.0	24.0	27.0	19.0	14.0
ON6	5′-CCCCTT**5**TCTTTTTT	46.0	38.0	27.5	31.5	33.5	26.5
ON9	5′-CCC**5**CTTTCTTTTTT	41.0	52.5[e]	41.5	43.5	31.5	29.0

[a] C = 1.5 μM of **ON1**, **ON6**, **ON9**, and 1.0 μM of each strand of dsDNA in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0, duplex T_m = 55.0 °C (**D5**), 56.0 °C (**D6**), 57.0 °C (**D7**). [b] The meltings are also given for parallel duplexes (C = 1.0 μM of **ON1**, **ON6**, **ON9**, **ON15** and **ON21** in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0.) with insertion of **5** in the purine stretch. [c] Prepared by Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), 1-ethynylpyrene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [d] Prepared by double treatment with Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), 1-ethynylpyrene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [e] Third strand and duplex melting overlaid.

Table 4. T_m [°C] Data for Antiparallel Duplex[a] Melting Taken from UV Melting Curves (λ = 260 nm)

		ON25 DNA 5′-AGCTTGCTTGAG	**ON26**[b] DNA 5′-AGCTTG**5**CTTGAG	**ON27** RNA 5′-AGCUUGCUUGAG
ON22	3′-TCGAACGAACTC	47.5	32.0	40.5
ON23[b]	3′-TCGAAC**5**GAACTC	39.5	36.0	30.5
ON24[c]	3′-TCGAAC**5**G**5**AACTC	34.0	22.5	25.0

[a] C = 1.0 μM of each oligonucleotide in 140 mM NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0. [b] Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), 1-ethynylpyrene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [c] Double treatment with Sonogashira reaction mixture: $Pd(PPh_3)_4$ (7.5 mM), 1-ethynylpyrene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h.

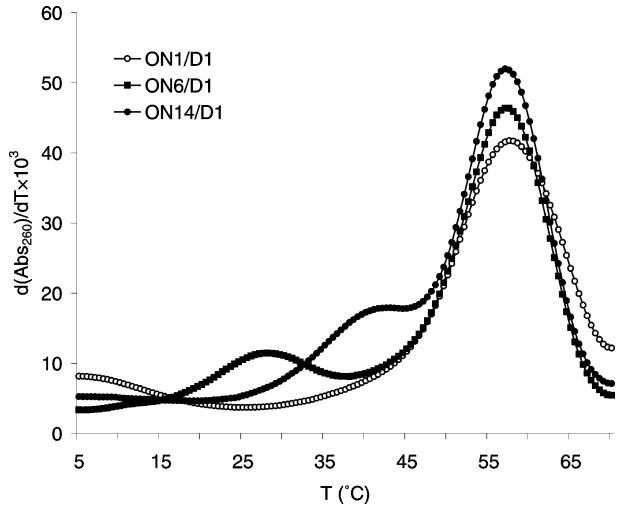


Figure 1. First derivative plots of triplex melting recorded at 260 nm versus increasing temperature (1 °C/min) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.2.

Thus, a single insertion of (*R*)-1-*O*-[4-(1-pyrenylethynyl)-phenylmethyl]glycerol as a bulge in a less stable parallel triplex (**ON6**/**D1**) at pH 6.0 stabilized the triplex to the level of a Watson−Crick duplex (**ON6**/**ON16**) with the same nucleotide content. The thermal stability for different TINAs prompted us to investigate the properties of the 1-pyrenylethynyl-containing TINA more closely.

Some fluctuation in the thermal stability of Hoogsteen's triplexes and duplexes was seen on placing 1-pyrenylethynyl at different positions in the TFO. When cytosine was neighboring either the 5′-side or the 3′-side (**ON7**−**9**), both the parallel triplex and the parallel duplex were less stabilized than when **5** was placed between two thymidines at pH 6.0 (**ON6**). This could be a result of the interaction of the aromatic structure with the positively charged pair of C^+·G. Interestingly, at pH 7.2, when cytosine was not protonated, the lowest triplex hybridization affinity was detected for TFO with **5** at the 5′-dangling end (**ON10**) among the tested TFOs with single insertion of 1-pyrenylethynyl (**ON6**−**10**). It was a surprise that the lid effect[16] was absent here. This could be a consequence of generally lower stability of C-rich regions of TFO with the target dsDNA under physiological conditions.[17] However, it is an important observation that efficient hybridization affinity could be achieved by placing **5** in the middle of the C-rich region (**ON9**) in neutral media. One can speculate whether intercalation will make protonation more likely in the triplex structure at physiological pH because the intercalator is separating two positively charged triples.

The dependence of the distance between multiple bulged insertions of the pyrene intercalator **5** on thermal stability was investigated using **ON11**−**14** (Table 1). In the case of overlapping triplex and duplex transitions, melting experiments were performed at 373 nm. However, sometimes not very well-defined transitions were observed at 373 nm. In these cases, the meltings at pH 6.0 of the triplexes at temperatures close to those of the duplexes were based on comparison with meltings at pH 7.2 which were measured at 260 nm. When the intercalator **5** was inserted as a next nearest neighbor (**ON12**), the Hoogsteen triplex and duplex were stabilized compared to the unmodified **ON1** at pH 6.0. However, the stabilities in both cases were lower than for the single insertion of **5** (**ON6**), and no triplex formation was observed at pH 7.2. This could be due to the large interruption of the double and triple helixes by two bulged (*R*)-1-*O*-methylglycerol linkers positioned very close to each other. When the two insertions of **5** were separated by two or three nucleobases (**ON13** and **ON14**, respectively), the complexes with **D1** and **ON15** were more stable than those with single insertions. At pH 7.2, the T_m for the triplexes was even higher than the physiological temperature 37 °C (see **ON14**/**D1** in Figure 1). Like double insertions of **5** in the middle, double insertions with one insertion at the 5′-end with six base-pairs between the insertions (**ON11**) considerably stabilized the

(17) Hildbrand, S.; Blaser, A.; Parel, S. P.; Leumann, C. J. *J. Am. Chem. Soc.* **1997**, *119*, 5499−5511.

Hoogsteen-type duplex and triplex at pH 6.0. Opposite to the Hoogsteen helixes, antiparallel duplexes with double insertions of **5** (**ON12**−**14**/**ON16**) showed decreased stabilities when compared with the wild-type duplex **ON1**/**ON16**, especially when one or three nucleobases were between the two insertions. When comparing thermal stabilities of parallel and antiparallel duplexes with double insertions of **5** at pH 5.0, Hoogsteen duplexes **ON11**/**ON15** and **ON14**/**ON15** were even more stable than the corresponding Watson−Crick duplexes (**ON11**/**ON16** and **ON14**/**ON16**). The stabilization of parallel triplexes and parallel duplexes upon addition of an intercalator was first reported by C. Escude et al.[18] for benzopyridoindole (BPI) derivatives. The reorganization of a nonperfectly matched Watson−Crick DNA duplex into a perfectly matched Hoogsteen paired DNA duplex has been detected when BPI was added to the aqueous solution of the oligodeoxynucleotides. In cases of multiple insertions of **5** into oligodeoxynucleotides, the similar reorganization to a fully matched parallel duplex is also anticipated depending on pH and salt concentration.

The extraordinary stabilization of parallel triplexes was observed at pH 5.0. High content of cytosines in the TFO shifted the melting of the unmodified triplex (T_m = 55.0 °C) close to the melting of the duplex. However, this value was still lower than that of the duplex melting at pH 5.0 ($T_{m(\mathbf{D1})}$ = 56.5 °C). Single bulged insertion of 1-naphthalenylethynyl derivative **4** in the TFO slightly increased the triplex stability ($\Delta T_{m(\mathbf{ON5/D1}-\mathbf{ON1/D1})}$ = 2.0 °C). However, bulged insertion of **5** in all cases led the dissociation of the whole complex at temperatures which were higher than T_m for the dsDNA (**D1**). The clear transition for **ON11** was observed at 373 nm at the same temperature as at 260 nm, which confirmed that the triplex and the duplex melted together. The same dependence of thermal stability for double insertion of **5** in TFO as at pH 6.0 was observed at pH 5.0. Thus, the double insertion of **5** as next-nearest neighbors (**ON12**) and insertions of **5** in the middle and at the 5-end (**ON11**) were the least and the most stabilized triplexes, respectively. These results support the idea of placing the intercalator into the dsDNA part of the triple helix which under appropriate conditions can increase the thermal stability of the duplex itself. At pH 5.0, the triplex **ON14**/**D1** was 16.5 and 20.5 °C more stable than the corresponding parallel and antiparallel duplexes, respectively. Importantly, even at pH 7.2 oligonucleotide **ON14** forms more stable Hoogsteen-type triplex (T_m = 43.0 °C, **ON14**/**D1**) than the corresponding Watson Crick dsDNA (T_m = 38.0 °C, **ON14**/**ON16**). At pH 7.2, the melting temperature for the parallel duplex (**ON14**/**ON15**) is supposed to be lower than 38.0 °C observed at pH 6.0 since this duplex is pH-sensitive. These data clearly demonstrate the ability of oligonucleotides with multiple insertions of **5** in the middle of the sequence separated by three bases to discriminate well between dsDNA and ssDNA.

The sensitivity to mismatches was studied for parallel triplexes and duplexes with bulged insertion of **5** in the middle and at the 5′-end of the sequence (Table 2). In the case of triplexes, the sensitivity to mismatch was dependent on the site of insertion of the intercalator. The smallest value of ΔT_m = 11.5 °C between matched and mismatched triplexes was detected when adenine was replaced by guanine in the purine strand on the 3′-site of the intercalator (**ON6**/**D3** and **ON11**/**D3**, Table 2). In all other cases, the dropping of T_m was in the range 14.0−22.0 °C. In the work of Zhou et al.,[8a] the bulged insertion of 2-methoxy-6-chloro-9-aminoacridine via a flexible linker in the middle of TFO was used to stabilize triplexes with mismatches to the level of the perfectly matched triplexes. The ΔT_m values were within 10 °C. In our case, the ΔT_m values are higher, which is believed to be sufficient to distinguish between matched and single mismatched triplexes. To reduce the false positives which could come from binding of the TFO possessing **5** to mismatched or shorter dsDNAs, the structured probes should be used.[19] The benefit of structured probes over linear nucleic acids has been already presented for specific DNA triplex formation.[19c] Mismatched parallel duplexes with a single insertion of **5** were destabilized in the range 8.0−13.0 °C which was in the same range as mismatched wild type parallel duplexs. For comparison, the least sensitive mismatched unmodified duplex showed a ΔT_m of 9 °C at pH 6.0 ($T_{m(\mathbf{ON1/ON15})} - T_{m(\mathbf{ON1/ON18})}$).

We studied the luminescent characteristics of the TFO possessing (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**5**) which was the most effective TINA to form triplexes and to discriminate mismatches to the duplex. The introduction of **5** into oligonucleotides resulted in a characteristic monomeric fluorescence spectrum, with maxima at 400 and 421 nm upon excitation at 373 nm (Figure 2A, black curve), which was similar to previously published data for 4-[4-(1-pyrenylethynyl)phenyl]-1,3-butanediol inserted into DNA.[20] In all cases, a 4 nm shift of monomeric fluorescence was detected upon formation of triplexes or duplexes. Formation of the fully matched triplex led to approximately 1.5-fold increased monomeric fluorescence (Figure 2A, **ON6**/**D1**) compared to the single-stranded **ON6**. For nonperfectly matched triplexes, the fluorescence intensity depended on the sequence of dsDNA. Thus, an almost 2-fold increase was detected for a TA inversion site (**ON6**/**D2**) compared to **ON6**. On the contrary, when a cytosine or a guanine base was mismatching in dsDNA to the TFO near the insertion of **5** (**D3** and **D4**), a decrease of monomeric fluorescence was seen in comparison with the perfectly matched triplex (Figure 2A). Especially guanine gave a large effect with 2-fold lower fluorescence intensity for the mismatched triplex **ON6**/**D3**.

Interestingly, a considerable increase in monomer fluorescence was detected upon formation of the antiparallel duplex (**ON6**/**ON16**, Figure 2C), whereas the formation of the parallel duplex (**ON6**/**ON15**) resulted in only a slightly increased fluorescence when compared with the single-strand fluorescence (Figure 2C). When a second 4-(1-pyrenylethynyl)phenyl residue appeared as a next-nearest neighbor in **ON12**, the monomeric fluorescence of the single-strand decreased approximately 3-fold (Figure 2C, comparison of **ON6** and **ON12**), and an excimer fluorescence with a maximum at 500 nm and with an intensity half of that of the monomeric intensity could be observed (Figure 2B). A considerable decrease of the monomeric fluorescence and disappearance of the excimer band was observed for the

(18) (a) Escude, C.; Mohammadi, S.; Sun, J.-S.; Nguyen, C.-H.; Bisagni, E.; Liquier, J.; Taillandier, E.; Garestier, T.; Helene, C. *Chem. Biol.* **1996**, *3*, 57−65.

(19) (a) Demidov, V. V.; Frank-Kamenetskii, M. D. *Trends Biochem. Sci.* **2004**, *29*, 62−71. (b) Bonnet, G.; Tyagi, S.; Libchaber, A.; Kramer, F. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6171−6176. (c) Roberts, R. W.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9397−9401.

(20) Malakhov, A. D.; Skorobogatyi, M. V.; Prokhorenko, I. A.; Gontarev, S. V.; Kozhich, D. T.; Stetsenko, D. A.; Stepanova, I. A.; Shenkarev, Z. O.; Berlin, Y. A.; Korshun, V. A. *Eur. J. Org. Chem.* **2004**, 1298−1307.

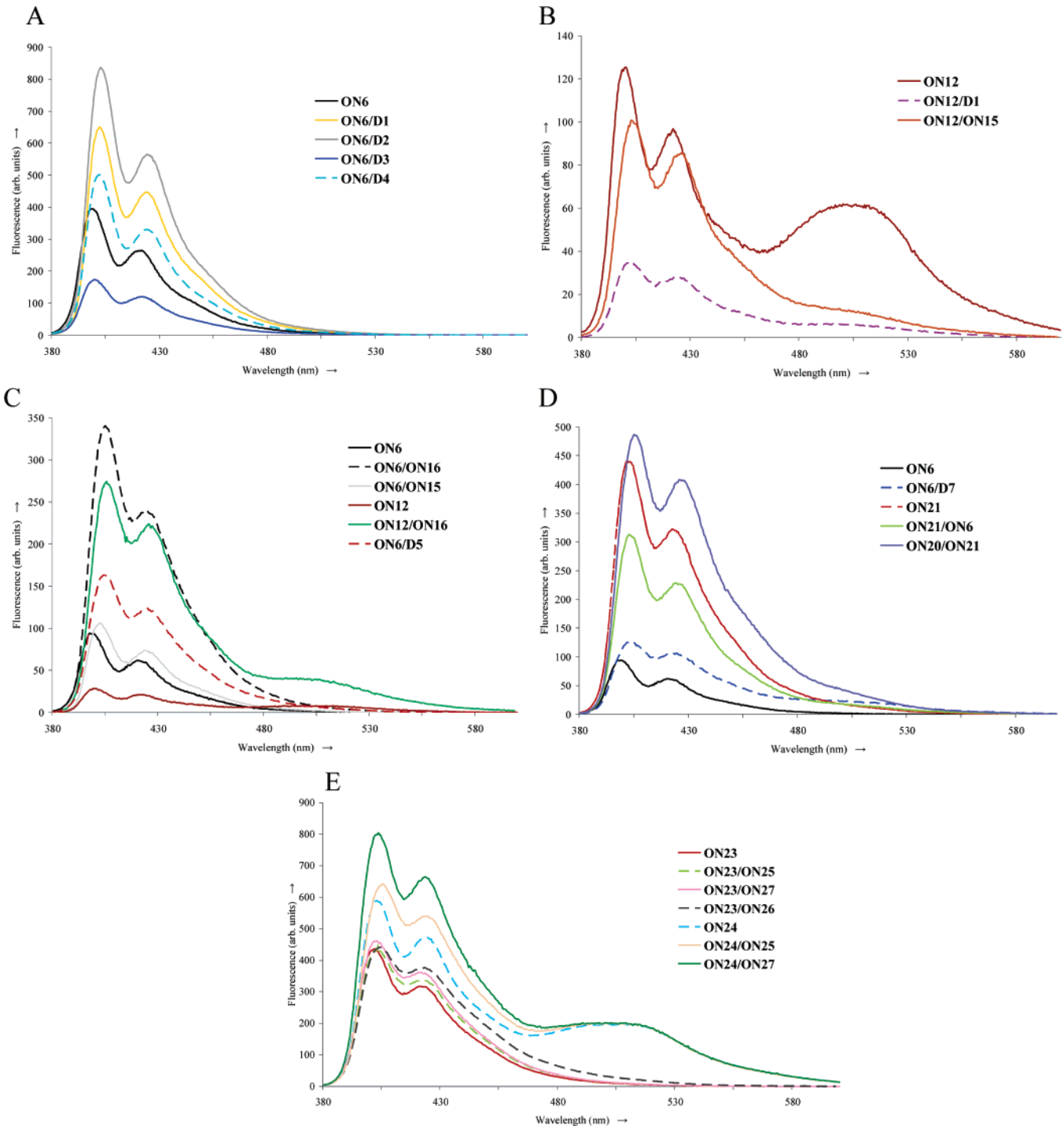


Figure 2. Fluorescence spectra of single-strands, antiparallel and parallel duplexes, and parallel triplexes. Measurement conditions: 1 μM of each strand in a buffer at 10 °C (20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0), excitation 373 nm (excitation slit 4.0 nm), emission 380−600 nm (emission slit 2.5 nm for A, B, E, and 0.0 nm for C and D). **ON6** and **ON12** were used as references in spectra recorded under different conditions.

same oligo in a matched triplex (Figure 2B, **ON12**/**D1**). This means that the pyrene moieties were not in the sufficient proximity needed for excimer formation upon binding to dsDNA in the environment of the triplex helix. Similarly, the excimer band disappeared when **ON12** formed a Hoogsteen-paired dsDNA with **ON15** (Figure 2B). On the contrary, very high monomeric fluorescence intensity and increased excimer fluorescence were observed for the antiparallel duplex (**ON12**/**ON16**) when compared with fluorescence intensities of the single-stranded **ON12** (Figure 2C). This indicates that the two pyrenyls in the same strand were still in close contact with each other after formation of the Watson−Crick dsDNA although this seems not the case in the Hoogsteen-type dsDNA. In this way, the different properties of TINA toward Watson−Crick- and Hoogsteen-type helixes were reflected by both hybridization and fluorescence properties. Moreover, fluorescence data for (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**5**) as bulged next-nearest neighbors in a pyrimidine-rich strand can be summarized as follows: single-strand **ON12**, medium monomer fluorescence at 400 and 421 nm and excimer band at 500 nm;

parallel triplex **ON12**/**D1**, low monomer fluorescence and no excimer band; parallel duplex **ON12**/**ON15**, medium monomer fluorescence and no excimer band; antiparallel duplex **ON12**/**ON16**, high monomer fluorescence and excimer band.

The ability of structure **5** to affect the stability of the parallel triple helix upon its incorporation into the Watson−Crick duplex part of the triplex is presented in Table 3. The triplex was stabilized in all cases when **5** was inserted as a bulge in the pyrimidine strand of the duplex (**ON1**, **ON6**, and **ON9** toward **D5**) when compared to the unmodified triplex (**ON1**/**D1**). Two transitions (T_m = 36.5 and 55.5 °C) were detected for the triplex **ON1**/**D1** in the thermal denaturation experiment at 373 nm [λ_{max} of **5**] which corresponded to triplex and duplex meltings, respectively. Detection of the meltings by the 373 nm absorbance indicated that the intercalator was involved in both the duplex and triplex formation. The insertion of **5** as bulges in both Watson−Crick and Hoogsteen pyrimidine strands opposite to each other (**ON6**/**D5**) did not change the melting of the triplex when compared with a triplex with an intercalator only in the duplex part of the triplex (**ON1**/**D5**). When two pyrene moieties **5**, one in each of the pyrimidine strands, were placed as bulges separated by three base-pairs (**ON9**/**D5**), the triplex melting was very close to the duplex melting temperature, which was also observed above for the double incorporation of **5** into TFO. Decreased triplex and parallel duplex stabilities compared to the unmodified complexes were observed when compound **5** was inserted in the purine strand as a bulge (**ON1** toward **D6** and toward **ON21**).

We also studied the hybridization affinity of TINA possessing **5** toward mixed purine/pyrimidine sequences of ssDNA and ssRNA in Watson−Crick-type duplexes (Table 4) using the same sequence and conditions as have been described for INA.[9a,d] Considerable destabilization of TINA/DNA (ΔT_m in the range −8.0 to −15.5 °C) and TINA/RNA (ΔT_m = −10.0 °C) was observed for **5** as a bulge in the middle of the sequence when compared with the wild-type duplexes. The insertion of the second intercalator **5** as a next-nearest neighbor into DNA (**ON24**) led to further destabilization of the duplex (**ON24**/**ON25** and **ON24**/**ON27**). The incorporation of **5** opposite to each other into two complementary mixed purine−pyrimidine strands, as the complex **ON23**/**ON26**, resulted in a T_m value of 36.0 °C which was at the same level of magnitude as TINA/DNA duplexes (**ON23**/**ON25** and **ON22**/**ON26**). However, when INA was inserted in the same positions in INA/INA duplexes, they were less stable (T_m = 43.6 °C) than INA/DNA (T_m = 51.5 °C).[9d]

The fluorescence properties of complexes with the 4-(1-pyrenylethynyl)phenyl moiety in the Watson−Crick dsDNA as a duplex alone and as a part of the triplex are shown in Figure 2C−E. The monomer fluorescence was considerably increased when **5** was inserted into the purine strand (ss**ON21**) compared with the insertion into the pyrimidine strand (ss**ON6**, Figure 2D). Slightly decreased fluorescence intensity was seen upon assembling of the triplexes and duplexes with unmodified DNA and ss**ON21** (data not shown). It was a surprising finding that the strong sensitivity of the monomer fluorescence of 4-(1-pyrenylethynyl)phenyl moieties in homopyrimidine sequences upon the formation of antiparallel duplexes completely disappeared for mixed sequences (**ON23**/**ON25**, **ON23**/**ON27**, Figure 2E). This differs also from previous results reported for bulged insertions of (*R*)-1-*O*-(1-pyrenylmethyl)glycerol using the same sequences.[9a,d]

When two pyrenyl intercalators **5** were separated by one base-pair, an excimer band observed for the ss**ON24** (Figure 2E) did not disappear upon formation of the antiparallel duplex (**ON24**/**ON25** and **ON24**/**ON27**, Figure 2E). This observation is opposite to the above observations for parallel triplex and parallel duplex with TINA and is also contrary to the previously obtained results for INA.[9b] The presence of the excimer band upon formation of the antiparallel duplex in both homopyrimidine and mixed pyrimidine/purine strands (**ON12**/**ON16** and **ON24**/ **ON25**, respectively) with bulged **5** as next-nearest neighbors indicates that two pyrenyl residues were positioned very closely and communicated with each other and were not fully embedded into stacking interactions with neighboring Watson−Crick base-pairs. This can also explain the decrease of the antiparallel duplex stability upon incorporation of **5** as a bulge.

We then checked whether an excimer bond could be formed for duplexes and triplexes if two or three dyes were placed opposite to each other, in each of their complementary strand. No excimer band was observed in either parallel duplex **ON21**/**ON6** (Figure 2D) or antiparallel duplexes **ON20**/**ON21** (Figure 2D) and **ON23**/**ON26** (Figure 2E). This result correlates with the work of A. Malakhov et al.[20] when 4-[4-(1-pyrenylethynyl)-phenyl]-1,3-butanediol was positioned opposite one another in the complementary strands of antiparallel dsDNA with mixed sequences. Only for the triplex with three 4-(1-pyrenylethynyl)-phenyl moieties placed opposite to each other in all three strands was a weak excimer band at 500 nm detected (**ON6**/**D7**, Figure 2D). The conclusion is that the communication of the 4-(1-pyrenylethynyl)phenyl moieties positioned in different strands of the parallel and antiparallel duplexes and parallel triplex is impeded, which makes zipping of intercalators together with excimer formation unlikely, contrary to what was found for INA. Thus, zipping of two pyrene moieties of INA situated opposite to each other in a duplex have been observed in an NMR structure,[9f] and this duplex structure led to formation of an excimer band at 480 nm in a steady-state fluorescence spectra upon excitation at 343 nm (unpublished data).

The differences in fluorescence spectra and hybridization properties of the two different pyrene intercalating nucleic acids INA and TINA in Watson−Crick-type duplexes clearly illustrate the consequence of adding an extra 1-phenylethynyl moiety to the aromatic part of (*R*)-1-*O*-(1-pyrenylmethyl)glycerol (INA). By this work we have succeeded in placing pyrene appropriately in the Hoogsteen-type triplex. The ability of intercalators to stabilize parallel triplex structures with only little influence on the stability of dsDNA is known. Thus, addition of 2-(2-naphthyl)quinolin-4-amine and analogues thereof[6e,f] lead to considerable stabilization of triplex DNA [ΔT_m = 35.6 °C for 2-(2-naphthyl)quinolin-4-amine] with only a little increased hybridization affinity of duplex DNA (ΔT_m = 5.5 °C). Y. Aubert et al.[8e] have in a similar work reported the synthesis and hybridization properties of oligodeoxynucleotides with perylene coupled either directly or via a propyl linker to the anomeric position of a 2′-deoxyribose residue. One of the advantages of TINA with polycyclic moieties over monomeric triplex-specific intercalators is that TINA can be inserted several times into desired positions of the sequence instead of using excess of the

intercalator in the solution. Moreover, high parallel triplex and duplex stabilization together with destabilization of antiparallel duplexes as described here for TINA have never been observed hitherto for other intercalating systems covalently attached to the oligodeoxynucleotides. In this context, TINA, when incorporated as multiple bulge insertions into oligodeoxynucleotides, is a unique molecule with the ability to discriminate dsDNA over ssDNA. This feature is clearly seen for **ON14** when their triplex and antiparallel duplex stabilities are compared at pH 6.0 and 7.2 (Table 1). This opens up the possibility of reducing the number of false positives coming from duplex formation when parallel triplex formation is to be detected. This could for example be the case for fluorescence in situ hybridization (FISH) on genomes under nondenaturing conditions[21] and for the purification of plasmid DNA using triple-helix affinity chromatography[22] or triple-helix affinity precipitation[23] which can be performed at pH 6.0 or 5.0. This type of discrimination of parallel triplex formation over duplex formation cannot be achieved with triplex forming oligos such as PNA, LNA, or N3′→P5′ phosphoramidates which are also known to stabilize antiparallel duplexes.

Using the Sonogoshira-type postsynthetic modification of oligonucleotides possessing (*R*)-1-*O*-(4-iodophenyl)methylglycerol, we screened several twisted intercalating nucleic acids (TINAs) for their ability to increase the thermal stability of Hoogsteen-paired duplexes and triplexes. The insertion of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**5**) as a bulge in oligodeoxynucleotides was found to be the most effective TINA with good discriminating properties between matched and mismatched sequences. The Watson−Crick-type DNA/DNA and DNA/RNA duplexes were destabilized upon insertion of TINA in the middle of the sequence compared with native duplexes. We believe that TINA is the first intercalating system covalently attached to oligodeoxynucleotides as a bulge showing increased affinity toward Hoogsteen-type base-pairing and decreased affinity toward Watson−Crick-type helixes. The short synthetic route to phosphoramidite **8** and postsynthetic Sonogashira modification of oligonucleotides are competitive advantages of TINA over other triplex-stabilizing nucleic acids. From studying double insertions of TINA (**5**) in one strand, it could be concluded that placing of three nucleobases between two bulged (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]-glycerols may be an optimum for high thermal stability of Hoogsteen DNA helixes. On the other hand, the different luminescence properties (excimer band formation) upon insertion of **5** as next-nearest bulged neighbors in the pyrimidine DNA sequence could be used to distinguish between parallel triplex, parallel dsDNA, and antiparallel dsDNA. Increasing the thermal stability in the range 12−19 °C for TINA with single bulged insertion of **5** can be applied to reduce the required length of the TFO. Moreover, good thermal stability for Hoogsteen-type duplexes and triplexes could be obtained at pH 7.0 even in the presence of several cytosines in the sequence (up to 36% in the present work). The multiple insertions of **5** can be used to increase the melting temperature of less stable Hoogsteen duplexes to the level of Watson−Crick duplexes of the same length under proper conditions (sequence, pH, salt concentration, etc.). Considering the development of modified nucleic bases with high affinity for C−G[24] and T−A[25] inversion sites in dsDNA along with alternate-stranded triplexes,[7e,26] we think such improvements of triplex formation will expand the applicability of TINA. The ability to stabilize the triplex upon insertion of **5** into the pyrimidine strands of circular oligodeoxynucleotides or clamps to target ssDNA and ssRNA is also an obvious possibility. As a next step, studies are devoted to the influence of insertion of TINA and INA on the stability of nucleic acid helixes different from the classical Watson−Crick and Hoogsteen complexes. Thus, there is still limited availability of nucleic acid analogues which can stabilize reverse-Hoogsteen base-pairing, i-motifs (C−C$^+$ base-pairs), or quadruplexes (G-rich sequences). We believe that the ability of TINA to stabilize parallel triplexes and duplexes along with discrimination of Hoogsteen-type over Watson−Crick-type nucleic acid helixes can make TINA very useful in the design of DNA-based tools in bio- and nanotechnology where specific recognition, high thermal stability, and self-organization or reorganization are vital.[27]

Experimental Section

General Methods. NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The internal standard used in ^{1}H NMR spectra was TMS (δ: 0.00) for $CDCl_3$; in ^{13}C NMR the standard was $CDCl_3$ (δ: 77.0). Accurate ion mass determinations were performed using the 4.7 T Ultima Fourier transform (FT) mass spectrometer (Ion Spec, Irvine, CA). The $[M + Na]^+$ ions were peak matched using ions derived from the 2,5-dihydroxybenzoic acid matrix. Thin-layer chromatography (TLC) analyses were carried out with use of TLC plates 60 F_{254} purchased from Merck and were visualized in 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.

Synthesis of Target Compound 8. (*S*)-1-(4,4′-Dimethoxytriphenylmethyloxy)-3-(4-iodo-benzyloxy)-propan-2-ol. (*S*)-(+)-2,2-Dimethyl-1,3-dioxolane-4-methanol (**6**, 1.17 g, 8.9 mmol) and 4-iodobenzylbromide (2.5 g, 8.4 mmol) were refluxed under Dean−Stark conditions in toluene (80 mL) in the presence of KOH (8.8 g, 154.0 mmol) for 12 h. The reaction mixture was allowed to cool, and H_2O (30 mL) was added. After separation of the phases, the water layer was washed with toluene (2 × 15 mL). Combined organic layers were washed with H_2O (30 mL) and concentrated in vacuo. The residue was treated with 80% aq AcOH (25 mL) for 48 h at room temperature.

(21) (a) Anguiano, A. *J. Clin. Lig. Assay* **2000**, *23*, 33−42. (b) Hausmann, M.; Winkler, R.; Hildenbrand, G.; Finsterle, J.; Weisel, A.; Rapp, A.; Schmitt, E.; Janz, S.; Cremer, C. *BioTechniqes* **2003**, *35*, 564−577.
(22) (a) Wils, P.; Escriou, V.; Warnery, A.; Lacroix, F.; Lagneaux, D.; Ollivier, M.; Crouzet, J.; Mayaux, J.-F.; Scherman, D. *Gene Ther.* **1997**, *4*, 323−330. (b) Schuelp, T.; Cooney, C. L. *Nucl. Acids Res.* **1998**, *26*, 4524−4528.
(23) (a) Costioli, M. D.; Fisch, M. D.; Garret-Flaudy, F.; Hilbrig, F.; Freitag, R. *Biotechnol. Bioeng.* **2003**, *81*, 535−545.
(24) Huang, C.-Y.; Cushman, C. D.; Miller, P. S. *J. Org. Chem.* **1993**, *58*, 5048−5049. (b) Prevot-Halter, I.; Leumann, C. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2657−2660. (c) Obika, S.; Hary, Y.; Sekiquchi, M.; Imanishi, T. *Chem. Eur. J.* **2002**, *8*, 4796−4802. (d) Buchini, S.; Leumann, C. J. *Angew. Chem.* **2004**, *116*, 4015−4018; *Angew. Chem., Int. Ed.* **2004**, *43*, 3925−3928. (e) Ranasinghe, R. T.; Rusling, D.; Powers, V. E. C.; Fox, K. R.; Brown, T. *Chem. Commun.* **2005**, 2555−2557.
(25) (a) Guianvarc'h, D.; Benhida, R.; Fourrey, J. L.; Maurisse, R.; Sun, J. S. *Chem. Commun.* **2001**, 1814−1815. (b) Guianvarc'h, D.; Fourrey, J. L.; Maurisse, R.; Sun, J. S.; Benhida, R. *Bioorg. Med. Chem.* **2003**, *11*, 2751−2759.
(26) (a) Ono, A.; Chen, C.-N.; Kan, L.-S. *Biochemistry* **1991**, *30*, 9914−9921. (b) Froehler, B. C.; Terhorst, T.; Shaw, J.-P.; McCurdy, S. N. *Biochemistry* **1992**, *31*, 1603−1609. (c) de Bizemont, T.; Duval-Valentin, G.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Helene, C. *Nucleic Acids Res.* **1996**, *24*, 1136−1143. (d) de Bizemont, T.; Sun, J.-S.; Garestier, T.; Helene, C. *Chem. Biol.* **1998**, *5*, 755−762. (e) Ueno, Y.; Mikawa, M.; Hoshika, S.; Matsuda, A. *Bioconjugate Chem.* **2001**, *12*, 635−642.
(27) (a) Wengel, J. *Org. Biomol. Chem.* **2004**, *2*, 277−280. (b) Samori, B.; Zuccheri, C. *Angew. Chem.* **2005**, *117*, 1190−1206; *Angew. Chem., Int. Ed.* **2005**, *44*, 1166−1181.

The solvent was removed in vacuo, and the residue was coevaporated twice with toluene/EtOH (30 mL, 5:1, v/v). The residue was dried under diminished pressure to afford (*R*)-3-(4-iodo-benzyloxy)-propane-1,2-diol (**7**, 100%, 2.3 g) as yellowish oil that was used in the next step without further purification.

This oil (2.3 g, 8.4 mmol) was dissolved in anhydrous pyridine (25 mL), and 4,4′-dimethoxytrityl chloride (3.5 g, 10.4 mmol) was added under nitrogen. After 24 h, MeOH (2 mL) followed by EtOAc (150 mL) were added, and the mixture was extracted with saturated aq $NaHCO_3$ (40 mL × 2). The water phase was extracted with EtOAc (20 mL × 2). The combined organic layers were dried (Na_2SO_4), filtered, and evaporated under diminished pressure. The residue was coevaporated twice with toluene/EtOH (25 mL, 1:1, v/v). The residue was adsorbed on silica gel (3.0 g) from EtOAc (30 mL) and purified using dry column vacuum chromatography with EtOAc (0−30%, v/v) in petroleum ether to afford compound (*S*)-1-(4,4′-dimethoxytriphenylmethyloxy)-3-(4-iodo-benzyloxy)-propane-2-ol (70%, 3.6 g) as a yellow foam. ^{1}H NMR ($CDCl_3$) δ 2.42 (br s, 1H), 3.20 (m, 2H), 3.56 (m, 2H), 3.78 (s, 6H), 3.97 (m, 1H), 4.43 (s, 2H), 6.78 (d, 4H, J = 8.5 Hz), 7.00 (d, 2H, J = 8.0 Hz), 7.30−7.45 (m, 9H), 7.63 (d, 2H, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 55.2, 62.2, 69.9, 71.6, 72.6, 86.1, 93.1, 129.4, 137.4, 137.7, 113.1, 126.7, 127.8, 128.1, 130.0, 135.9, 144.7, 158.5. HR-MALDI-MS calcd for $C_{31}H_{31}IO_5Na$ $[M + Na]^+$ *m*/*z* 633.1108; found *m*/*z* 633.1116.

(*S*)-2-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-1-*O*-(4,4′-dimethoxy triphenylmethyl)-3-*O*-(4-iodo-benzyl)glycerol (8). (*S*)-1-(4,4′-Dimethoxytriphenylmethyloxy)-3-(4-iodo-benzyloxy)-propane-2-ol (2.0 g, 3.3 mmol) was dissolved under nitrogen in anhydrous CH_2Cl_2 (50 mL). *N*,*N*-Diisopropylammonium tetrazolide (0.850 g, 5.0 mmol) was added followed by dropwise addition of 2-cyanoethyl tetraisopropylphosphordiamidite (1.1 g, 3.7 mmol) under external cooling with an ice−water bath. After 16 h, analytical TLC showed no more starting material, and the reaction was quenched with H_2O (30 mL). Layers were separated, and the organic phase was washed with H_2O (30 mL). 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 solvents were removed under reduced pressure. The residue was purified using silica gel dry column vacuum chromatography with NEt_3(0.5%, v/v)/EtOAc(0−25%,)/petroleum ether. Combined UV-active fractions were evaporated in vacuo affording the final compound **8** (1.8 g, 67%) as a foam that was used in ODN synthesis. ^{32}P NMR ($CDCl_3$) δ 149.8, 149.9 in ratio 1:1. HR-ESI-MS calcd for $C_{40}H_{46}IO_6N_2PLi$ $[M + Li]^+$ *m*/*z* 817.2449, found *m*/*z* 817.2447.

Synthesis and Purification of TINAs. ODNs were synthesized on an Expedite nucleic acid synthesis system model 8909 from Applied Biosystems using 4,5-dicyanoimidazole as an activator and an increased deprotection time (100 s) and coupling time (2 min) for 0.075 M solution of the phosphoramidite **8** in a 1:1 mixture of dry MeCN/CH_2Cl_2. After the DNA synthesis, the columns with CPG-supports and DMT-on oligonucleotides possessing 4-iodophenyl moieties were flushed with argon (2 min) prior to the coupling reaction. Sonogashira-coupling reagent mixture containing $Pd(PPh_3)_4$ or $Pd(PPh_3)_2Cl_2$ (7.5 mM), an aromatic structure possessing a terminal acetylene (22.5 mM), and CuI (7.5 mM) in dry DMF/Et_3N (3.5/1.5, 500 μL) was prepared in a 1 mL plastic syringe under dry conditions at room temperature. Syringes were also flushed with argon prior to use. The syringe with Sonogashira-coupling reagent mixture was attached to the column with the CPG, and another empty syringe was connected from another side of the column. The CPG-support with modified oligonucleotide was washed with the reaction mixture several times by syringes. After every 45 min, the last operation was repeated. After 3−4 h, the reaction mixture was removed from the support, and columns were washed with DMF (2 × 0.5 mL) and CH_3CN (2 × 1 mL), and dried. In the cases of **ON12**−**14**, **ON21**, and **ON24**, CPG-supports were treated one more time with freshly prepared Sonogashira-coupling reaction mixture. Afterward, the 5′-DMT-on oligonucleotides were cleaved off from the solid support (room temperature, 2 h) and deprotected (55 °C, overnight) using 32% aqueous ammonia. Purification of 5′-*O*-DMT-on TINAs was accomplished using a reverse-phase semipreparative HPLC on a Waters Xterra MS C_{18} column. The ODNs were DMT deprotected in 100 μL 10% aq acetic acid (30 min), diluted with 32% aqueous ammonia (1 mL), and purified again on HPLC. Corresponding fractions with ODNs were evaporated and diluted with 1 M aq NaOAc (150 μL), and ODNs were precipitated from ethanol (550 μL). The modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite biospectrometry research station from PerSeptive Biosystems. The purity of the final TFOs was checked by ion-exchange chromatography using a LaChrom system from Merck Hitachi on a GenPak-Fax column (Waters).

Melting Temperature Measurements. Melting temperature measurements were performed on a Perkin-Elmer UV−vis spectrometer Lambda 35 fitted 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 cooled to room temperature, 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 temperature. The melting temperature (T_m, °C) was determined as the maximum of the first derivative plots of the melting curves obtained by measuring absorbance at 260 nm against increasing temperature (1.0 °C per min). A lower speed of increasing the temperature (0.5 °C per min) resulted in the same curves. Experiments were also done at 373 nm. All melting temperatures are within the uncertainty ± 0.5 °C as determined by repetitive experiments.

Fluorescence Measurements. Fluorescence measurements were performed on a Perkin-Elmer luminescence spectrometer LS-55 fitted with a Julabo F25 temperature controller. The triplexes and duplexes were formed in the same way as for T_m measurements except that only 1.0 μM of TFOs were used in all cases. The spectra were recorded at 10 °C in the buffer 20 mM sodium cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$ at pH 6.0.

Acknowledgment. The Nucleic Acid Center is funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

Note Added in Proof. TINA oligonucleotides and their monomeric phosphoramidites have been made commerical available from University of Southern Denmark after manuscript submission. See http://www.sdu.dk/Nat/Chem/INFO/DNAmonomers.pdf.

Supporting Information Available: MALDI-TOF MS and reverse-phase (DMT-on) and ion-exchange (DMT-off) HPLC analysis of oligonucleotides synthesized. This material is available free of charge via the Internet at http://pubs.acs.org.

JA053645D