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1-, 2-, and 4-Ethynylpyrenes in the Structure of Twisted Intercalating Nucleic Acids: Structure, Thermal Stability, and Fluorescence Relationship

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Abstract: A postsynthetic, on-column Sonogashira reaction was applied on DNA molecules modified by 2- or 4-iodophenylmethylglycerol in the middle of the sequence, to give the corresponding *ortho*- and *para*-twisted intercalating nucleic acids (TINA) with 1-, 2-, and 4-ethynylpyrene residues. The convenient synthesis of 2- and 4-ethynylpyrenes started from the hydrogenolysis of pyrene that has had the sulfur removed and separation of 4,5,9,10-tetrahydropyrene and 1,2,3,6,7,8-hexahydropyrene, which were later converted to the final compounds by successive Friedel–Crafts acetylation, aromatization by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, and a Vilsmeier–Haack–Arnold transformation followed by a Bodendorf fragmentation. Significant alterations in thermal stability of parallel triplexes and antiparallel duplexes were observed upon changing the attachment of ethynylpyrenes from *para* to *ortho* in homopyrimidine TINAs. Thus, for *para*-TINAs the bulge insertion of an intercalator led to high thermal stability of Hoogsteen-type parallel triplexes and duplexes, whereas Watson–Crick-type duplexes were destabilized. In the case of *ortho*-TINA, both Hoogsteen and Watson–Crick-type complexes were stabilized. Alterations in the thermal stability were highly influenced by the ethynylpyrene isomers used. This also led to TINAs with different changes in fluorescence spectra depending on the secondary structures formed. Stokes shift of approximately 100 nm was detected for pyren-2-ylethynylphenyl derivatives, whereas values for 1- and 4-ethynylpyrenylphenyl conjugates were 10 and 40 nm, respectively. In contrast with *para*-TINAs, insertion of two *ortho*-TINAs opposite each other in the duplex as a pseudo-pair resulted in formation of an excimer band at 505 nm for both 1- and 4-ethynylpyrene analogues, which was also accompanied with higher thermal stability.

Keywords: DNA structures · fluorescence · nucleic acids · thermal stability · triplex molecules

Introduction

Chemically modified nucleic acids are of considerable interest in the areas ranging from molecular biology and medicinal chemistry to bio- and nanotechnology. Covalent attachment of hydrophobic structures, known as intercalating moieties, is one of the attractive modifications of nucleic acids. Nowadays, intercalators can be introduced in any desired site of the sequence and, depending on the molecule's design, aromatic moieties can be positioned differently in the interior of DNA secondary structures. As a result of such research, we gain a deeper knowledge and understanding of nucleic acid structures, which is pivotal for future progress within nucleic acid technology and for the development of novel approaches in nanotechnology and drug design.

The pyrene residue has been studied a lot in nucleic acids thanks to its π–π stacking interactions with nucleobases (pyrene may occupy the area (220 Å^2) that is usually covered by two natural purine:pyrimidine base pairs (269 Å^2)[1]), its fluorescence sensitivity to the microenvironment (fluorescence is quenched in low polarity environments and increased in high polarity environments), and its ability to participate in charge-transfer complexes[2–5] (see reference [6] on electron transfer processes in DNA). An-

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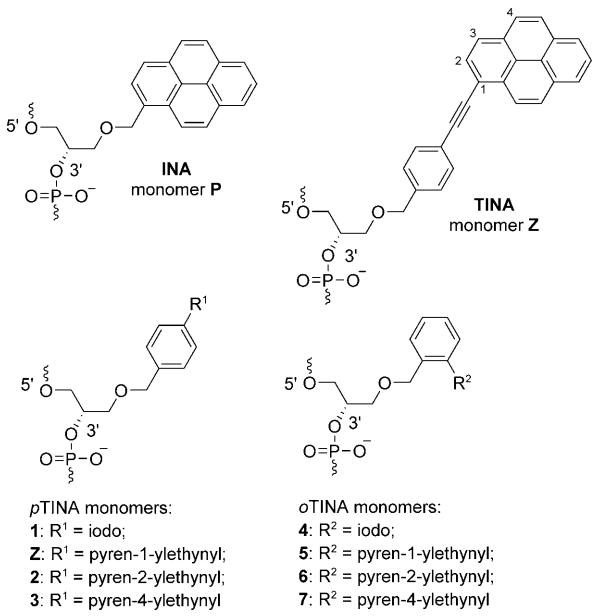
other important feature of pyrene is the formation of an excimer, when two fluorescent molecules are positioned in a close proximity to each other (≈3.4 Å). This is of particular importance for studies of biological supramolecular molecules. Pyrene is widely used in the design of probes for recognition of DNA,[7–10] RNA[11–14] sequences, DNA duplexes,[15–18] as well as for the detection of point mutations in DNA (see reference [19]). Recently, several groups reported the synthesis of nucleic acid structures with continuous helical arrangement of several pyrene moieties, which resulted in the formation of the strong excimer band.[20–26] As highlighted in several articles, the spectroscopic properties of alkynylpyrene analogues are derived from those of the parent pyrene.[27–30] Thus, substitutions of pyrene at positions 1, 3, 6, and/or 8 via an acetylene bridge leads to a bathochromic shift in the absorption/emission spectra accompanied by high fluorescence quantum yields, even in the presence of oxygen.[27–29,31] Shifts to the longer wavelengths in the fluorescence spectra are especially useful in molecular biology applications because biomolecules can also be excited upon irradiation at the same wavelength as pyrene.[27]

Introduction of a slightly modified intercalating moiety into a DNA structure can lead to significant changes in properties. Recently, we have developed two types of pyrene-containing nucleic acids that exhibit a totally different thermal-stability gain depending on the DNA secondary structure. Bulged insertions of (*R*)-1-*O*-(1-pyrenylmethyl)-glycerol (**P**, Scheme 1) in the middle of oligonucleotides (ONs), known as intercalating nucleic acids (INAs), resulted in significantly increased affinities towards complementary single-stranded (ss) DNA, whereas INA/RNA duplexes and the Hoogsteen-type triplex and duplex were destabilized.[8,32] In contrast to INAs, the extraordinary high thermal stability of Hoogsteen-type triplexes and duplexes was observed upon bulge insertions of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (monomer **Z**) into the middle of homopyrimidine ONs. At the same time, Watson–Crick-type duplexes of an identical nucleotide content possessing monomer **Z** were destabilized.[15] It was assumed that the pyrene moiety of monomer **Z** is situated in the double-stranded (ds) DNA part of the triplex, whereas the phenyl is supposed to coaxially stack with the nucleobases of the triplex-forming oligonucleotides (TFOs). A twist around the triple bond allows the phenyl and pyrene groups to fit properly inside the triplex, promoting stabilizing stacking interactions. Because of these interactions, such phenylethynylpyrene intercalators have been called twisted intercalating nucleic acids (TINAs).[15] We were curious therefore, as to the effect of different attachments of pyrene on binding affinity and spectral properties of the TINA conjugates. Recently, a 2-pyrenyl residue was attached to the 5-position of 2′-deoxyuridine and compared with the 5-(1-pyrenyl)-2′-deoxyuridine in the DNA duplex.[33] Herein, we present the synthesis, thermal stability, and fluorescence properties of TINAs possessing 1-, 2-, and 4-ethynylpyrene residues at the *para* or *ortho* positions of (*R*)-1-*O*-phenylmethylglycerol (*p*TINA and *o*TINA, respectively, Scheme 1) and compare them with the properties of initial INA and TINA molecules.

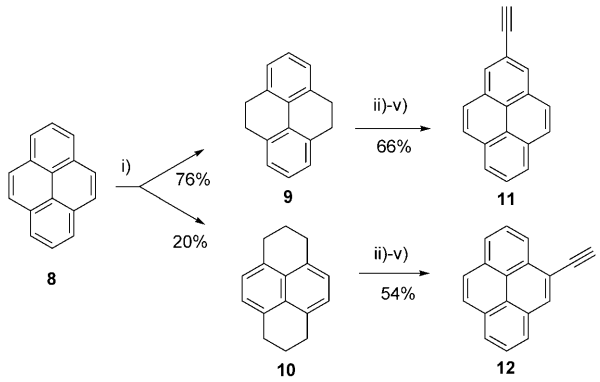


Scheme 1. Structure of intercalating nucleic acids with pyrene and phenylethynylpyrene moieties.

Results and Discussion

The primary evaluation of aromatic structures in nucleic acids is easier to perform by using postsynthetic oligonucleotide modification, which is less time-consuming than the routine preparation of several pseudo-nucleoside phosphoramidites. Previously, the palladium(0)-catalyzed Sonogashira reaction was successfully applied in derivatization on a solid support of ONs with 5-iodopyrimidines,[34,35] 8-bromopurines,[36,37] as well as 4-iodobenzyl- and 4-ethynylbenzylglycerols.[15,38] The conversion during the Sonogashira coupling was found to be dependent on Pd catalysis and on the reactivity of aromatic iodides and acetylenes. Importantly, no side reactions were observed for native nucleobases with protective groups.[34,35] Herein we decided to investigate different isomers of phenylethynylpyrene in the structure of TINA through the introduction of 1-, 2-, or 4-ethynylpyrenes into the *para*- or *ortho*- positions of (*R*)-1-*O*-phenylmethylglycerol inserted in ONs (Scheme 1). We assumed that placing ethynylpyrene into the *ortho* position could lead to TINAs, which should have properties similar to INAs, that is, increased binding affinity towards ssDNA but decreased affinity towards ssRNA and dsDNA. At the same time, more-attractive fluorescence properties might also be obtained.

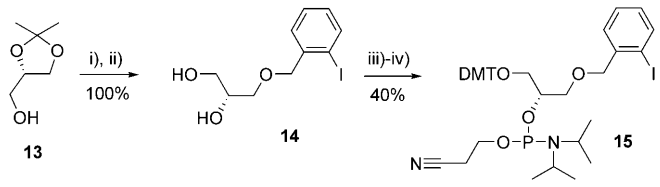
Synthesis of 2- and 4-ethynylpyrenes and their incorporation into ONs: 2- and 4-Substituted pyrenes are not readily available owing to the fact that electrophilic substitution on pyrene (**8**, Scheme 2) is directed to the electron-rich posi-



Scheme 2. Reagents and conditions: i) H_2 (160 atm), 10% Pd/C, EtOAc, 60°C, 24 h; ii) Ac_2O, $AlCl_3$, CH_2Cl_2, 5°C; iii) 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), toluene, 110°C, 1 h; iv) DMF, $POCl_3$; v) KOH, dioxane, reflux, 2 h.

tion 1.[39] Pyrene derivatives substituted in positions 2[40–43] and 4[44,45] can be prepared from 4,5,9,10-tetrahydropyrene (**9**) and 1,2,3,6,7,8-hexahydropyrene (**10**), respectively, by electrophilic substitution followed by aromatization. Although **10** is commercially available, it is rather expensive. Tetrahydropyrene **9** can be prepared by Pd/C hydrogenolysis of commercial pyrene that has to be purified by column chromatography on silica[43] or have the sulfur removed over Raney nickel[42] prior to the reaction. In our experiments, the hydrogenolysis of the pyrene that has had the sulfur removed by Raney nickel gave a mixture of **9** and **10** (≈3.5:1). We found that the mixture is easily separable by chromatography on aluminum oxide. Compounds **9** and **10** were converted into the corresponding 2-ethynylpyrene (**11**) and 4-ethynylpyrene (**12**) by using successive acetylation, aromatization, Vilsmeier–Haack–Arnold transformation, and Bodendorf fragmentation (Scheme 2), a reaction sequence recently used for the synthesis of 1-ethynylpyrene.[46] The method affords ethynylpyrenes **11** (66% yield from **9**) and **12** (54% from **10**) and is more convenient in comparison with the previously published procedures for 2- and 4-ethynylpyrenes.[47–50]

The phosphoramidite **15** required for the preparation of modified ONs was prepared in four steps from 2-iodobenzylbromide and (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (**13**) in 40% overall yield by using a similar route to that described earlier for the phosphoramidite of 4-iodobenzylglycerol[15] (Scheme 3). After completion of the DNA synthesis without the final deprotection of the 4,4-dimethoxytrityl group (DMT on ONs), the control-pore glass (CPG) supports with ONs possessing iodophenyl moieties were treated with a Sonogashira-coupling reagent mixture containing $[Pd(PPh_3)_4]$ (7.5 mM), CuI (7.5 mM), and the corresponding ethynylpyrene (22.5 mM) in dry *N*,*N*-dimethylformamide (DMF)/Et_3N (3.5/1.5, 500 μL) in 1 mL syringes under dry conditions at RT. Plastic syringes and solvents were flushed with argon prior to the reaction. After the coupling reaction (3–4 h), the CPGs were flushed with DMF (2×2 mL) and CH_3CN (2×2 mL) and then dried by evaporation under a stream of argon. The treatment with the Sonogashira reaction mixture was repeated for ONs possessing double insertions of the TINA-precursor monomers and for couplings with 2-ethynylpyrene. Then ONs were cleaved from the CPG support with 32% NH_4OH (2 h) and deprotected at 55°C (overnight). Semipreparative HPLC on a C_{18} column was used to isolate the target TINA oligonucleotides, which had longer retention times than unreacted ONs. After the separation, DMT-on oligonucleotides were treated with 80% aqueous AcOH (20 min), purified again on HPLC, and precipitated from ethanol. The composition was verified by MALDI-TOF and purity was found to be over 90% using ion-exchange chromatography analysis. Conversion of ONs during the Sonogashira reaction by using 1- and 4-ethynylpyrenes was found to be 80–85% for homopyrimidine sequences and 50–60% for sequences possessing purines. This is in agreement with our previous observations.[15] However, in the case of 2-ethynylpyrene, we observed a considerable amount of sparingly soluble by-product of Glaser oxidative dimerization that was formed during the reaction, which resulted in a very low conversion (<50%) and yield for the final ONs, especially in the presence of purines in the sequence. We have previously seen, through our first attempts at performing the Sonogashira reaction on oligonucleotides, that formation of 1,4-bis(1-pyrenyl)buta-1,3-diyne in the presence of atmospheric oxygen leads to complete cleavage of DNA, so no oligonucleotide sequences were observed during HPLC purification after the reaction. This can be explained by DNA cleavage in the presence of O_2 and copper ions.[51]



Scheme 3. Reagents and conditions: i) 2-iodobenzylbromide, KOH, toluene, reflux; ii) 80% aq. CF_3COOH, RT; iii) DMTCl, pyridine, RT; iv) $NC(CH_2)_2OP(NiPr_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 0°C to RT, overnight.

Thermal stabilities of the modified duplexes and triplexes: The thermal stability of parallel triplexes, DNA/DNA, and DNA/RNA duplexes with the synthesized ONs was assessed by thermal denaturation experiments. The melting temperatures (T_m °C) determined as the first derivatives of the melting curves are listed in Tables 1 and 2.

Parallel triplexes and duplexes were still stabilized when the attachment of ethynylpyrenes was changed from the *para* (**ON2**–**ON4**, *p*TINA, Table 1) to *ortho* position (**ON6**–**ON8**, *o*TINA) in comparison with unmodified sequences at pH 6.0 (**ON1** toward **D1** and **ON11**, respectively). However, the increase in thermal stability was lower for *o*TINA than for *p*TINA. Having 1-ethynylpyrene in the structure of the TINA at both *para* (*p*TINA) and *ortho* (*o*TINA) positions

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

No.		Parallel triplex[a] 3′-CTGCCCCTTTCTTTTTT 5′-GACGGGGAAAGAAAAAA (**D1**)		Parallel duplex[b] 5′-GACGGGGAAAGAAAAAA (**ON11**)	Antiparallel duplex[c] 3′-GGGGAAAGAAAAAA (**ON12**)
		pH 6.0	pH 7.2	pH 6.0	pH 6.0
ON1	5′-CCCCTTTCTTTTTT	27.5	<5.0	19.0	48.0
ON2[d]	5′-CCCCTT**Z**TCTTTTTT	46.0	28.0	33.5	46.5
ON3[e]	5′-CCCCTT**2**TCTTTTTT	40.0	18.5	24.5	44.5
ON4[e]	5′-CCCCTT**3**TCTTTTTT	44.5	23.5	28.5	45.0
ON5[d]	5′-CCCCTT**Z**T**Z**CTTTTTT	40.0	<5.0	26.5	41.0
ON6[f]	5′-CCCCTT**5**TCTTTTTT	37.5	15.0	21.5	51.0
ON7[f]	5′-CCCCTT**6**TCTTTTTT	30.5	<5.0	21.0	50.5
ON8[f]	5′-CCCCTT**7**TCTTTTTT	30.0	7.5	19.0	50.0
ON9[f]	5′-CCCCTT**5**T**5**CTTTTTT	29.5	<5.0	25.0	45.0
ON10[f]	5′-CCCCTT**7**T**7**CTTTTTT	23.5	<5.0	21.5	43.5

[a] Concentration of ONs = 1.5 μM of **ON1**–**ON10** 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] $C = 1.0$ μM of each strand in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0. [d] See reference [15]. [e] Prepared by a Sonogashira reaction mixture with ON-possessing monomer **1**: $[Pd(PPh_3)_4]$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [f] Prepared by a Sonogashira reaction mixture with ON possessing monomer **4**: $[Pd(PPh_3)_4]$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h.

Table 2. T_m [°C] data for antiparallel duplex[a] melting, taken from UV-melting curves ($\lambda = 260$ nm).

		DNA 5′-AGCTTGCTTGAG (**ON21**)	DNA 5′-AGCTTG**X**CTTGAG X = **P**[b] (**ON22**)	X = **Z**[c] (**ON23**)	X = **2**[c] (**ON24**)	X = **3**[c] (**ON25**)	X = **5**[d] (**ON26**)	X = **7**[d] (**ON27**)	RNA 5′-AGCUUGCUUGAG (**ON28**)
ON13	3′-TCGAACGAACTC	46.0	48.0	32.0	29.5	33.5	44.5	–	40.5
ON14[b]	3′-TCGAAC**P**GAACTC	49.5	43.0	–	–	–	–	–	38.0
ON15[c]	3′-TCGAAC**Z**GAACTC	38.0	–	36.0	–	–	–	–	30.5
ON16[c]	3′-TCGAAC**2**GAACTC	37.0	–	–	33.5	–	–	–	30.5
ON17[c]	3′-TCGAAC**3**GAACTC	39.5	–	–	–	39.5	–	–	32.5
ON18[d]	3′-TCGAAC**5**GAACTC	46.5	–	–	–	–	42.0	–	34.0
ON19[d]	3′-TCGAAC**6**GAACTC	46.5	–	–	–	–	–	–	33.5
ON20[d]	3′-TCGAAC**7**GAACTC	47.5	–	–	–	–	–	45.5	36.0

[a] Concentration of ONs = 1.0 μM of each oligonucleotide in 140 mM NaCl, 10 mM sodium phosphate buffer solution, 1 mM EDTA, pH 7.0. [b] See reference [52]. [c] Prepared by a Sonogashira reaction mixture with ON-possessing monomer **1**: $[Pd(PPh_3)_4]$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h. [d] Prepared by a Sonogashira reaction mixture with ON-possessing monomer **4**: $[Pd(PPh_3)_4]$ (7.5 mM), corresponding acetylene (22.5 mM), CuI (7.5 mM), dry DMF/Et_3N (3.5/1.5, 500 μL), 3 h.

was the most beneficial for Hoogsteen base pairing among all of the ethynyl pyrenes studied in each series. It is an interesting observation that changing the attachment of pyrene from position 1 to 2 in *p*TINA resulted in a considerable drop of triplex thermal stability from 46.0 to 40.0 °C and from 28.0 to 18.5 °C at pH 6.0 and 7.2, respectively. T_m values for 4-ethynylpyrene (**ON4**) were in the range between 1- and 2-ethynylpyrene derivatives. In all cases, destabilization of Watson–Crick-type duplexes was observed for *p*TINA ($\Delta T_m = -1.5 \cdots -3.5$ °C, **ON2**–**ON4**/**ON12**). Contrary to that, a bulge insertion of *o*TINA monomers resulted in the stabilization of antiparallel duplexes, with the highest value being obtained for the 1-ethynylpyrene derivative ($\Delta T_{m(\mathbf{ON6}/\mathbf{ON12}-\mathbf{ON1}/\mathbf{ON12})} = +3.0$ °C). Our expectations that attachments of ethynylpyrenes in the *ortho* position of TINAs would provide a molecule with the ability to induce thermal stabilization were correct. Indeed, bulge insertion of *o*TINA into homopyrimidine sequences led to increased thermal stability of both Hoogsteen- and Watson–Crick-type complexes, whereas *p*TINA showed discriminative binding to Hoogsteen-type duplexes and triplexes over Watson–Crick-type duplexes. It is impressive to see the difference in thermal stability observed for different ethynylpyrenes. Thus, 1-ethynylpyrene in the structure of *o*TINA (**ON6**) showed higher stabilization for the parallel triplex and duplex in comparison with its isomers (**ON7** and **ON8** toward **D1** and **ON11**), whereas for antiparallel duplexes, the T_m values for all ethynylpyrenes were of the same range (**ON6**–**ON8**/**ON12**). For all substituents, bulged insertion of the second TINA monomer in the sequence as a next-nearest neighbor (**ON5**, **ON9**, **ON10**) resulted in decreased thermal stability in comparison with sequences possessing only one bulged intercalator (**ON2**, **ON6**, and **ON8**, respectively). This could be due to the large interruption of the double and triple helices by two bulged (*R*)-1-*O*-methylglycerol linkers positioned very close to each other. We have previously seen that duplexes and triplexes were more stable if two insertions of the monomer **Z** were separated by two or three nucleobases.[15]

As was expected from the previous data for monomer **Z**,[15] we observed a considerable drop in T_m values for all mixed purine/pyrimidine antiparallel duplexes with bulged *p*TINA monomers (**ON15**–**ON17** toward **ON21** and **ON28**, Table 2) as compared with the wild-type duplexes (**ON13**/**ON21** and **ON13**/**ON28**). Changing the position of ethynylpyrenes in TINAs from *para* to *ortho* led to ONs with discriminative binding: duplexes with complementary DNA (**ON21**) were slightly stabilized, whereas duplexes with RNA (**ON28**) were strongly destabilized. Thus, *o*TINA in the context of mixed purine/pyrimidine sequences showed properties similar to INAs (**ON14**) though a thermal stability gain for DNA/DNA duplexes was lower for *o*TINA. The latter also resulted in a slightly destabilized duplex **ON26**/**ON13**, whereas the duplex-containing INA monomer showed higher thermal stability (**ON22**/**ON13**) than the reference (**ON13**/**ON21**). All duplexes with INA and TINA monomers that interacted with RNA targets were destabilized. We have recently observed that placing two INA monomers opposite each other in the duplex led to the formation of so-called easily denaturing nucleic acids[53,54] in which each of the INA sequences has a preference in binding to the complementary unmodified DNA of the same length rather than forming a duplex with each other.[26,52,53] A similar tendency was observed for *o*TINA with 1-ethynylpyrene, that is, the duplex with an inserted pair of **5** (**ON18**/**ON26**) had lower T_m (42.0 °C) than duplexes with the complementary DNA molecules (**ON18**/**ON21** and **ON26**/**ON13** with T_m of 46.5 and 44.5 °C, respectively). None of the duplexes with a bulged pair of intercalators in the middle of the sequence showed an increased thermal stability in comparison with the wild-type duplex. However, one duplex, **ON20**/**ON27**, showed a T_m value of 45.5 °C, which is very close to the T_m of the unmodified duplex **ON13**/**ON21** (T_m = 46.0 °C). The T_m value for the duplex **ON16**/**ON24** of 33.5 °C was the lowest among the studied duplexes with pyrenes positioned across from each other. This observation along with the fluorescence properties of these duplexes discussed below highlights the importance of the proper design of intercalating moieties as a pseudo-pair in the middle of the DNA duplex.

UV and fluorescence measurements: The introduction of the fluorescent labels into DNA was also confirmed by the appearance of extra maxima peaks in the UV/Vis spectra in the range 300 to 400 nm in addition to the absorption maxima at approximately 260 nm, which is the peak location inherent to ONs (Figure 1). As expected, differences in the UV/Vis spectra were observed between ethynylpyrenes (Figure 1 A) and their corresponding phenylethynylpyrene isomers in the DNA structures **ON18**–**ON20** (Figure 1 B). The long-wave maxima at 357 and 339 nm observed for 1-ethynylpyrene were shifted to 336 and 321 nm for 2- and 4-ethynylpyrenes, respectively (Figure 2 A). For **ON18** possessing pyren-1-ylethynylphenyl residue we observed two peaks at 371 and 395 nm, which is in agreement with previous observations for DNA molecules with 4-(pyren-1-ylethynyl)-

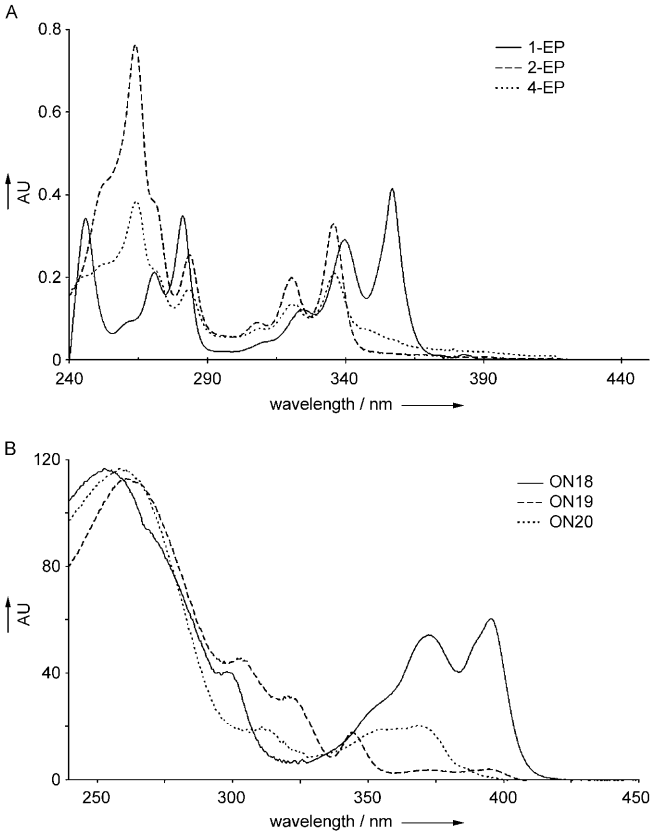


Figure 1. A) UV/Vis absorption spectra of 1-, 2-, and 4-ethynylpyrenes (1-, 2-, and 4-EP) in 0.4% DMSO/ethanol (v/v), concentration of each compound 10^{-5} M. B) UV/Vis absorption spectra of single-stranded ONs with 1-, 2-, and 4-ethynylpyrenes in the structure of *o*TINA at 1.0 μM in a buffer solution at 10 °C (140 mM NaCl, 10 mM sodium phosphate buffer solution, 1 mM EDTA, pH 7.0). DMSO = dimethyl sulfoxide, EDTA = ethylenediaminetetracacetic acid.

phenyl.[15,28] A blue shift of approximately 20 nm and lower intensity in absorbance were detected for **ON20** in comparison with **ON18** with 4- and 1-ethynylpyrene, respectively. Taking into account the similarity of the UV spectra for 2- and 4-ethynylpyrenes above 300 nm, one could expect the UV profiles for **ON19** and **ON20** to also be similar. However, considerably lower absorbance in the region of 350–400 nm and several shoulders at 302, 320, and 343 nm were observed in UV spectrum of single-stranded **ON19** with 2-ethynylpyrene. Comparing these results with the UV spectra of the non-nucleoside model compounds with 4-(pyren-1(2 or 4)-ylethynyl)benzyl residues (unpublished results, see the Supporting Information), we suspect that the through-space interactions between pyren-2-ylethynylphenyl and the surrounding nucleobases led to the decrease in the absorbance between 350 and 400 nm for **ON19**. A more-detailed conclusion might be obtained only upon insertion of these model compounds into the structure of nucleosides and then into oligonucleotide sequences, which is a subject of our ongoing studies. In any case, the considerable changes in the UV/Vis absorbance spectra upon substitution of pyrene as an individual monomer at different positions in the DNA se-

quence represents a difference in the electronic structure of these dyes.

Next, we studied the changes in the fluorescence spectra upon formation of a variety of complexes of ONs with phenylethynylpyrene isomers with complementary strands in a buffer solution. Profiles for fluorescence excitation and emission spectra, as already expected from the UV spectra, were different for each ethynylpyrene isomer. An excitation maximum for the 1-ethynylpyrene analogue **ON18** upon observation of the emission at 405 nm was detected at 392 nm with a peak of lower intensity at 373 nm (Figure 2). Two

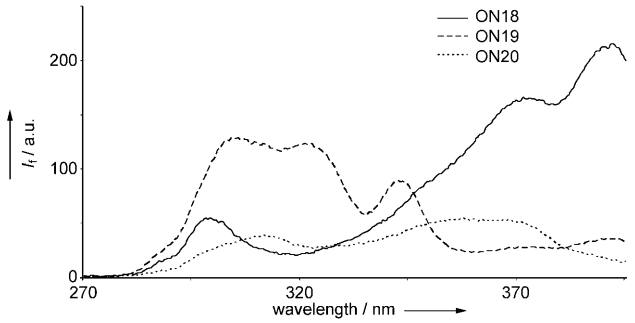


Figure 2. Fluorescence excitation spectra of single-stranded ONs with 1-, 2-, and 4-ethynylpyrenes in the structure of *o*-TINA. Measurement conditions: 1 μM of each strand in a buffer solution at 20 °C (140 mM NaCl, 10 mM sodium phosphate buffer solution, 1 mM EDTA, pH 7.0), emission 405 nm (emission slit 0.0 nm), excitation 270–395 nm (excitation slit 4.0 nm). I_f = fluorescence intensity.

other ethynylpyrene derivatives showed their maxima at shorter wavelengths. Thus, excitation maxima for 2-ethynylpyrene *o*TINA (**ON19**) were detected at 304, 321, and 343 nm. A very broad peak with a plateau in between 350 and 370 nm was observed for the 4-ethynylpyrene analogue **ON20**. From a practical point of view and taking into account the excitation spectra of **ON18–ON20**, exceptionally large Stokes shifts of pyren-2-ylethynylphenyl (ca. 100 nm) were calculated as the difference between positions of the band maxima of the absorption and emission spectra. Values for 1- and 4-ethynylpyrene derivatives are 10 and 40 nm, respectively. Large Stokes shifts may result from excitation into higher energy electronic states followed by a relaxation to the common emitting states of phenylethynylpyrenes. The position of substituents on the pyrene ring determines the nature of the higher-energy electronic states. For convenience, we performed all fluorescence emission spectra upon excitation at 373 nm, where all analogues absorb energy to differing extents. In the fluorescence emission spectra of 2-ethynylpyrenyl derivatives, we observed a shoulder at 445–465 nm in addition to the monomeric fluorescence maxima at 405 and 425 nm (**ON3**, **ON7**, **ON16**, and **ON19**; Figure 3 and Figure 4). The latter maxima are similar to those of ssONs with 1-ethynylpyrene. Spectra of ssONs containing 4-ethynylpyrene are characterized by a broad peak of low intensity in the range of 380–430 nm (**ON4**, **ON8**, **ON17,** and **ON20**, Figure 3, Figure 4). Upon addition of the complementary strands, the fluorescence intensity of labeled ONs changed depending on the phenylethynylpyrene isomer used and the secondary DNA structure formed. A red shift of 3–5 nm and an increase in fluorescence intensity were observed upon formation of parallel triplexes/duplexes and antiparallel duplexes for both *para*- and *ortho*-TINAs with 1- and 2-ethynylpyrenes in comparison with ssONs (Figure 3 A–D). For homopyrimidine ONs possessing 1- and 4-ethynylpyrenes, a considerably higher fluorescence intensity was observed upon antiparallel duplex formation relative to the parallel triplexes and duplexes, except for *o*TINA with 4-ethynylpyrene (Figure 3 A, B and 4 E, F). In the latter case, both duplexes had approximately the same fluorescence intensity (Figure 3 F). For 4-ethynylpyrene derivatives, a high increase in fluorescence was also accompanied by formation of more discrete peaks at 395 and 411 nm (Figure 3 E, F). The range of these values is lower than the range of monomeric fluorescent peaks of 1- and 2-ethynylpyrenes and higher than those of the parent pyrene in the INA structure (380 and 405 nm[8]).

The formation of Watson–Crick duplexes by purine–pyrimidine sequences with TINA isomers resulted in a different fluorescence response in comparison with the homopyrimidine sequences described above. Thus, only marginal changes in the fluorescence spectra were observed for 1-ethynylpyrene *p*TINA (**ON15**) upon hybridization with complementary DNA and RNA and with an ON with the same dye as the pseudo-pairs (Figure 4 A), whereas formation of the antiparallel duplex **ON2**/**ON12** induced an increase in fluorescence intensity (Figure 2 A). The same was valid for 4-ethynylpyrene derivatives (Figure 3 E and F and Figure 4 E and F), which represents the influence of the oligonucleotide content on changes in the fluorescence spectra. For 2-ethynylpyrene derivatives, a more than three-fold increase in fluorescence was observed for *p*TINA upon formation of the duplex with complementary DNA and RNA (Figure 4 C), whereas no changes in fluorescence intensity could be seen for *o*TINA (Figure 4 D). Notably, even in the single-stranded form, TINA monomers responded differently to the sequence contents.

Marginal changes in fluorescence spectra were observed for 1-ethynylpyrene analogues of *p*TINA (**ON15** and **ON23**), while almost a fourfold difference in intensity was detected between **ON16** and **ON24** possessing 2-ethynylpyrene. Taking into account that the next nearest neighbors in **ON15** and **ON16** are purines and not pyrimidines as in **ON23** and **ON24**, we suspect that purines might result in a more effective stacking of the monomer **2** with the neighboring and/or next nearest neighboring purines than with the monomer **Z**. Another explanation might be the different response of monomers **2** and **Z** to the redox potentials of the neighboring nucleobases in conjunction with the flanking sequences. This is also the case for *o*TINA analogues with 1- and 4-ethynylpyrene, as higher fluorescence intensities were observed for the sequence 5′-AAG**X**CAA-3′ than for the sequence 5′-TTG**X**CTT-3′.

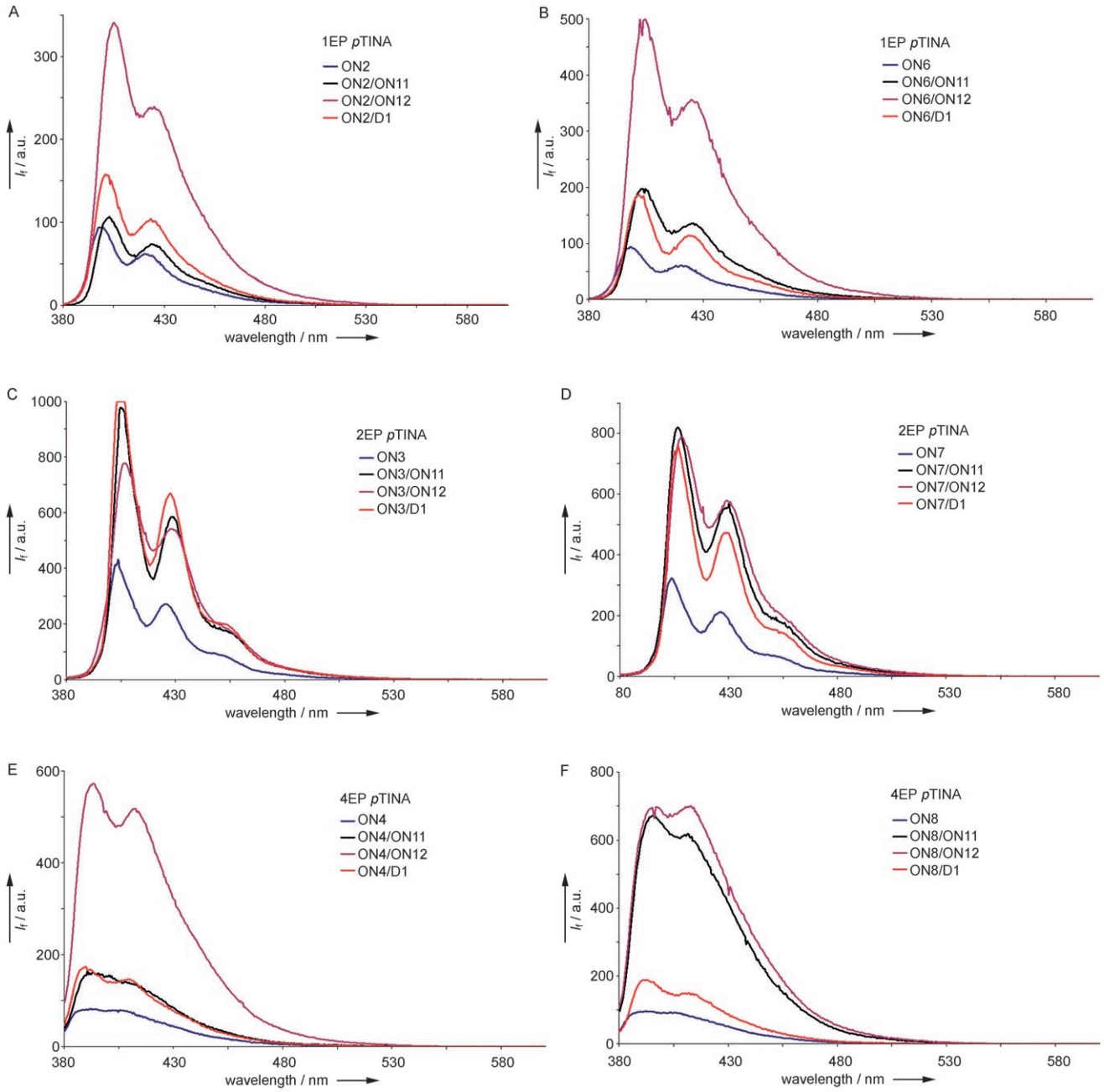


Figure 3. Fluorescence emission spectra of single-strands, antiparallel and parallel duplexes, and parallel triplexes formed by homopyrimidine ONs with 1-ethynylpyrene (1EP, Figures 2A and B), 2-ethynylpyrene (2EP, Figures 3C and D), and 4-ethynylpyrene (4EP, Figures 3E and F) in the structure of *para*-TINA (*p*TINA, Figures 3A, C, and E) and *ortho*-TINA (*o*TINA, Figures 3B, D, and F). Measurement conditions: 1 μM of each strand in a buffer solution 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 C, D, E, and F and 0.0 nm for A and B).

None of the ethynylpyrene derivatives of *p*TINA displayed formation of an excimer in duplexes with bulged identical dyes positioned opposite each other in the middle of the duplex (**ON15/ON23**, **ON16/ON24**, **ON17/ON25**), which is in agreement with previous observations for 4-(pyren-1-ylethynyl)phenyl dyes.[15,28] However, upon changing the attachment of ethynylpyrenes from *para*- to *ortho*-TINA, the excimer band was detected at approximately 505 nm for 1- and 4-ethynylpyrene dyes (Figure 4B and F, respectively). Interestingly, the ratio between monomeric and excimer fluorescence bands was different for these two dyes in the duplex structure, that is, monomeric fluorescence had a higher intensity than the excimer band for 1-ethynylpyrene *o*TINA, whereas for 4-ethynylpyrene *o*TINA, it was opposite. There is also a correlation between the thermal stability and the formation of an excimer band. Thus, T_m values of duplexes showing an excimer band (**ON14/ON22**,[52,53] **ON18/ON26**, and **ON20/ON27**) are higher than

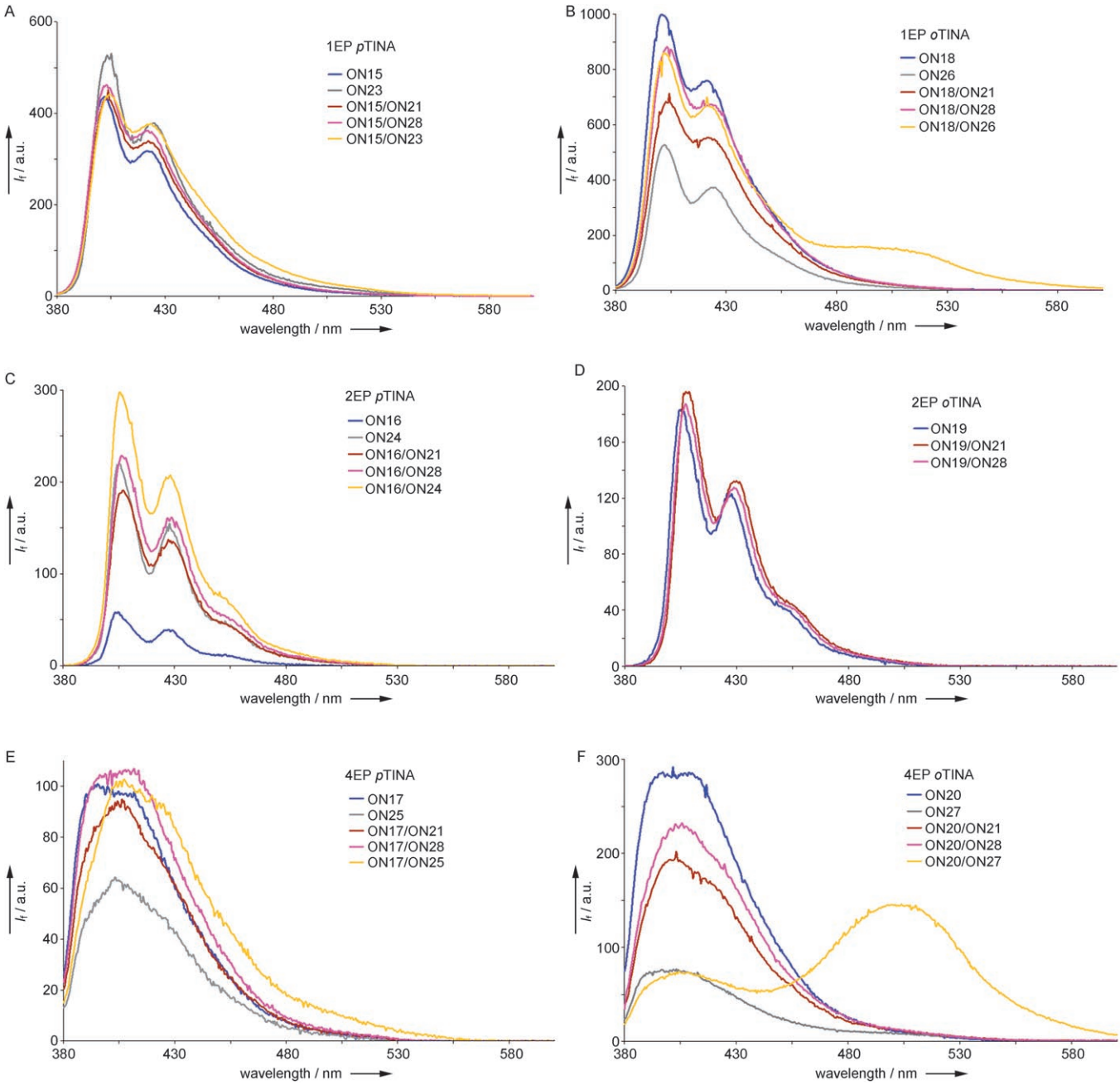


Figure 4. Fluorescence emission spectra of single strands and antiparallel duplexes formed by mixed purine–pyrimidine ONs with 1-ethynylpyrene (1EP, Figures 4A and B), 2-ethynylpyrene (2EP, Figures 4C and D) and 4-ethynylpyrene (4EP, Figures 4E and F) in the structure of *para*-TINA (*p*TINA, Figures 4A, C, and E) and *ortho*-TINA (*o*TINA, Figures 4B, D, and F). Measurement conditions: 1 μM of each strand in a buffer solution at 10 °C (140 mM NaCl, 10 mM sodium phosphate buffer solution, 1 mM EDTA, pH 7.0), excitation 373 nm (excitation slit 4.0 nm), emission 380–600 nm (emission slit 2.5 nm for A, B, E, and F and 0.0 nm for C and D).

the T_m values of duplexes with no excimer band in fluorescence spectra (**ON15**/**ON23**, **ON16**/**ON24**, and **ON17**/**ON25**). This means that proper interstrand overlapping/stacking of pyrene residues as a pseudo-pair in the middle of the DNA duplex results in the formation of an excimer band. This gives the thermodynamic gain that compensates for the distortion of the backbone arising from the use of a flexible glycerol linker. Owing to the difficulties with derivatization of ONs by 2-ethynylpyrene, we could not obtain an ON that was intended to be a pair for **ON19**. Previously, formation of an excimer as a result of interstrand stacking interactions of pyrenes in the core of a DNA duplex was observed for INA (monomer **P**) at 480 nm (λ_{ex} = 340 nm[53]) and for bis-substituted 1,8-pyrene at 493 nm (λ_{ex} = 354 nm[21]). Next, an interstrand pyrene excimer band was found to be useful in signaling full DNA complementarity[53,55–57] and as evidence of the assembly of intermolecular DNA triplexes in which a bis-substituted 1,8-pyrene deriva-

tive extended π–π stacking between two DNA triplexes.[16] An excimer band was also observed upon insertion of more than one dye into the DNA strand in close proximity to each other. For 4-(pyren-1-ylethynyl)phenyl derivatives, a strong pyrene excimer at approximately 505 nm was observed for ssONs with two intercalators inserted as neighbors[58] and as next-nearest neighbors.[15] A relatively higher excimer band was observed for ssON with *p*TINA monomers as next-nearest neighbors in comparison with the *o*TINA analogues (**ON5** and **ON9**, Figure 4A and B). Interestingly, in contrast with phenylethynylpyrenes, there is no typical red-shifted emission for pyrene excimers of 5-(pyren-1-ylethynyl)-2′-deoxyuridine, which exhibits only a moderate red shift in its fluorescence spectra from 455 nm for a single insertion to 467 nm when placing two fluorescent nucleotides as neighbors or next-nearest neighbors.[59,60]

As we have already seen for *para*- and *ortho*-TINAs with 1-ethynylpyrene, formation of the antiparallel duplex from homopyrimidine probes with two TINAs as next-nearest neighbors (**ON5** and **ON9** toward **ON12**, Figure 5C and D,

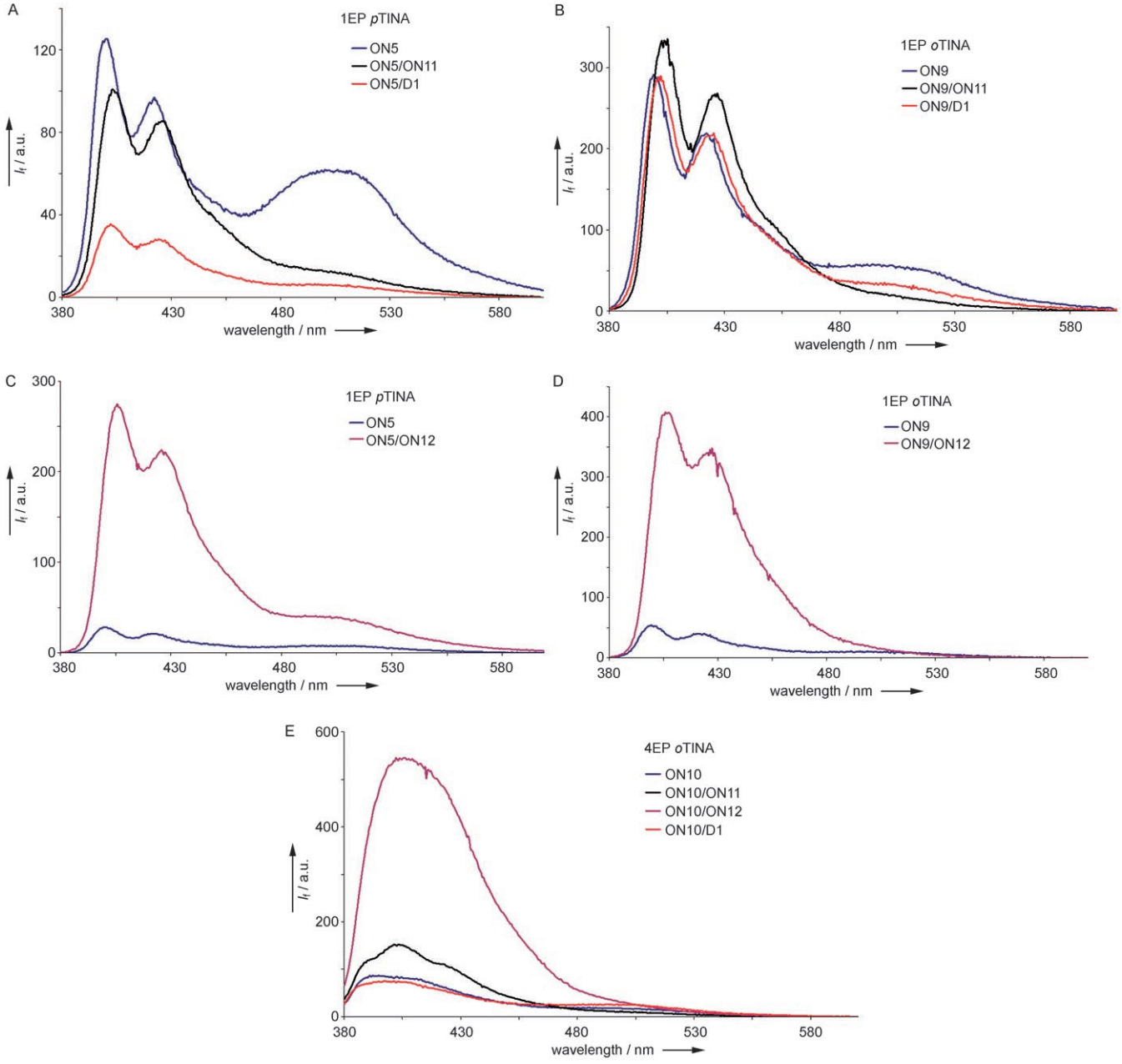


Figure 5. Fluorescence emission spectra of single strands, antiparallel and parallel duplexes, and parallel triplexes formed by homopyrimidine ONs with two dyes as next-nearest neighbours, that is, 1-ethynylpyrene (1EP, Figures 5A–D), and 4-ethynylpyrene (4EP, Figures 5E) in the structure of *para*-TINA (*p*TINA, Figures 5A and C) and *ortho*-TINA (*o*TINA, Figures 5B, D, and E). Measurement conditions: 1 μM of each strand in a buffer solution 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, and E and 0.0 nm for C and D). **ON5** and **ON9** were used as references in spectra recorded under different conditions.

respectively) also resulted in considerable fluorescence enhancement. Interestingly, it was accompanied with the disappearance of an excimer band for *o*TINA, whereas it still remained visible in the case of *p*TINA. This could mean that after duplex formation, no or little overlapping of pyrene was possible when 1-ethynylpyrene was positioned in the *ortho* position. Remarkably, an increase in fluorescence intensity and the disappearance of an excimer band were observed upon formation of an antiparallel duplex for 4-ethynylpyrene *o*TINAs as next-nearest neighbors. The excimer signal was, however, slightly higher for the triplex relative to the intensity of the single-stranded probe, which is contrary to that found for 1-ethynylpyrene-labeled *o*TINA. Another interesting observation was the formation of the peak at 403 nm for the parallel duplex **ON10**/**ON11** (Figure 5E). In general, the fluorescence properties of 4-ethynylpyrene derivatives in the DNA structure differ from those of 1- and 2-ethynylpyrenes and from the parent pyrene. However, formation of different complexes with complementary DNA molecules had a different effect on the fluorescence properties. This means that derivatives of 4-ethynylpyrene, as well as pyrene and 1-ethynylpyrene, are sensitive to microenvironmental changes. Low fluorescence intensity and unstructured peaks of monomeric fluorescence for single-stranded ONs and Watson–Crick duplexes with 4-ethynylpyrene might be a result of efficient charge transfer, which could be even more efficient than for 1-ethynylpyrene derivatives.[61]

Conclusion

By using the Sonogashira-type postsynthetic modification of ONs with either (*R*)-1-*O*-(4-iodophenyl)methylglycerol or (*R*)-1-*O*-(2-iodophenyl)methylglycerol, we screened twisted intercalating nucleic acids (TINAs) with 1-, 2-, and 4-ethynylpyrenes for their ability to increase the thermal stability of Hoogsteen-type triplexes/duplexes and Watson–Crick-type duplexes. We found that the bulged insertion of *p*TINA derivatives is more efficient for Hoogsteen-type triplexes and duplexes, whereas *o*TINA analogues can stabilize both Hoogsteen- and Watson–Crick-type complexes. 1-Ethynylpyrene derivatives showed the highest increment in thermal stabilities among the ethynylpyrenes studied, except for antiparallel duplexes that were formed by mixed purine–pyrimidine sequences (the use of 4-ethynylpyrene resulted in slightly higher T_m values). UV/Vis and fluorescence properties of each ethynylpyrene isomer were different from each other. A remarkably large Stokes shift (ca. 100 nm) was observed for pyren-2-ylethynylphenyl, whereas the Stokes shifts for 1- and 4-ethynylpyrene conjugates were 10 and 40 nm, respectively. An interstrand excimer band at 505 nm was observed for *o*TINA derivatives, when the dyes were positioned opposite each other in the middle of the duplex as bulges, whereas an excimer band could not be detected for any of the duplexes with a pair of *p*TINAs. The current study highlights the importance of intercalator positioning within the core of DNA duplexes. Tiny structural alterations, as, for example, the use of another ethynylpyrene isomer, can lead to significant changes in the properties of the resulting nucleic acid analogues. The design of DNA-based tools is based on specificity, self-assembly, and high thermal stability, and therefore knowledge of specific positioning of reporter groups in the interior of DNA complexes is vital for further development of DNA-based bio- and nanotechnology.

Experimental Section

General methods: 500 MHz ^{1}H and 125.7 MHz ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer and referenced to $CDCl_3$ ($\delta = 7.25$ ppm for ^{1}H and $\delta = 77.00$ ppm for ^{13}C). ^{1}H–^{13}C gradient-selected HMQC and HMBC spectra were obtained by using 2048 (t_2) × 256 (t_1) complex point data sets, zero-filled to 2048 (F_2) × 1024 (F_1) points. The spectral widths were 13 ppm and 200 ppm for ^{1}H and ^{13}C dimensions, respectively. HMBC spectra were measured with 50 ms delay for evolution of long-range couplings. ^{1}H–^{13}C HMQC and HMBC spectra were used for the assignment of signals in the ^{13}C NMR spectra of ethynylpyrenes **11** and **12**. Melting points were determined by using a Boetius heating table and are uncorrected. Analytical thin-layer chromatography was performed on Kieselgel 60 F_{254} precoated aluminum plates (Merck), spots were visualized under UV light (254 nm). Silica gel column chromatography was performed by using Merck Kieselgel 60 0.040–0.063 mm. Reagents and solvents obtained from commercial suppliers were used without further purification unless otherwise noted. Dichloromethane was always used freshly distilled over CaH_2.

Synthesis of 2- and 4-ethynylpyrene: **4,5,9,10-tetrahydropyrene (2) and 1,2,3,6,7,8-hexahydropyrene (3)**: Pyrene (21.0 g, 104 mmol) was purified by short column chromatography on silica gel in $CHCl_3$, then dissolved in EtOAc (250 mL), and the sulfur removed by stirring over Raney nickel (10 g) for 48 h.[42] Nickel was filtered off, and the solution was hydrogenated in the presence of 10% Pd/C (4 g) at 160 atm and 60 °C for 24 h. The mixture was filtered and evaporated to give a crude mixture of **10** and **9** ($R_f = 0.65$ and 0.56, respectively; aluminum oxide TLC plate, petroleum ether). The mixture was chromatographed on Al_2O_3 in petroleum ether to yield **9** and **10**. 4,5,9,10-Tetrahydropyrene (**9**, 16.2 g, 76%), colorless crystals, m.p. 120–123 °C (96% aq. ethanol; lit. m.p. 126–127 °C,[40] 137–138 °C,[43] 119–120 °C[41]); ^{1}H NMR ($CDCl_3$): $\delta = 7.12$ (m, 2H, $J_{1,2} = 7.1$ Hz, 2-H, 7-H), 7.08 (m, 4H, $J_{1,2} = 7.1$ Hz, 1-H, 3-H, 6-H, 8-H), 2.89 ppm (s, 8H, CH_2). 1,2,3,6,7,8-Hexahydropyrene (**10**, 4.3 g, 20%), colorless crystals, m.p. 128–130 °C (96% aq. ethanol; lit. m.p. 127 °C,[62,63] 129–130 °C,[64] 132–133 °C[65]); ^{1}H NMR ($CDCl_3$): $\delta = 7.13$ (s, 4H, 4-H, 5-H, 9-H, 10-H), 3.07 (t, 8H, $J_{1,2} = 6.1$ Hz, 1-H, 3-H, 6-H, 8-H), 2.06 ppm (quintet, 4H, $J_{1,2} = 6.1$ Hz, 2-H, 7-H).

Synthesis of 2-ethynylpyrene (11): A solution of Ac_2O (4.82 mL, 51 mmol) in CH_2Cl_2 (60 mL) was added dropwise over 30 min to a stirred, ice-cooled solution of 4,5,9,10-tetrahydropyrene (**9**, 10.0 g, 48.5 mmol) and $AlCl_3$ (14.46 g, 108 mmol) in CH_2Cl_2 (200 mL). The mixture was kept at 0 °C for 1 h, then the cooling was removed, and the mixture was stirred at ambient temperature for 3 h (the TLC analysis showed the disappearance of the starting hydrocarbon). The mixture was poured into the mixture of concentrated HCl (80 mL) in cold water (500 mL). The organic layer was separated and the aqueous layer was extracted with DCM (100 mL). The organic solutions were combined and washed with water (300 mL), 5% $NaHCO_3$ (300 mL) followed by water (300 mL), then dried over $CaCl_2$ and evaporated. The residue was chromatographed on silica gel in toluene and then recrystallized from petroleum ether to yield 2-acetyl-4,5,9,10-tetrahydropyrene (12.04 g, 98%) as yellow crystals, m.p. 107–108 °C (lit. m.p. 113–114 °C,[40] 110.5–112 °C[66]), $R_f = 0.42$ (5% EtOAc/toluene, v/v). ^{1}H NMR ($CDCl_3$): $\delta = 7.67$ (s, 2H, 1-H, 3-H), 7.18 (m, 1H, $J_{6,7} = 7.4$ Hz, 7-H), 7.10 (m, 2H, $J_{6,7} = 7.4$ Hz, 6-H, 8-H), 2.92 (m, 8H, CH_2), 2.60 ppm (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): $\delta =$

198.00 (CO), 136.19 (2C), 135.59, 135.48 (2C), 135.30, 129.79, 128.36, 126.15 (2C), 126.13 (2C), 126.11, 28.23 (2C), 28.13 (2C), 26.60 ppm (CH_3). One portion of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 5.06 g, 22.3 mmol) was added to a solution of 2-acetyl-4,5,9,10-tetrahydropyrene (2.38 g, 9.6 mmol) in toluene (150 mL) and the solution was refluxed for 1 h, cooled, and then passed through an Al_2O_3 layer (4 cm) in toluene to yield pure 2-acetylpyrene (2.0 g, 85%), m.p. 121–124 °C (AcOH/H_2O) (lit. m.p. 145–147 °C[50] and 145–146 °C[40]), R_f = 0.37 (5% EtOAc/toluene, v/v). ^{1}H NMR ($CDCl_3$): δ = 8.72 (s, 2H, 1-H, 3-H), 8.20 (d, 2H, $J_{6,7}$ = 7.7 Hz, 6-H, 8-H), 8.12 (m, 4H, $J_{4,5}$ = 8.9 Hz, 4-H, 5-H, 9-H, 10-H), 8.06 (t, 1H, $J_{6,7}$ = 7.7 Hz, 7-H), 2.89 ppm (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ = 198.69 (CO), 134.21, 131.81 (2C), 131.04 (2C), 128.29 (2C), 127.88 (2C), 127.11 (C7), 127.01, 125.49 (2C), 124.47 (2C), 124.27, 27.11 ppm (=CH_3). The 2-acetylpyrene was transformed further into 3-(2-pyrenyl)-3-chlor-2-propenal by using the following procedure: $POCl_3$ (5.0 mL, 54 mmol) was added dropwise to ice-cooled DMF (4.3 mL, 55 mmol) over 1 h. The mixture obtained was added dropwise at RT to a solution of 2-acetylpyrene (1.86 g, 7.6 mmol) in DMF (70 mL) and the reaction mixture was left to stir overnight. Then the reaction mixture was poured into cold water (500 mL), and solid AcONa·3H_2O was added until the pH value reached 6. The mixture was extracted with $CHCl_3$ (200 mL), the organic layer was washed with water (3×300 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel in toluene to yield 3-(2-pyrenyl)-3-chlor-2-propenal (1.86 g, 84%) as a yellow solid. R_f 0.46 (5% EtOAc-toluene v/v). NMR spectroscopy showed the compound to be the mixture of *E*- (97%) and *Z*- (3%) stereoisomers. ^{1}H NMR ($CDCl_3$): δ = 10.34 (d, 0.97H, J = 6.8 Hz, (*E*)-CHO), 9.56 (d, 0.03H, J = 7.6 Hz, (*Z*)-CHO), 8.48 (s, 2H, 1-H, 3-H), 8.20 (d, 2H, $J_{6,7}$ = 7.8 Hz, 6-H, 8-H), 8.13–8.02 (m, 5H, $J_{4,5}$ = 9.0 Hz, 4-H, 5-H, 7-H, 9-H, 10-H), 6.98 (d, 0.97H, J = 6.8 Hz, (*E*)-C*H*CHO), 6.74 ppm (d, 0.03H, J = 7.6 Hz, (*Z*)-C*H*CHO). To a solution of 3-(2-pyrenyl)-3-chlor-2-propenal (648 mg, 2.23 mmol) in dioxane (90 mL), pulverized KOH (394 mg, 7.0 mmol) was added with stirring under argon and the reaction mixture was refluxed for 2 h, cooled, and then the pH value was adjusted to 3 through the addition of 5% aq. citric acid. Solvents were removed in vacuo and the residue was dissolved in toluene (150 mL), washed with water (3×200 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel by using a step gradient of 50→70→90→100% (v/v) of toluene in petroleum ether to give 2-ethynylpyrene (**11**) as colorless crystals. Yield 480 mg (95%). R_f 0.6 ($CHCl_3$). m.p. 112–114 °C (ethanol; lit. m.p. 125–127 °C,[50] 110–112 °C,[49] and 103–104 °C[47]). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.27 (s, 2H, 1-H, 3-H), 8.16 (d, 2H, $J_{6,7}$ = 7.6 Hz, 6-H, 8-H), 8.06 (m, 2H, $J_{4,5}$ = 9.1 Hz, 5-H, 9-H), 8.00 (m, 3H, 4-H, 7-H, 10-H), 3.24 ppm (s, 1H,=CH); ^{13}C NMR ($CDCl_3$): δ = 131.26 (2C, C5a, C8a), 131.00 (2C, C3a, C10a), 128.16 (2C, C1, C3), 128.12 (2C, C5, C9), 126.82 (2C, C4, C10), 126.41 (C7), 125.40 (2C, C6, C8), 124.46 (C10b), 124.29 (C10c), 119.39 (C2), 84.28 (Ar*C*≡), 77.43 ppm (≡CH).

Synthesis of 4-ethynylpyrene (12): For a preliminary report, see reference [67]. In a manner similar to that described in the above procedure, 4-acetyl-1,2,3,6,7,8-hexahydropyrene was prepared from **10** (8.06 g, 39 mmol), $AlCl_3$ (11.37 g, 85 mmol), and Ac_2O (4.03 mL, 43 mmol). The ketone was purified by chromatography on silica gel in toluene. Yield 8.93 g (92%), yellow crystals. R_f = 0.31 (toluene). m.p. 78–79 °C (benzene; lit. m.p. 85–86 °C[49]). ^{1}H NMR ($CDCl_3$): δ = 7.38 (s, 1H, 5-H), 7.19 (m, 2H, J = 7.1 Hz, 9-H, 10-H), 3.28 (t, 2H, J = 6.0 Hz, 3-H), 3.12–3.03 (m, 6H, 1-H, 6-H, 8-H), 2.64 (s, 3H, CH_3), 2.10–1.97 (m, 4H, 2-H, 7-H) ppm; ^{13}C NMR ($CDCl_3$): δ 203.31 (CO), 136.15, 134.73, 134.03, 134.00, 133.48, 131.27, 130.22, 125.38, 124.47, 122.40, 31.51, 31.30, 31.27, 30.56, 29.32, 23.19, 23.01 ppm. 4-Acetyl-1,2,3,6,7,8-hexahydropyrene (770 mg, 3.08 mmol) was aromatized with DDQ (2.27 g, 10 mmol) in toluene (20 mL) to give 4-acetylpyrene as yellow crystals. Yield 560 mg (75%). R_f = 0.45 (toluene), m.p. 136–138 °C (toluene; lit. m.p. 132.5–133.5 °C[45]). ^{1}H NMR ($CDCl_3$): δ = 9.09 (d, 1H, J = 8.0 Hz, 3-H), 8.52 (s, 1H, 5-H), 8.25–8.17 (m, 3H, 1-H, 6-H, 8-H), 8.08–7.97 ppm (m, 4H, 2-H, 7-H, 9-H, 10-H); ^{13}C NMR ($CDCl_3$): δ = 201.64 (CO), 134.90, 131.64, 131.11, 131.02, 129.17, 128.17, 127.34, 127.24, 126.71, 126.64, 126.48, 126.17, 125.92, 125.54, 125.27, 124.47, 29.83 ppm (CH_3). 3-(4-Pyrenyl)-3-chlor-2-propenal was prepared from 4-acetylpyrene (1.338 g, 5.5 mmol) and Vilsmeier reagent ($POCl_3$ (3.6 mL, 39 mmol) with DMF (3.0 mL, 39 mmol)). Yield 1.49 g (94%), yellow solid. R_f = 0.4 (5% EtOAc/toluene, v/v). ^{1}H NMR ($CDCl_3$): δ = 10.46 (d, 0.46H, J = 7.0 Hz, (*E*)-CHO), 9.43 (d, 0.54H, J = 7.4 Hz, (*Z*)-CHO), 8.45–8.05 (m, 9H, ArH), 6.90 (d, 0.54H, J = 7.4 Hz, (*Z*)-C*H*CHO), 6.71 ppm (d, 0.46H, J = 7.0 Hz, (*E*)-C*H*CHO). 3-(4-Pyrenyl)-3-chlor-2-propenal (291 mg, 1.0 mmol) in dioxane (10 mL) with KOH (177 mg, 3.16 mmol) was converted to 4-ethynylpyrene (**12**), colorless crystals, m.p. 103–104 °C (lit. m.p. 103–105 °C[47]). Yield 190 mg (84%). R_f = 0.6 (toluene). ^{1}H NMR ($CDCl_3$): δ = 8.69 (d, 1H, J = 7.8 Hz, 3-H), 8.38 (s, 1H, 5-H), 8.22 (d, 1H, J = 7.8 Hz, 1-H), 8.20 (d, 1H, J = 7.8 Hz, 8-H), 8.15 (d, 1H, J = 7.3 Hz, 6-H), 8.11–8.04 (m, 3H, 2-H, 9-H, 10-H), 8.00 (m, 1H, 7-H), 3.55 ppm (s, 1H, ≡CH); ^{13}C NMR ($CDCl_3$): δ = 133.26 (C5), 131.22 (C10a), 131.03 (C8a), 130.30 (C3a), 130.07 (C5a), 127.61 (C10), 127.20 (C9), 126.25 (C8), 126.20 (C2), 126.12 (C7), 125.75 (C1), 125.23 (C6), 124.40 (2C, C10b, C10c), 123.72 (C3), 119.28 (C4), 82.01 (Ar*C*≡), 81.69 ppm (≡CH).

Synthesis of DMT-protected *o*TINA precursor: **(*S*)-1-(4,4′-dimethoxytriphenylmethyloxy)-3-(2-iodobenzyloxy)-propan-2-ol (DMT-14)**: (*S*)-(+)-2,2-Dimethyl-1,3-dioxolane-4-methanol (**13**, 1.17 g, 8.9 mmol) and 2-iodobenzylbromide (2.5 g, 8.4 mmol) were refluxed under Dean–Stark conditions in toluene (125 mL) in the presence of KOH (4.4 g, 77.0 mmol) for 7 h. The reaction mixture was allowed to cool and H_2O (50 mL) was added. After separation of the phases, the water layer was washed with toluene (2×25 mL). The combined organic layers were washed with H_2O (30 mL) and concentrated in vacuo. The residue was treated with 80% aq. CF_3CO_2H (25 mL) for 4 h at RT. The solvent was removed in vacuo and the residue was coevaporated twice with toluene/EtOH (30 mL, 5:1, v/v) and then with dry pyridine (20 mL). The residue was dried under diminished pressure to afford (*R*)-3-(2-iodobenzyloxy)propane-1,2-diol (**14**, 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 then added under nitrogen. After 24 h, ethanol (2 mL) followed by EtOAc (150 mL) were added and the mixture was extracted with saturated aqueous $NaHCO_3$ (3×40 mL). The water phase was extracted with EtOAc (50 mL). 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 by using dry column vacuum chromatography with EtOAc (0–25%, v/v) in cyclohexane to afford (*S*)-1-(4,4′-dimethoxytriphenylmethyloxy)-3-(2-iodobenzyloxy)propane-2-ol (62%, 3.0 g) as a yellow foam. ^{1}H NMR ($CDCl_3$): δ = 2.46 (d, 1H, J = 5.0 Hz; OH), 3.25 (dd, 2H, J = 1.9, 5.0 Hz; CHOHCH_2OCH$_2$), 3.67 (m, 2H; CH_2ODMT), 3.75 (s, 6H, 2×CH_3), 4.00 (m, 1H; *CH*OH), 4.50 (s, 2H; CH_2Ar), 6.81 (d, 4H, J = 8.5 Hz; DMT), 6.97 (m, 1H; phenyl), 7.20–7.30 (m, 9H; phenyl+DMT), 7.41 (m, 2H; phenyl), 7.80 ppm (d, 1H, J = 7.9 Hz; phenyl). ^{13}C NMR ($CDCl_3$): δ = 55.17, 55.19 (OCH_3), 64.39 (CH2ODMT), 69.93 [CH(OH)CH_2OCH$_2$], 72.04 (*C*HOH), 76.9 (CH_2-phenyl), 86.09 (CAr_3), 97.79 (*C*-1) , 113.10 (DMT), 126.76 (DMT), 127.81 (iodophenyl), 128.11 (DMT), 128.17 (DMT), 128.87 (iodophenyl), 129.25 (iodophenyl), 130.04 (DMT), 135.96 (DMT), 139.17 (iodophenyl), 140.21 (iodophenyl), 144.8 (DMT), 158.45 ppm (DMT). HR-MALDI-MS: calcd for $C_{31}H_{31}IO_5Na$ [*M*+Na]$^+$: *m*/*z* 633.1108; found: *m*/*z* 633.1082.

Synthesis of (*S*)-2-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-1-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*O*-(2-iodobenzyl)glycerol (15): (*S*)-1-(4,4′-Dimethoxytriphenylmethyloxy)-3-(2-iodobenzyloxy)propane-2-ol (1.5 g, 2.46 mmol) was dissolved under nitrogen in anhydrous CH_2Cl_2 (50 mL). *N*,*N*-Diisopropylammonium tetrazolide (0.660 g, 3.85 mmol) was added followed by dropwise addition of 2-cyanoethyl tetraisopropylphosphordiamidite (0.854 g, 2.82 mmol) with external cooling with an ice-water bath. After stirring overnight, analytical TLC showed no more starting material, and the reaction was quenched with H_2O (45 mL). The 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 by using silica gel dry column vacuum chromatography with Et_3N (0.5%)/EtOAc (0–25%, v/v)/petroleum

ether. Combined UV-active fractions were evaporated in vacuo to afford the final compound **15** (1.28 g, 64%) as a foam that was used in DNA synthesis. ^{32}P NMR ($CDCl_3$): $\delta = 150.3$, 150.4 ppm (1:1 ratio). HR-MALDI-MS: calcd for $C_{40}H_{49}IO_6N_2PNa$ $[M+Na]^+$: m/z 833.2187; found: m/z 833.2157.

Synthesis and purification of TINAs: DNA molecules were synthesized on an Expedite nucleic acid synthesis system model 8909 from Applied Biosystems by using 4,5-dicyanoimidazole as an activator and an increased deprotection time (100 s) and coupling time (2 min) for a 0.075 M solution of the phosphoramidite **15** or the phosphoramidite of (*S*)-1-(4,4′-dimethoxytriphenylmethyloxy)-3-(4-iodobenzyloxy)propane-2-ol[12] in a 1:1 mixture of dry MeCN/CH_2Cl_2. After the DNA synthesis, the columns with CPG-supports and DMT-on oligonucleotides with 4-iodophenyl or 2-iodophenyl moieties were flushed with argon (2 min) prior to the coupling reaction. The Sonogashira coupling reagent mixture containing $[Pd(PPh_3)_4]$ (7.5 mM), one of the ethynylpyrenes (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 RT. 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 full 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 then dried. In the cases of **ON3**, **ON5**, **ON7**, **ON9**, **ON10**, **ON16**, and **ON19** 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 (RT, 2 h) and deprotected (55 °C, overnight) by using 32% aqueous ammonia. Purification of 5′-*O*-DMT-on TINAs was accomplished by using a reverse-phase semipreparative HPLC on a Waters Xterra MS C18 column. The ONs were DMT deprotected in 100 μL 80% aqueous acetic acid (30 min), diluted with 32% aqueous ammonia (1 mL), and purified again on HPLC. Corresponding fractions with ONs were evaporated and diluted with 1 M aqueous NaOAc (150 μL), and ONs were precipitated from ethanol (550 μL). The modified ONs were confirmed by MALDI-TOF analysis on a Voyager Elite biospectrometry research station from PerSeptive Biosystems. The purity of the final ONs was checked by ion-exchange chromatography by using a LaChrom system from Merck Hitachi on a GenPak-Fax column (Waters) and was found to be over 90%.

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 mixing the two strands of the Watson–Crick duplex, each at a concentration of 1.0 μM followed by addition of the third (TFO) strand (1.5 μM) in the corresponding buffer solution. The solution was heated to 80 °C for 5 min, cooled to RT, 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 RT. 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. 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 and in the same buffer solution as for the T_m measurements, except that only 1.0 μM of TFOs were used in all cases. The single-stranded and hybridized samples were adjusted to an identical optical density at an excitation wavelength of 373 nm. Fluorescence excitation spectra of single-stranded ONs were performed on a Perkin-Elmer luminescence spectrometer LS-50B. The selection of the emission slit width of 0.0 nm gives a resolution of less than 2 nm on the above mentioned spectrometers.

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