DOI: 10.1002/chem.200700053

Stabilization of Parallel Triplexes by Twisted Intercalating Nucleic Acids (TINAs) Incorporating 1,2,3-Triazole Units and Prepared by Microwave-Accelerated Click Chemistry

Imrich Géci,[a] Vyacheslav V. Filichev,[a, b] and Erik B. Pedersen*[a]

Abstract: A highly efficient method for postsynthetic modification of unprotected oligonucleotides incorporating internal insertions of (*R*)-1-*O*-(4-ethynylbenzyl)glycerol has been developed through the application of click chemistry with water-insoluble pyren-1-yl azide and water-soluble benzyl azide and acceleration by microwave irradiation. The twisted intercalating nucleic acids (TINAs) obtained in these reactions, possessing bulged insertions of (*R*)-3-*O*-{4-[1-(pyren-1-yl)-1*H*-1,2,3-triazol-4-yl]benzyl}glycerol (**7**), formed parallel triplexes with thermal stabilities of 20.0, 34.0, and 40.0 °C at pH 7.2 in the cases of one, two, or three insertions of **7**, respectively, separated by three nucleic bases. An oligonucleotide with four insertions of **7**—each between three nucleic bases in the sequence—was unable to form complexes with complementary single- or double-stranded DNAs, as a result of self-aggregation of the pyrene moieties. This assumption was supported by the formation of a very strong excimer band at 460 nm in the fluorescence spectra. Molecular modeling of the parallel triplex with bulged insertion of the monomer **7** in the triplex-forming oligonucleotide (TFO) showed that only the pyrene moiety was stacking between the bases of the dsDNA, whereas 1,2,3-triazole did not participate in the triplex stabilization. Thermal denaturation studies of the duplexes and triplexes, as well as the fluorescence properties of TINA-triazole **7**, are discussed and compared with previous studies on TINA.

Keywords: click chemistry · DNA · microwave-assisted chemistry · oligonucleotides · triplex stabilization

Introduction

The ability of double-stranded DNA (dsDNA) to form triple helical structures has been exploited in intensive research into triplex-forming oligonucleotides (TFOs) as promising therapeutics and as tools for bionanotechnology.[1] To achieve high binding affinities and target specificities of TFOs to dsDNA, chemical modification of nucleic acids is required. In order to screen large numbers of different substituents in nucleic acid structures, postsynthetic approaches are more convenient than the time-consuming preparation of an individual phosphoramidite for each modification. We have therefore screened several aromatic substituents in the structures of twisted intercalating nucleic acids (TINAs; **1** and **2** in Figure 1) by use of postsynthetic Sonogashira-type reactions with oligonucleotides (ONs) incorporating 4-iodophenylmethylglycerol or 4-ethynylphenylmethylglycerol moieties.[2,3] High thermal stabilities of parallel triplexes have been observed upon bulged insertion of **1** and **2** in the middles of TFOs. The ability of structure **1** to form Hoogs-

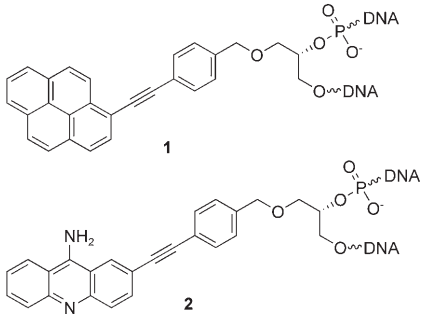


Figure 1. TINA **1** and TINA-acridine **2**.

[a] I. Géci, Dr. V. V. Filichev, Prof. E. B. Pedersen
Nucleic Acid Center, Department of Physics and Chemistry
University of Southern Denmark
Campusvej 55, 5230, Odense M (Denmark)
Fax: (+45) 6615-8780
E-mail: ebp@chem.sdu.dk

[b] Dr. V. V. Filichev
Institute of Fundamental Sciences
Massey University (New Zealand)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

teen-type triplexes that were thermally more stable than Watson–Crick-type duplexes at neutral pH and had the capability to discriminate between matched and mismatched sequences has also been shown.[2] Further postsynthetic functionalization was achieved by replacement of the pyrene moiety with acridine, which allowed us to evaluate the effects of the presence of primary amines in TINA structures.[3] Computation of simulations of parallel triplexes showed the intercalators to be positioned between the bases of dsDNA, whereas the phenyl groups were stacking between the bases of TFO. Moreover, the twisting of intercalators around the triple bond of the TINA monomer structure has been determined.

In our ongoing study we decided to evaluate the effects of substitution of the triple bond with a five-membered aromatic ring—1,2,3-triazole—on the abilities of TINAs to form triplexes and duplexes. We assumed that the presence of a third aromatic ring could provide thermal stabilization at least as good as that seen in TINA through the contribution of the 1,2,3-triazole to intercalation in the dsDNA part of the triplex. Moreover, we were able to use Huisgen 1,3-dipolar cycloadditions of azides and alkynes catalyzed by Cu^{I} in the presence of ascorbic acid or sodium ascorbate—as one of the highly efficient click reactions—to provide 1,4-regioisomeric 1,2,3-triazoles.[4–6] This bioorthogonal reaction[4] was also found to be useful for postsynthetic modification of ONs. Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloadditions between organic azides and nucleic acids have been used in the synthesis of fluorescent single-stranded DNA,[7] DNA-templated reactions,[8] and labeling of DNA after methyltransferase-mediated site-specific alkylation of DNA.[9] However, the number of reports relating to "click" chemistry and DNA is still limited, due to the required long reaction times (from hours to days), elevated temperatures (37–80 °C), and unavailability of an easy procedure for coupling of water-insoluble organic azides. This is crucial for multiple modifications and for sterically hindered acetylenes in the structure of ONs, as illustrated by the observation that galactosyl azide derivatives reacted more efficiently with DNA incorporating 5-(hexa-1,5-diynyl)uridine moieties than with those containing 5-ethynyluridines.[10] It should also be mentioned that copper ions in the presence of O_2 induce DNA cleavage,[11,12] so the development of a method that will allow fast, efficient, and multiple cycloadditions of water-insoluble organic azides to DNA containing terminal acetylenes is highly desirable.

The times required for [2+3]-cycloaddition reactions for the synthesis of organic compounds can be reduced to 10–15 min by application of microwave irradiation,[13] and microwave conditions have also been found to be compatible with ONs in postsynthetic modifications.[14–17] During the preparation of this manuscript, Bouillon et al.[18] reported microwave-assisted cycloadditions between galactosyl azide derivatives and homopyrimidine sequences incorporating three alkyne groups, but no analogous reactions with mixed-mer sequences and water-insoluble aromatic azides have yet been reported. In this paper we report on microwave-accelerated 1,3-dipolar cycloadditions between aromatic azides and ONs incorporating several insertions of (*R*)-1-*O*-(4-ethynylbenzyl)glycerol (**4**) in aqueous buffer solution. The thermal stabilities of Hoogsteen-type triplexes and duplexes and fluorescence properties are discussed.

Results and Discussion

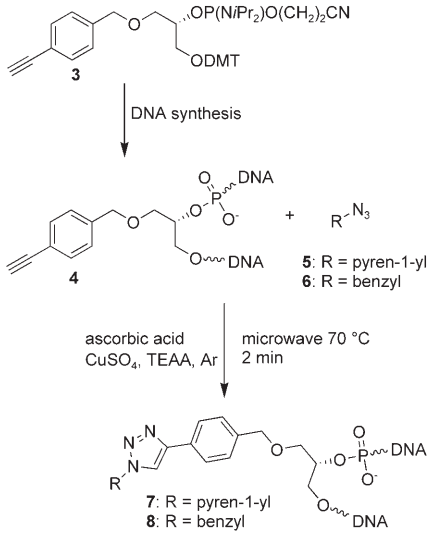
Synthesis of TINA-triazole: For the postsynthetic click reactions we prepared aqueous solutions of fully deprotected ONs (Table 1, **ON1**–**ON6**), containing from one to four internal insertions of (*R*)-1-*O*-(4-ethynylbenzyl)glycerol (**4**). The phosphoramidite required for the oligonucleotide synthesis was prepared in our previous work on the synthesis of TINA-acridine **2**.[3]

Table 1. ONs synthesized, MALDI-TOF MS, and yield of click reactions in a microwave cavity.

No.	Oligonucleotide	*m/z* [Da] calcd	found	Postsynth. yield [%]
ON1	5′-CCCCTT**4**TCTTTTTT	4390	4388	–
ON2	5′-**4**CCCCTTTCTTTTTT	4390	4390	–
ON3	3′-TCGAAC**4**GAACTC	3880	3882	–
ON4	5′-CCCCTT**4**TCT**4**TTTTT	4658	4654	–
ON5	5′-CCC**4**CTT**4**TCT**4**TTTTT	4926	4923	–
ON6	5′-CCC**4**CTT**4**TCT**4**TTT**4**TT	5194	5196	–
ON7	5′-CCCCTT**7**TCTTTTTT	4633	4632	45[a]
ON8	5′-**7**CCCCTTTCTTTTTT	4633	4633	36[a]
ON9	3′-TCGAAC**7**GAACTC	4123	4124	34[a]
ON10	5′-CCCCTT**7**TCT**7**TTTTT	5144	5146	31[b]
ON11	5′-CCC**7**CTT**7**TCT**7**TTTTT	5655	5658	29[b]
ON12	5′-CCC**7**CTT**7**TCT**7**TTT**7**TT	6166	6170	23[b]
ON13	5′-CCCCTT**8**TCTTTTTT	4520	4520	32[a]
ON14	5′-**8**CCCCTTTCTTTTTT	4520	4520	31[a]
ON15	3′-TCGAAC**8**GAACTC	4016	4016	20[a]

[a] Reaction mixtures irradiated for 5 min. [b] Reaction mixtures irradiated for 15 min.

We investigated microwave assistance of the 1,3-dipolar cycloaddition between **ON1**—containing one insertion of **4** in the middle of the backbone—and pyren-1-yl azide (**5**; Scheme 1). Azide **5** (1.0 μmol) was dissolved in DMSO in a microwave tube and precipitated immediately after dilution with aq. $CuSO_4$ and triethylammonium acetate buffer (TEAA, 0.2 M). Argon was bubbled through the suspension for one min, which was followed by the addition of aqueous solutions of ascorbic acid and **ON1** (0.05 μmol). The suspension was again consistently degassed with argon for the next three minutes. The use of TEAA buffer and degassing of the reaction mixture was found to be important for avoidance of DNA cleavage caused by copper ions and oxygen.[9] The closed tube was placed in the microwave instrument (Emrys Creator) and heated to 70 °C for two minutes. Compound **ON7** was separated by semipreparative HPLC on a C_{18} column, precipitated from EtOH (99 %), and characterized by MALDI-TOF MS. The isolated yield of the product, as determined by UV spectroscopy, was 45 %.



Scheme 1. Microwave-accelerated click chemistry.

The same procedure was successfully applied to **ON2**, with the monomer **4** at the 5′-end, and also to the mixed purine–pyrimidine **ON3**, with monomer **4** in the middle of the sequence. Products **ON8** and **ON9** were isolated in 36 and 34% yield, respectively. The latter reaction is a demonstration of the applicability of the developed method to purine–pyrimidine mixed DNAs.

To examine the time dependence of the microwave-induced click reaction between the azide **5** and **ON1**, we performed this reaction with different reaction times (2, 5, 10, 15, and 20 min). As can be seen from Figure 2, we did not observe any difference in the retention times and intensities of the peaks in HPLC profiles of the reaction mixtures after the 2 and the 20 min microwave-assisted couplings. Moreover, no side products were observed. The click reaction between **ON1** and pyren-1-yl azide (**5**) in the absence of microwave irradiation afforded **ON7** in 31% isolated yield (stirring at 30°C for 30 min followed by stirring at room temperature for 24 h).

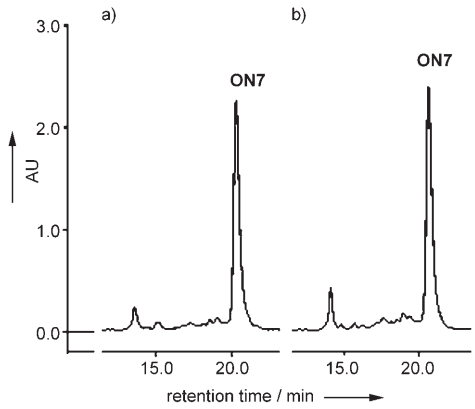


Figure 2. HPLC profiles of the reaction mixture obtained after the microwave-accelerated click reaction between **ON1** and azide **5**. a) Heating for 2 min. b) Heating for 20 min.

To determine the limitations of this approach for multiple modifications of ONs, we used **ON4**–**ON6**, containing two, three, and four insertions of the monomer **4**, respectively. We found that longer microwave irradiation times had to be applied to achieve full conversion of ONs containing several insertions of **4**. Thus, after 5 min of microwave heating of **ON6** with azide **5**, considerable amounts of products containing two and three couplings were observed (Figure 3a), as was confirmed by MALDI-TOF. After 15 min, however, only **ON12**, containing all four **7** monomers, was obtained (Figure 3b). The click reaction between **ON6** and azide **5** in the absence of microwave irradiation, with stirring of the reaction mixture at 30°C for 30 min, followed by stirring at room temperature for the next 24 h, gave the desired **ON12** contaminated with several side products (Figure 3c), which are most probably incompletely converted ONs. In order to check the coupling between the azide **5** and **ON6** under conventional heating conditions, we prepared the reaction mixture in the same way as described above and placed it in a preheated oil bath (70°C) for 15 min. The efficiency of this reaction was much lower than that of the microwave-induced reaction (Figure 3d and b, respectively).

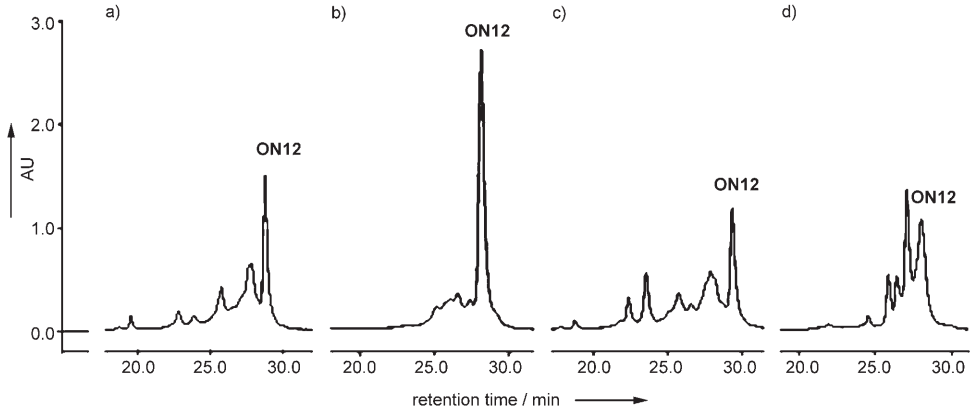


Figure 3. HPLC profiles of the reaction between the azide **5** and **ON6** under the following conditions. a) Microwave heating at 70°C for 5 min. b) Microwave heating at 70°C for 15 min. c) Room temperature for 24 h. d) Conventional heating at 70°C for 15 min.

To verify the universality of the microwave assistance for the postsynthetic click chemistry on ONs we also performed the established procedure with benzyl azide (**6**) and the same series of ONs. The reaction performance and the product isolation were easier than those for the pyrenyl azide, due to the liquid nature of benzyl azide. The synthesized ONs (**ON13**, **ON14**, **ON15**) incorporating monomer **8** were isolated by RP HPLC and characterized by MALDI-TOF (Table 1).

Thermal stability and fluorescence properties of TINA-triazole derivatives: Melting temperatures (T_m) as the first derivatives of the melting profiles of triplexes and duplexes containing from one to four insertions of TINA-triazole monomer **7** are shown in Tables 2 and 3 and are compared with the T_m values of unmodified ONs and previously studied TINAs containing monomers **1** and **2**.

The insertion of TINA-triazole **7** into the middles of TFOs as a bulge and also at the 5′-ends resulted in stabilization of the triplexes (**ON7** and **ON8** with **D1**) relative to the unmodified **ON16**/**D1**. However, modification **7** gave less stable triplexes than the monomer **1** (**ON17**/**D1**). The same tendency was observed in parallel duplexes: at pH 6.0 these were also formed through Hoogsteen base pairing, but at pH 7.2 the modified parallel duplexes **ON7**/**ON19** and **ON8**/**ON19** were slightly destabilized in relation to **ON16**/**ON19**. As was to be expected from our previous studies on TINAs,[2,3] the destabilization of homopyrimidine and mixed-mer Watson–Crick-type antiparallel duplexes (Tables 2 and 3) was observed with all TINA monomers. The fluorescence properties of the TINA-triazole **7** at pH 6.0 are shown in Figure 4a–d. The intensity of monomeric fluorescence for **ON8** upon binding with **D1** was quenched significantly, whereas for **ON7**, with the monomer **7** in the middle of the strand, the fluorescence intensity was increased (Figure 4a). Moreover, the fluorescence maxima of the triplexes were shifted to 395 and 415 nm (in relation to the single strands appearing at 380 and 400 nm). Such differences in the fluorescence properties of monomer **7**, depending on its position in the TFO, can be explained in terms of the presence of different surrounding bases in dsDNA. Thus, in **ON7** the neighboring bases of monomer **7** in the dsDNA are adenosines, while in **ON8** there are guanosines. Moreover, upon binding of **ON8** to the dsDNA, the intercalator, located at the 5′-end of the TFO, can adjust its position in the core of dsDNA more easily than in the case of **ON7**.

As we can see in Table 2, by increasing the number of TINA-triazole monomers **7** we can obtain more thermally stable parallel triplexes at pH 6.0 and 7.2 (**ON10** and **ON11**, with two and three monomers **7** with **D1**). At the same time, further destabilization of the antiparallel duplexes (**ON10**, **ON11** to **ON20**) was observed. In **ON11**, the T_m value for the triplex at pH 7.2 was 11.0 °C higher than the T_m for the antiparallel duplex. This discrimination confirms that **ON11** prefers to bind to dsDNA and not to the complementary ssDNA upon incubation at 37.0 °C, a conventional temperature in molecular biological assays. Interestingly, every subsequent insertion of **7** gave a lower increase in the triplex

Table 3. T_m [°C] data for antiparallel duplex melting, taken from UV-melting curves at 260 nm.

		DNA 5′-AGCTTGCTTGAG **ON24**	RNA 5′AGCUUGCUUGAG **ON25**
ON21	3′-TCGAACGAACTC	47.5	40.5
ON22	3′-TCGAAC**1**GAACTC[a]	39.5	30.5
ON23	3′-TCGAAC**2**GAACTC[b]	44.5	36.5
ON9	3′-TCGAAC**7**GAACTC	43.5	33.0
ON15	3′-TCGAAC**8**GAACTC	33.0	31.5

[a] T_m values for triplexes and duplexes were taken from a previous study.[2] [b] values were taken from a previous study.[3]

Table 2. T_m [°C] data for triplex and duplex melting, taken from UV-melting curves at 260 nm.

		Parallel triplex 3′-CTGCCCCTTTCTTTTTT 5′-GACGGG-GAAAGAAAAAA **D1**		Parallel duplex 5′GACGGGGAAAGAAAAAA **ON19**		Antiparallel duplex 3′GGGGAAAGAAAAAA **ON20**	
		pH 6.0	pH 7.2	pH 6.0	pH 7.2	pH 6.0	pH 7.2
ON16	5′-CCCCTTTCTTTTTT	28.0	< 5.0	19.0	18.5	48.0	48.5
ON17	5′-CCCCTT**1**TCTTTTTT[a]	46.0	28.0	33.5	_[c]	46.5	_[c]
ON26	5′-CCCCTT**1**TCT**1**TTTTT[a]	56.5	43.0	38.0	_[c]	41.0	38.0
ON18	5′-CCCCTT**2**TCTTTTTT[b]	36.0	17.5	_[c]	_[c]	_[c]	_[c]
ON7	5′-CCCCTT**7**TCTTTTTT	37.0	20.0	24.5	17.0	44.0	45.0
ON8	5′-**7**CCCCTTTCTTTTTT	36.0	17.5	23.0	16.5	43.0	44.5
ON10	5′-CCCCTT**7**TCT**7**TTTTT	46.5	34.0	23.0	20.0	35.0	38.0
ON11	5′-CCC**7**CTT**7**TCT**7**TTTTT	48.5	40.0	20.0	23.5	25.0	29.0
ON12	5′-CCC**7**CTT**7**TCT**7**TTT**7**TT	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]
ON13	5′-CCCCTT**8**TCTTTTTT	< 5.0	< 5.0	_[c]	_[c]	_[c]	_[c]
ON14	5′-**8**CCCCTTTCTTTTTT	24.0	< 5.0	_[c]	_[c]	_[c]	_[c]

[a] T_m values for triplexes and duplexes were taken from a previous study.[2] [b] T_m values were taken from a previous study.[3] [c] Not determined. [d] Not applicable, due to self aggregation.

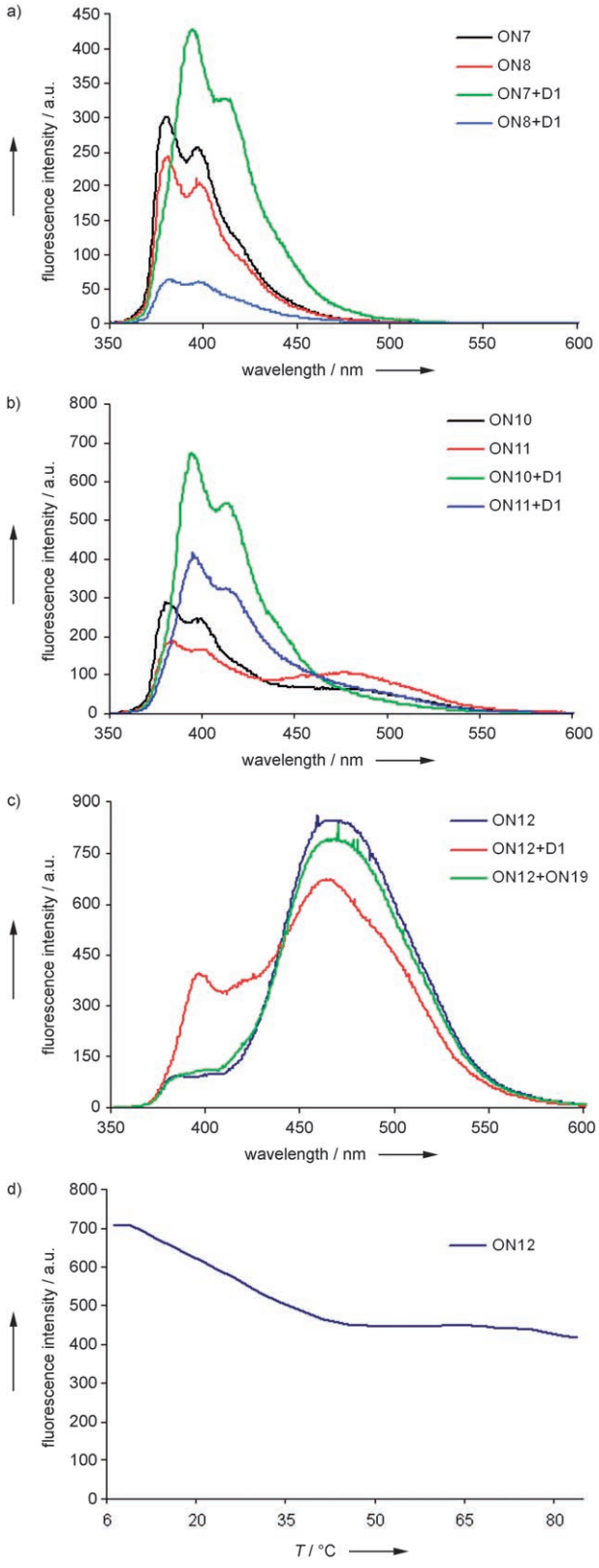


Figure 4. Fluorescence emission spectra of ONs incorporating monomer **7** upon excitation at 340 nm. A) Single-stranded **ON7**, **ON8**, and corresponding parallel triplexes. B) Insertion of two and three monomers **7** in the parallel triplex. C) **ON12** with four insertions of **7** and the corresponding mixtures with **D1** and **ON19**. D) Fluorescence melting profile (at 465 nm) of **ssON12** upon excitation at 340 nm.

T_m at pH 7.2 than the previous insertion: for example, ΔT_m (**ON7/D1–ON16/D1**) $>15.0\,°C$, ΔT_m (**ON10/D1–ON7/D1**) $=+14.0\,°C$, and ΔT_m (**ON11/D1–ON10/D1**) $=+6.0\,°C$.

With the antiparallel duplexes, unlike in the case of the parallel triplexes, greater destabilization was even observed on stepwise insertion of the monomer **7**: ΔT_m (**ON7/ON20–ON16/ON20**) $=-3.5\,°C$, ΔT_m (**ON10/ON20–ON7/ON20**) $=-7.0\,°C$, and ΔT_m (**ON11/ON20–ON10/ON20**) $=-9.0\,°C$. It should be mentioned that there were always three bases between insertions of **7**, because previous studies on TINAs had shown that a lower number of natural bases between the insertions did not result in improved stabilization of triplexes.[2,3]

Surprisingly, no triplex or duplex transitions were detected in UV-melting curves at 260 and 340 nm with the 14-mer sequence **ON12**, containing four TINA-triazole monomers **7**, which means that **ON12** was unable to bind to the complementary sequences. These results were also correlated by fluorescence spectroscopy. In all single-stranded ONs possessing more than one monomer **7**, an excimer band was observed at 460 nm. A considerable change in the ratio between the intensity of the monomeric and the excimer fluorescence was observed upon insertion of the fourth monomer **7** in the single-stranded DNA [see **ON10** and **ON11**, with two and three insertions of monomer **7**, respectively (Figure 4b) and **ON12**, with four monomers **7** in the sequence (Figure 4c)]. The intensity of the excimer band was reduced upon triplex formation in the cases of **ON10** and **ON11** (Figure 4b), while at the same time, increases and shifts in the monomeric fluorescence were observed, as had earlier been detected for **ON7**. On mixing of **ON12** either with **D1** or with **ON19**, only slight decreases in the excimer band were observed (Figure 4c). From these results we were able to conclude that self-aggregation of the intercalator was preventing the formation of complementary duplexes and triplexes. A slight decrease in the excimer band for **ON12** upon mixing with dsDNA (**D1**) and an increase in the monomeric fluorescence indicate that **ON12** was able to bind partially to the duplex, but that existence in the aggregate form was still preferred. This may be the result of a high content of lipophilic groups over a very short distance. We had previously observed a similar effect for TINAs **1** if they contained more than four intercalating monomers within short sequences (e.g. 15–20-mers; unpublished results). To avoid self-complexation we recommend not synthesizing oligonucleotides with contents of intercalators exceeding 28–30 % in the sequence.

To evaluate the stability of a self complex we monitored the excimer fluorescence (at 465 nm) of ss **ON12** versus temperature (Figure 4d). The excimer intensity decreased upon heating from 5 to 45 °C, afterwards remaining constant until 80 °C.

We also performed melting measurements with parallel triplexes and antiparallel duplexes containing monomer **8** (Tables 2 and 3). As the contribution of the phenyl ring to the π interaction with the bases of the dsDNA is too small, no triplex stabilization was observed. The smallest triplex

destabilization, of 4 °C, was observed when the monomer **8** was inserted at the 5′-end of the TFO (**ON14**) at pH 6.0. Similarly, incorporation of the monomer **8** into the antiparallel DNA/DNA and DNA/RNA duplexes resulted in destabilization of the corresponding duplexes. Here, it is interesting to compare two TINA analogues, monomers **1** and **8**, as the most stabilizing and the least stabilizing triplex molecules, respectively. In the case of the TINA/RNA duplexes the use of these intercalators resulted in similar destabilizing effects (**ON22** and **ON15** toward **ON25**).

From thermal stability and fluorescence studies we can conclude that substitution of the triple bond with a 1,2,3-triazole ring in the TINA structure results in: i) lower stabilization of triplexes with bulged insertions of the modified TINA monomer in the TFO (T_m (**ON17/D1**) = 46.0 °C and T_m (**ON7/D1**) = 37.0 at pH 6.0), and ii) monomeric fluorescence peaks shifted from 400 and 421 nm for monomer **1** to 380 and 400 nm for **7**, which is in a similar range to that of the unsubstituted pyrene.[19] Here it is worth mentioning a contrary effect of placing monomer **7** at the 5′-end of the sequence (**ON8**) on the stabilities of parallel triplexes and antiparallel duplexes. Generally, covalent attachment of intercalators at the 5′- or at the 3′-termini of oligonucleotides results in increased stability of the resulting complexes with DNA/RNA.[20,21] Thanks to stacking with the closest nucleic bases[22] and protection of the hydrogen bonds of the terminal base pair from the solvent,[23] the terminal base pair has an environment that is similar to that of internal base pairs. The effect of using such molecular caps is usually called lid effect. In our case, however, the parallel triplex is stabilized (ΔT_m(**ON8/D1–ON16/D1**) = +9.0 °C, pH 6.0) and the antiparallel duplex is destabilized (ΔT_m(**ON8/ON20–ON16/ON20**) = −5.0 °C, pH 6.0). With TINA monomer **1** we observed increased thermal stability in both these cases (+17.5 and +5.0 °C for parallel triplex and antiparallel duplex, respectively).[2] To understand these results we performed molecular modeling studies on the eight-mer parallel triplex containing the monomer **7** inserted as a bulge in the middle of the sequence, with MacroModel 8.0. The energy-minimized structure obtained from AMBER* calculation showed that the pyrene moiety was situated in the dsDNA part of the triplex, whereas the 1,2,3-triazole was not stacking with any of the bases (Figure 5a). Moreover, pronounced twisting of 35° between the pyrene and the 1,2,3-triazole was observed, while the 1,2,3-triazole and the phenyl ring were almost coplanar, with a torsion angle of 4.5° (Figure 5b). In the monomer **1**, a twisting of pyren-1-yl and phenyl residues about the triple bond was observed, but with a much lower torsion angle of 15.3°. Such pronounced twisting of the pyrenyl moiety around the phenyl/triazole system was most probably responsible for the imperfect stacking between the bases of the dsDNA. These might also explain the observed differences between monomers **1** and **7** in thermal stability and fluorescence properties.

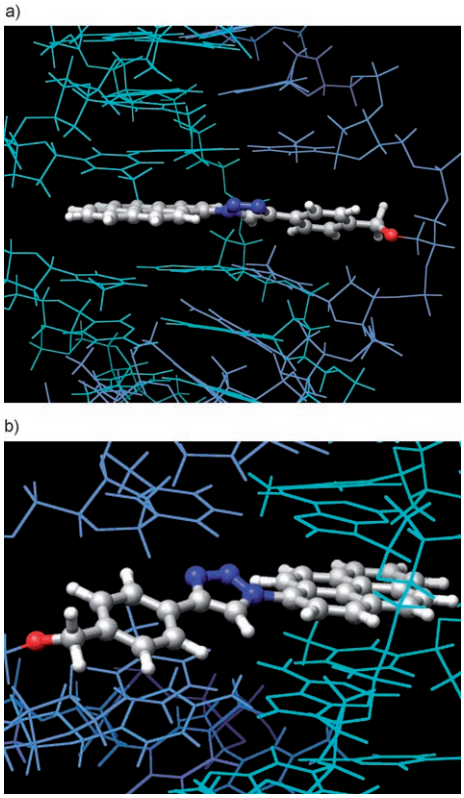


Figure 5. Representative structure of a triplex containing bulged insertion of **7** produced by AMBER* calculations. a) Side view on the monomer **7** shows the stacking of pyrene between the bases of dsDNA. b) Detailed view showing the twisting of the pyrene relative to the 1,2,3-triazole. dsDNA (cyan) and TFO (blue) are shown in stick form, while monomer **7** is presented in a ball-stick form.

Conclusion

We have developed microwave-accelerated 1,3-dipolar reactions between the water-insoluble pyren-1-yl azide (**5**) and ONs incorporating from one to four insertions of (*R*)-1-*O*-(4-ethynylbenzyl)glycerol (**4**). This highly efficient method allowed postsynthetic click reactions to be carried out on fully deprotected ONs in aqueous solution, affording 1,4-regioisomeric 1,2,3-triazoles in very short timescales (2–20 min). The universality of the method was shown with the click reaction with the water-soluble benzyl azide (**6**). This method allowed click reactions both on homopyrimidine and on mixed purine-pyrimidine sequences.

Melting studies showed that the substitution of the triple bond in TINA **1** by the 1,2,3-triazole moiety (monomer **7**) produced less stabilized Hoogsteen-type parallel triplexes and duplexes upon insertion of monomers in the middles of homopyrimidine sequences. The bulged insertions of two and three monomers **7** between three bases increased the triplex stability even further at pH 6.0 and 7.2. Watson–Crick-type duplexes were destabilized in all cases studied. No triplex and duplex melting was observed for oligonucleotides possessing four TINA-triazole monomers **7**. It is believed that this is due to the strong self-interactions of

pyrene moieties, which also resulted in the formation of a very strong excimer band at 460 nm in the fluorescence spectra of ss**ON12**. Molecular simulations showed a nonplanar structure for the aromatic part of the monomer **7** inserted as a bulge in the TFO, while the 1,2,3-triazole ring did not stack with any of the bases in the triplex structure.

Experimental Section

UV-melting and fluorescence experiments: Melting experiments were carried out with a Perkin–Elmer Lambda 35 UV/VIS spectrometer fitted with a PTP-6 temperature programmer. Antiparallel duplexes (Table 3) were formed by mixing of complementary ONs, each at a concentration of 1.0 μM in sodium phosphate buffer (10 mM) containing NaCl (140 mM) and EDTA (1 mM) at pH 7.0. Parallel triplexes (Table 2) were formed by mixing the two strands of the Watson–Crick duplex, each at a concentration of 1.0 μM, followed by addition of TFO at a concentration of 1.5 μM in a buffer consisting of sodium cacodylate (20 mM), NaCl (100 mM), and $MgCl_2$ (10 mM) at pH 6.0 or 7.2. Parallel and antiparallel duplexes in Table 2 were formed by mixing of complementary ONs, each at a concentration of 1.0 μM, in the cacodylate buffer described above. The solutions were heated at 80 °C for 5 min and cooled to 15 °C and were then kept at this temperature for 30 min. The melting temperature (T_m, °C) was determined as the maximum of the first derivative plots of the melting curves (see Supporting Information) obtained by measuring absorbance at 260 nm against increasing temperature (1.0 °C per min). Control experiments were also performed at 340 nm.

The fluorescence spectra were measured on a Perkin–Elmer LS-55 luminescence spectrometer fitted with a Julabo F25 temperature controller set at 10 °C. The excitation wavelength was set to 340 nm. Excitation and emission slits were set to 4 nm and 2.5 nm, respectively. The triplexes and duplexes were formed in the same way as for the T_m measurements, except that only 1.0 μM of TFOs were used in all cases.

Molecular simulations: Molecular modeling experiments were performed with the program MacroModel 8.0. The starting 8-mer parallel triplex was built by consecutive superimposition of triples (CGC and TAT). The triplex structure was energy-minimized after the insertion of the TINA-triazole monomer **7**. For the molecular simulations, the AMBER* force field method was used. The calculations were performed with the water solvent model and extended cut-off potential with the following settings: simulation temperature 300 K, simulation time 400 ps, and equilibration time 100 ps. The 250 structures generated by the Dynamics calculation were minimized in the next step by the Multiple Minimization method. The XCluster program was used to determine the lowest-energy representative structure of the conformations found.

Synthesis of ON1–ON6: DMT-on oligodeoxynucleotides **ON1–ON6** were synthesized at 1.0 μmol scales on CPG supports with an Expedite Nucleic Acid Synthesis System Model 8909 (Applied Biosystems), with use of 4,5-dicyanoimidazole as an activator and a solution (0.075 M) of the corresponding phosphoramidite **3**[3] in a mixture of dry $MeCN/CH_2Cl_2$ 1:1. For insertion of the monomer **3**, increased coupling (2 min) and deprotection (100 s) times were used. DMT-on oligonucleotides bound to CPG supports were treated with aq. ammonia (32 %, 1 mL) at room temperature for 2 h and then at 55 °C overnight. Purification of 5′-*O*-DMT-on ONs was accomplished by reversed-phase semipreparative HPLC on a Waters Xterra MS C_{18} column with a Waters Delta Prep 4000 Preparative Chromatography System {Buffer A [0.05 M triethyl ammonium acetate in H_2O (pH 7.0)] and Buffer B (75 % CH_3CN in H_2O)}. Flow 2.5 mL min^{-1}. Gradients: 2 min 100 % A, linear gradient to 70 % B in 38 min, linear gradient to 100 % B in 7 min, 100 % B in 3 min and then 100 % A in 10 min). DMT groups were cleaved with aq. AcOH (80 %, 100 μL) over 20 min. Afterwards, aq. AcONa (1 M, 50 μL) was added and the ONs were precipitated from EtOH (99 %, 550 μL). Their purities were found to be over 90 % by ion-exchange chromatography on a LaChrom system (Merck Hitachi) with a GenPak-Fax column (Waters). ONs were characterized by MALDI-TOF mass spectrometry on a Voyager Elite biospectrometry research station (PerSeptive Biosystems).

General procedure for microwave-assisted click chemistry with ONs and pyren-1-yl azide (5): $CuSO_4$ (0.5 μmol in 10 μL water) and triethylammonium acetate (TEAA, 50 μL, 0.2 M aq. solution) were added to a solution of pyren-1-yl azide (**5**,[24] 1 μmol, 0.24 mg) in DMSO (50 μL) in a microwave tube. The aromatic azide immediately precipitated from water. Ar was bubbled through the suspension for 1 min. Afterwards, freshly prepared ascorbic acid (10.9 μmol in 10 μL of water) and the oligonucleotide (0.05 μmol in 100 μL of water) were added and Ar was consistently bubbled through the mixture for 3 min. The microwave tube was placed in a microwave apparatus (Emrys Creator) and the following settings for the instrument were used: the heating temperature was set to 70 °C, the absorption level was set to very high, and a 1 min pre-stirring time was used. The reaction mixture was irradiated for 2–20 min, followed by intensive N_2 jet cooling to 40 °C. Afterwards, the mixture was transferred to the plastic vial and centrifuged at 13 000 g for 30 min. The solution was decanted and filtered, and the ON was isolated by reversed-phase semipreparative HPLC on a Waters Xterra MS C_{18} column with a Waters Delta Prep 4000 Preparative Chromatography System {Buffer A [0.05 M triethylammonium acetate in H_2O (pH 7.0)] and Buffer B (75 % CH_3CN in H_2O)}. Flow 2.5 mL min^{-1}. Gradients: 2 min 100 % A, linear gradient to 70 % B in 38 min, linear gradient to 100 % B in 7 min, 100 % B in 3 min, and then 100 % A in 10 min). Aq. AcONa (1 M, 50 μL) was added and the ON was precipitated from EtOH (99 %, 550 μL). The ON was characterized by MALDI-TOF MS. The purities of the synthesized **ON7–ON12** were found to be over 95 % by ion-exchange chromatography on a LaChrom system (Merck Hitachi) with a GenPak-Fax column (Waters).

General procedure for microwave-assisted click chemistry of ONs with benzyl azide (6): The preparation of the reaction mixture containing benzyl azide (**6**, Apollo Scientific Ltd.) (1 μmol, 0.087 μL), microwave-assisted click chemistry, and the product isolation and purification were achieved by the procedure for azide **5** described above. The purities of the synthesized **ON13–ON15** were found to be over 97 %.

Acknowledgements

This work was supported by the Sixth Framework Program Marie Curie Host Fellowships for Early Stage Research Training under contract number MEST-CT-2004—504018 and by the Nucleic Acid Center, which is funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

[1] J. M. Kalish, P. M. Glazer, *Ann. N. Y. Acad. Sci.* **2005**, *1058*, 151–161.
[2] V. V. Filichev, E. B. Pedersen, *J. Am. Chem. Soc.* **2005**, *127*, 14849–14858.
[3] I. Géci, V. V. Filichev, E. B. Pedersen, *Bioconjugate Chem.* **2006**, *17*, 950–957.
[4] V. V. Rostovstev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.
[5] Ch. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064.
[6] V. D. Bock, H. Hiemstra, J. H. Maarseveen, *Eur. J. Org. Chem.* **2006**, 51–68.
[7] T. S. Seo, Z. Li, H. Ruparel, J. Ju, *J. Org. Chem.* **2003**, *68*, 609–612.
[8] Z. J. Gartner, R. Grubina, Ch. T. Calderone, D. R. Liu, *Angew. Chem.* **2003**, *115*, 1408–1413; *Angew. Chem. Int. Ed.* **2003**, *42*, 1370–1375.
[9] R. L. Weller, S. R. Rajski, *Org. Lett.* **2005**, *7*, 2141–2144.
[10] G. A. Burley, J. Gierlich, M. R. Mofid, H. Nir, S. Tal, Y. Eichen, T. Carell, *J. Am. Chem. Soc.* **2006**, *128*, 1398–1399.

[11] A. K. Schrock, G. B. Schuster, *J. Am. Chem. Soc.* **1984**, *106*, 5234–5240.
[12] C. B. Chen, L. Milne, R. Landgraf, D. M. Perrin, D. S. Sigman, *ChemBioChem* **2001**, *2*, 735–740.
[13] P. Appukkuttan, W. Dehaen, V. V. Fokin, E. van der Eycken, *Org. Lett.* **2004**, *6*, 4223–4225.
[14] P. Kumar, K. C. Gupta, *Nucleic Acids Res.* **1997**, *25*, 5127–5129.
[15] P. Grünefeld, C. Richert, *J. Org. Chem.* **2004**, *69*, 7543–7551.
[16] T. Ernst, C. Richert, *Synlett* **2005**, 411–416.
[17] I. Velikyan, G. Lendvai, M. Välilä, A. Roivainen, U. Yngve, M. Bergström, B. Långström, *J. Labelled Compd. Radiopharm.* **2004**, *47*, 79–89.
[18] C. Bouillon, A. Meyer, S. Vidal, A. Jochum, Y. Chevolot, J.-P. Cloarec, J.-P. Praly, J.-J. Vasseur, F. Morvan, *J. Org. Chem.* **2006**, *71*, 4700–4702.
[19] U. B. Christensen, E. B. Pedersen, *Nucleic Acids Res.* **2002**, *30*, 4918–4925.
[20] U. Asseline, M. Delarue, G. Lancelot, F. Toulme, N. T. Thuong, T. Montenaygarestier, C. Hélène, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 3297–3301.
[21] S. Narayanan, J. Gall, C. Richert, *Nucleic Acids Res.* **2004**, *32*, 2901–2911.
[22] K. M. Guckian, B. A. Schweitzer, R. X. F. Ren, C. J. Sheils, P. L. Paris, D. C. Tahmassebi, E. T. Kool, *J. Am. Chem. Soc.* **1996**, *118*, 8182–8183.
[23] J. Isaksson, J. Chattopadhyaya, *Biochemistry* **2005**, *44*, 5390–5401.
[24] S. Goldstein, G. Czapski, *J. Am. Chem. Soc.* **1986**, *108*, 2244–2250.

Received: January 12, 2007
Published online: May 14, 2007

