Bridged Nucleic Acids

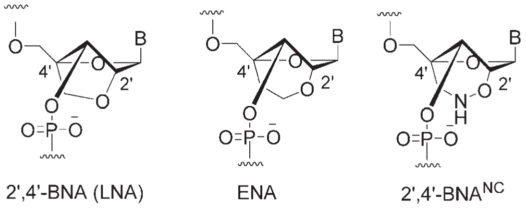
DOI: 10.1002/ange.200604857

Highly Stable Pyrimidine-Motif Triplex Formation at Physiological pH Values by a Bridged Nucleic Acid Analogue**

*S. M. Abdur Rahman, Sayori Seki, Satoshi Obika, Sunao Haitani, Kazuyuki Miyashita, and Takeshi Imanishi**

Formation of a stable triplex DNA molecule at physiological pH values is a highly desirable phenomenon in molecular biology and medicinal chemistry because of its great importance in regulation of gene expression, site-specific cleavage of DNA, gene mapping and isolation, maintenance of folded chromosome conformations, and gene-targeted mutagenesis.[1] In a pyrimidine-motif triplex DNA, the (homopyrimidine) triplex-forming oligonucleotide (TFO) binds with the homopurine tract of the target duplex DNA in a sequence-specific manner through Hoogsteen hydrogen bonds to form T·A:T and C^+·G:C triads. However, formation of the C^+·G:C triad is dependent on the cytosine protonation, which is only favorable at acidic pH values ($pK_a = 4.5$) and, therefore, homopyrimidine-motif triplexes are extremely unstable at physiological pH values, which severely restricts their biological application.

Although during the past few decades several efforts have been directed to the formation of stable triplex DNA, most of the investigations did not reach a practical level owing to instability of the triplexes at physiological pH values. Recently, our observation concluded that incorporation of a bridged nucleic acid (BNA),[2] such as 2′,4′-BNA[3] (also known as LNA;[4] Scheme 1) dramatically improved TFO affinity for the target duplex and formed a stable triplex at neutral pH values.[5] However, to our dismay, fully modified TFO failed to bind with double-stranded DNA (dsDNA). The optimum binding ability was found with TFOs composed of alternating BNA and DNA monomers. Ethylene-bridged nucleic acid (ENA), developed by us and Koizumi and co-workers,[6] also exhibited comparable or better triplex-forming ability than that obtained with 2′,4′-BNA. Although triplex formation with fully modified ENA was achieved at neutral pH values, partially modified TFOs provided variable results depending on the pH value (when compared with 2′,4′-BNA). As a result of our continued investigations of the BNA structure, we report herein a novel BNA molecule, 2′,4′-BNA^{NC}:[7] partially and fully modified TFOs[8] formed highly stable triplexes at physiological pH values. Their overall triplex-forming ability is superior to that of ENA and 2′,4′-BNA, which is the most widely used BNA (as LNA) for versatile genomic applications.[9]



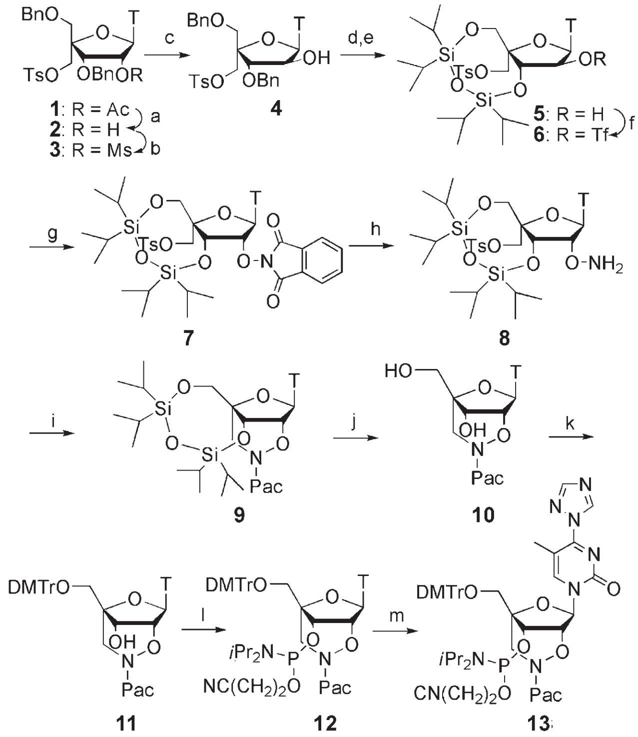
Scheme 1. Structures of 2′,4′-BNA (LNA), ENA, and 2′,4′-BNA^{NC}.

As shown in Scheme 2, the 2′,4′-BNA^{NC}–thymine and –5-methylcytosine phosphoroamidites **12** and **13**, respectively, were synthesized from the nucleoside derivative **1**.[10] The acetyl group was removed by aqueous methylamine and the resultant alcohol **2** was converted to a mesylate **3**, which was treated with alkali to give the stereochemically inverted alcohol **4** in very high yields. Debenzylation of **4** followed by reprotection with a cyclic disiloxy group afforded the bicyclic compound **5** in good yield.[11] The 2′-hydroxy group of **5** was transformed to the triflate **6** by the treatment of trifluoromethanesulfonic anhydride in the presence of DMAP and pyridine. The crude **6** was subjected to the S_N2 reaction with *N*-hydroxyphthalimide to yield the phthalimide derivative **7**, which was treated with hydrazine to deliver the aminoxy compound **8**. Exposure of **8** to phenoxyacetyl chloride in the presence of pyridine provided the desired cyclized product **9** in one step. Deprotection of the silyl groups furnished our target molecule, 2′,4′-BNA^{NC}–thymine monomer **10**, in excellent yields. Tritylation of the primary hydroxy group of **10** with 4,4′-dimethoxytrityl chloride gave **11**. Then, phosphitylation of the secondary hydroxy group of **11** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite yielded the desired thymine phosphoroamidite **12** in a very good yield. On treatment with 1,2,4-triazole in the presence of triethylamine and phosphoryl chloride,[12] compound **12** afforded the triazole derivative **13**, which was successfully used as a building block for the 5-methylcytidine unit of 2′,4′-BNA^{NC}. Various TFOs were synthesized from these phos-

[*] Dr. S. M. A. Rahman,[+] S. Seki, S. Haitani, Prof. K. Miyashita, Prof. T. Imanishi
Graduate School of Pharmaceutical Sciences
Osaka University
1-6 Yamadaoka, Suita, Osaka 565-0871 (Japan)
Fax: (+81) 6-6879-8204
E-mail: imanishi@phs.osaka-u.ac.jp
Dr. S. Obika
Graduate School of Pharmaceutical Sciences
Osaka University
and
PRESTO, JST
4-1-8 Honcho Kawaguchi, Saitama 332-0012 (Japan)
[+] On leave from the Faculty of Pharmacy
Dhaka University, Dhaka-1000 (Bangladesh)

[**] S.M.A.R. thanks the Japan Society of Promotion of Science (JSPS) for financial support.

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



Scheme 2. Synthesis of the 2′,4′-BNA^{NC} monomer **10** and the phosphoroamidites **12** and **13**. Reagents and conditions: a) 40% aqueous $MeNH_2$, THF, RT, 3 h (99%); b) MsCl, pyridine, RT, 1 h; c) 1 M NaOH, EtOH, RT, 1 h (95% from **2**); d) 20% $Pd(OH)_2$–C, cyclohexene, EtOH, reflux, 22 h; e) $TIPDSCl_2$, imidazole, DMF, RT, 5.5 h (67% from **4**); f) Tf_2O, DMAP, pyridine, RT, 7.5 h; g) *N*-hydroxyphthalimide, DBU, MeCN, RT, 12 h (61% from **5**); h) $H_2NNH_2 \cdot H_2O$, EtOH, RT, 15 min (73%); i) $PhOCH_2COCl$, Et_3N, CH_2Cl_2, RT, 2 h (57%); j) TBAF, THF, RT, 5 min (96%); k) DMTrCl, DMAP, pyridine, RT, 7 h (87%); l) $(iPr_2N)_2PO(CH_2)_2CN$, dicyanoimidazole, MeCN, RT, 4 h (85%); m) 1,2,4-triazole, $POCl_3$, Et_3N, MeCN, 0°C to RT, 5 h (95%). Bn = benzyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DMAP = 4-dimethylaminopyridine, DMF = N,N-dimethylformamide, DMTr = dimethoxytrityl = 4,4′-dimethoxytriphenylmethyl, Ms = methanesulfonyl, Pac = phenoxyacetyl T = thymin-1-yl, TBAF = tetra-*n*-butylammonium fluoride, Tf = trifluoromethanesulfonyl, TIPDS = tetraisopropyldisiloxane-l,3-diyl.

phoroamidites and natural amidite building blocks on an automated DNA synthesizer by using a conventional phosphoroamidite protocol. By using the usual workup procedure, the phenoxyacetyl group was removed and the triazole group was converted to an amino group to give 2′,4′-BNA^{NC}-modified TFOs. The TFOs were characterized by MALDI-TOF mass spectrometry (yields and the mass spectral data of the TFOs are provided in the Supporting Information).

The triplex-forming ability of 2′,4′-BNA^{NC}-modified TFOs against a 21-bp target DNA duplex at pH 7.0 was determined through UV melting curves (Figure 1 and UV melting curves in the Supporting Information). In the cases of the unmodified (**ON-0**) and slightly modified TFOs (such as **ON-1**), the usual two-phase dissociation curves were obtained. In contrast, only a strong transition was obtained for the extensively modified TFOs (**ON-5**, **ON-7**), which resulted from their very high triplex stability (triplex stability is higher than the target duplex stability). Unlike the usual curve, there is no transition

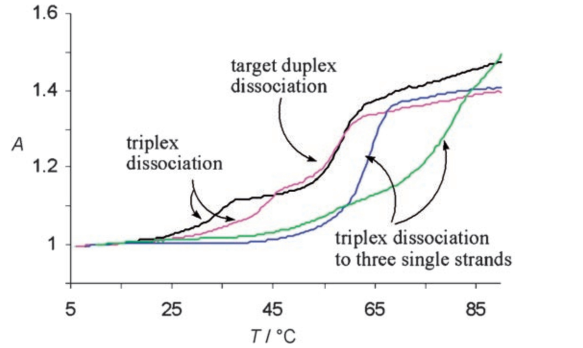


Figure 1. UV melting curves (260 nm) for triplexes formed by 2′,4′-BNA^{NC}-modified TFOs. **ON-0** ——, **ON-1** ——, **ON-5** ——, **ON-7** ——.

state for duplex dissociation and it might be concluded that triplexes were simultaneously converted to three different single strands.[13] Melting temperatures (T_m) of the triplexes formed by 2′,4′-BNA^{NC}-modified TFOs were compared with those formed by natural DNA, 2′,4′-BNA, and ENA-modified TFOs, and the results are summarized in Table 1. Modification of the natural DNA–TFO with a single 2′,4′-BNA^{NC}

Table 1: T_m values of triplexes containing 2,4,-BNA^{NC} (bold red), 2′,4′-BNA (bold blue), and ENA (bold black).[a,b]

TFO	Sequence (5′...3′)	T_m [°C]	ΔT_m [°C]	ΔT_m/mod
ON-0	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	33	–	–
ON-1	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	44	+11	+11.0
BNA-1	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	44	+11	+11.0
ENA-1	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	42	+9	+9.0
ON-2	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	60	+27	+9.0
BNA-2	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	59	+26	+8.7
ENA-2	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	56	+23	+7.7
ON-3	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	59	+26	+8.7
BNA-3	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	52	+19	+6.3
ENA-3	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	57	+24	+8.0
ON-4	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	58	+25	+6.3
BNA-4	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	57	+24	+6.0
ENA-4	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	57	+24	+6.0
ON-5	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	64	+31	+6.2
BNA-5	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	65	+32	+6.4
ENA-5	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	58	+25	+5.0
ON-6	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	78	+45	+6.4
BNA-6	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	67	+34	+4.9
ENA-6	TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT	72	+39	+5.6
ON-7	**TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT**	80	+47	+3.1
BNA-7	**TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT**	<5	<−28	<−2

[a] Target duplex: 5′-d(GCTAAAAAGAAAGAGAGATCG)-3′/3′-d(CGATTTTTCTTTCTCTCTAGC)-5′; underlined portion indicates the target site for triplex formation. [b] Conditions: 7 mM Na_2HPO_4 buffer solution containing 140 mM KCl; strand concentration = 1.5 μM; scan rate 0.5 °C min^{-1}. T_m = melting temperatures, ΔT_m = changes in melting temperature, ΔT_m/mod = changes in melting temperature per single modification; ^{m}C = 5-methylcytidine.

monomer (**ON-1**) increased the T_m value by 11 °C, which is equal to that of 2′,4′-BNA-modified TFO (**BNA-1**) and slightly higher than that of the corresponding ENA-modified TFO (**ENA-1**). Further modifications greatly enhanced the triplex thermal stability. For example, by increasing the number of modifications from one to three, T_m values increased to 60 °C ($\Delta T_m = +27$ °C) and 59 °C ($\Delta T_m = +26$ °C) for **ON-2** and **ON-3**, respectively. Therefore, it is noteworthy that both the values of the 2′,4′-BNA^{NC}-modified TFOs with either interrupted or continuous 2′,4′-BNA^{NC} residues are very high and the same, whereas the T_m value of **BNA-3** (52 °C) containing three continuous modifications was found to decrease by 7 °C compared with that of **BNA-2** (59 °C). The corresponding ENA–TFOs (**ENA-2** and **ENA-3**) showed lower T_m values than the 2′,4′-BNA^{NC}–TFOs. Interestingly, their triplex-forming behavior is in agreement with that of 2′,4′-BNA^{NC}, clarifying that continuous six-membered bridged structures are well tolerated by dsDNA. These observations were also consistent with the results of other TFOs (such as, **ON-4** and **ON-6** versus **BNA-4**, **BNA-6** versus **ENA-4** and **ENA-6**, and **ON-5** versus **BNA-5**) with the exception of **ENA-5**. In the cases of **ON-4/BNA-4/ENA-4** and **ON-5/BNA-5** where modifications are located far apart from each other, all the 2′,4′-BNA^{NC}–, 2′,4′-BNA–, and ENA–TFOs furnished similar T_m values for triplex dissociation. The reason for the lower T_m value for **ENA-5** is not clear. In contrast, with relatively congested BNA residues, 2′,4′-BNA^{NC}–TFO (**ON-6**) provided T_m values as high as 78 °C, which is 11 °C and 6 °C higher than those provided by the corresponding 2′,4′-BNA– and ENA–TFOs (**BNA-6** and **ENA-6**), respectively. Thus, with continuous modifications or with an increased number of modifications, 2′,4′-BNA^{NC}-modified TFOs showed higher T_m values than those of 2′,4′-BNA- and ENA-modified TFOs. These interesting characteristics of 2′,4′-BNA^{NC} prompted us to synthesize a fully modified TFO, **ON-7**, which formed a very stable triplex with a T_m value as high as 80 °C ($\Delta T_m = +47$ °C; ΔT_m per modification = +3.1 °C). The corresponding 2′,4′-BNA-modified TFO, **BNA-7**, failed to form a triplex.[14,15]

Next, triplex formation was evaluated by an electrophoretic mobility shift assay (EMSA) at pH 6.8 (Figure 2). Each TFO was incubated with the target dsDNA at a ratio of 1:1 at 4 °C in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid buffer solution (HEPES; pH 6.8) and subjected to a 20% polyacrylamide gel electrophoresis at 4 °C and room temperature.[16] To confirm the triplex formation, the target duplex without TFO and TFO with excess duplex (TFO/duplex = 1:2) were also run together (Figure 1, lanes 1 and 3, respectively). It was found that all the modified TFOs formed stable triplexes at the stoichiometric ratio under the experimental conditions. The natural DNA (**ON-0**) was unable to form a triplex at room temperature even though it showed triplex formation at 4 °C. In contrast, the TFO with only a single 2′,4′-BNA^{NC} modification (**ON-1**) can form a stable triplex at room temperature. These results correlate with the T_m data and it might be expected that extensively modified TFOs would promote stable triplex formation at higher temperatures. The fully modified TFO, **ON-7**, also formed a stable triplex with a clear and intense band. The electrophoretic mobilities of the triplexes formed by 2′,4′-BNA^{NC}-modified TFOs were slightly lower and that of the fully modified TFO (**ON-7**) was remarkably lower.[17]

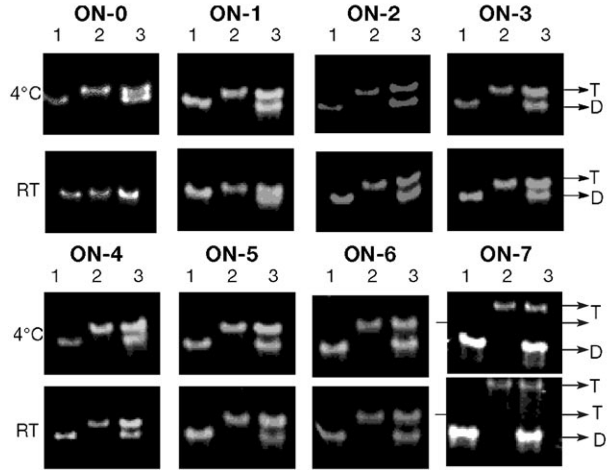


Figure 2. Electrophoretic mobility shift assay of the triplexes on 20% nondenaturing polyacrylamide gel at pH 6.8. Lanes 1, 2, and 3 in all cases represent a target duplex (control), stoichiometric mixture (6.5 pmol) of target duplex and TFO, and TFO with excess duplex (TFO/duplex = 1:2), respectively. T = triplex, D = duplex.

The extraordinarily high triplex-forming ability of 2′,4′-BNA^{NC} might result from the combined effects of restricted N conformation[5,18] and protonation of the N atom, which might cause electrostatic interactions between the positively charged TFO and the negatively charged phosphodiester linkage of the target duplex.[8c,19–21] Moreover, in contrast with the fully modified BNA–TFOs, which are too rigid in the overall structure,[5b,22] the 2′,4′-BNA^{NC}-modified TFOs, bearing a six-membered bridged structure like ENA, might pose suitable conformational flexibility in their overall structure, which facilitates stable triplex formation as well. The above facts are the reason for which the 2′,4′-BNA^{NC}-modified TFOs act as excellent TFOs for the recognition of the homopurine–homopyrimidine tract of dsDNA. The predominance of 2′,4′-BNA^{NC} over ENA and 2′,4′-BNA essentially lies with the role of the protonated nitrogen to neutralize the negatively charged phosphate backbone of the purine strand.

In conclusion, we have synthesized a novel bridged nucleic acid analogue, 2′,4′-BNA^{NC}, and demonstrated that the TFOs composed of 2′,4′-BNA^{NC} formed highly stable pyrimidine-motif triplexes at physiological pH values. The overall triplex-forming ability is higher than that of 2′,4′-BNA/LNA- and ENA-modified TFOs. Unlike the 2′,4′-BNA-modified TFOs, these TFOs eliminate the requirement of placing alternating DNA monomers for optimum efficacy.[23] More interestingly, fully modified TFOs still formed a highly stable triplex. These promising properties of 2′,4′-BNA^{NC} will be helpful for developing oligonucleotide-based technologies for the postgenome era.

Experimental Section

UV melting experiments: UV melting experiments were carried out on a Beckman DU-650 spectrometer equipped with a T_m analysis accessory. Equimolecular amounts of the target duplex and TFO were dissolved in 7 mM sodium phosphate buffer solution (pH 7.0) containing 140 mM KCl to give a final strand concentration of 1.5 μM. The strands were annealed by heating the samples at 90 °C for 5 minutes followed by slow cooling to room temperature. Then the samples were stored at 4 °C for 1 h. The melting profile was recorded at 260 nm from 10 to 85 °C at a scan rate of 0.5 °C min^{-1}. The T_m was calculated as the temperature of the half dissociation of the formed triplexes, which is determined by the first derivative of the melting curve.

Full experimental details are described in the Supporting Information.

Received: November 30, 2006
Revised: March 26, 2007
Published online: April 27, 2007

Keywords: bridged nucleic acids · oligonucleotides · synthesis design · thermal stability · triplexes

[1] a) H. E. Moser, P. B. Dervan, *Science* **1987**, *238*, 645; b) M. D. Frank-Kamenetskii, S. M. Mirkin, *Annu. Rev. Biochem.* **1995**, *64*, 65; c) V. N. Soyfer, V. N. Potaman, *Triple-Helical Nucleic Acids*, Springer, New York, **1996**, p. 220; d) M. M. Seidman, P. M. Glazer, *J. Clin. Invest.* **2003**, *112*, 487; e) S. Buchini, C. J. Leumann, *Curr. Opin. Chem. Biol.* **2003**, *7*, 717.

[2] T. Imanishi, S. Obika, *Chem. Commun.* **2002**, 1653.

[3] S. Obika, D. Nandu, Y. Hari, K. Morio, Y. In, T. Ishida, T. Imanishi, *Tetrahedron Lett.* **1997**, *38*, 8735.

[4] S. K. Singh, P. Nielsen, A. A. Koshkin, J. Wengel, *Chem. Commun.* **1998**, 455.

[5] a) S. Obika, Y. Hari, T. Sugimoto, M. Sekiguchi, T. Imanishi, *Tetrahedron Lett.* **2000**, *41*, 8923; b) S. Obika, T. Uneda, T. Sugimoto, D. Nanbu, T. Minami, T. Doi, T. Imanishi, *Bioorg. Med. Chem.* **2001**, *9*, 1001; c) H. Torigoe, Y. Hari, M. Sekiguchi, S. Obika, T. Imanishi, *J. Biol. Chem.* **2001**, *276*, 2354; d) S. Obika, Y. Hari, M. Sekiguchi, T. Imanishi, *Angew. Chem.* **2001**, *113*, 2149; *Angew. Chem. Int. Ed.* **2001**, *40*, 2079; e) S. Obika, Y. Hari, M. Sekiguchi, T. Imanishi, *Chem. Eur. J.* **2002**, *8*, 4796.

[6] a) K. Morita, C. Hasegawa, M. Kaneko, S. Tsutsumi, J. Sone, T. Ishikawa, T. Imanishi, M. Koizumi, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 73; b) M. Koizumi, K. Morita, M. Daigo, S. Tsutsumi, K. Abe, S. Obika, T. Imanishi, *Nucleic Acids Res.* **2003**, *31*, 3267.

[7] This BNA is defined as 2′,4′-BNA^{NC} because the bridge between 2′-*O* and 4′-*C* is constituted by N and C atoms.

[8] The other fully modified TFOs formed triplexes at around the physiological pH value, see: a) M. Bolli, C. Leumann, *Angew. Chem.* **1995**, *107*, 694; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 694; b) C. Escudé, C. Giovannangeli, J.-S. Sun, D. H. Lloyd, J.-K. Chen, S. M. Gryaznov, T. Garestier, C. Hélène, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4365; c) B. Cuenoud, F. Casset, D. Husken, F. Natt, R. M. Wolf, K.-H. Altmann, P. Martin, H. E. Moser, *Angew. Chem.* **1998**, *110*, 1350; *Angew. Chem. Int. Ed.* **1998**, *37*, 1288; d) S. M. Gryaznov, H. Winter, *Nucleic Acids Res.* **1998**, *26*, 4160; e) N. Kumar, K. E. Nielsen, S. Maiti, M. Petersen, *J. Am. Chem. Soc.* **2006**, *128*, 14.

[9] a) M. Petersen, J. Wengel, *Trends Biotechnol.* **2003**, *21*, 74; b) J. S. Jespen, J. Wengel, *Curr. Opin. Drug Discovery Dev.* **2004**, *7*, 188; c) B. Vester, J. Wengel, *Biochemistry* **2004**, *43*, 13233; d) L. Wang, C. J. Yang, C. D. Medley, S. A. Benner, W. Tan, *J. Am. Chem. Soc.* **2005**, *127*, 15664.

[10] A. A. Koshkin, V. K. Rajwanshi, J. Wengel, *Tetrahedron Lett.* **1998**, *39*, 4381.

[11] We had to replace the benzyl groups with a cyclic disiloxy group because we experienced cleavage of the N−O bridged structure during debenzylation of the corresponding dibenzyl derivative of **9**.

[12] a) K. Shah, H. Wu, T. M. Rana, *Bioconjugate Chem.* **1994**, *5*, 508; b) M. Scherr, C. Klebba, R. Häner, A. Ganser, J. W. Engels, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1791.

[13] Similar triplex-to-single-strand dissociation was also reported recently by Leumann, see: S. Buchini, C. J. Leumann, *Angew. Chem.* **2004**, *116*, 4015; *Angew. Chem. Int. Ed.* **2004**, *43*, 3925.

[14] Similar results were obtained by using two different BNA–TFOs in our previous investigation. The TFOs also failed to bind with a 30-bp target duplex.[5b]

[15] The T_m value of the corresponding ENA–TFO could not be determined because of the difficulties in purification of the TFO.

[16] In the case of room temperature EMSA, the triplexes were warmed to room temperature after incubating at 4 °C and kept at room temperature for two hours before applying onto the gel. The gel was run at room temperature at a constant voltage of 70 V.

[17] This type of lower mobility was also found in the case of the recently reported α-L-LNA-modified triplexes.[8e] Variation of triplex mobility was also noted by Roberts and Crothers, see: R. W. Roberts, D. M. Crothers, *Science* **1992**, *258*, 1463.

[18] J. L. Asensio, R. Carr, T. Brown, A. N. Lane, *J. Am. Chem. Soc.* **1999**, *121*, 11063.

[19] S. Rhee, Z.-J. Han, K. Liu, H. T. Miles, D. R. Davis, *Biochemistry* **1999**, *38*, 16810.

[20] T. P. Prakash, A. Puschl, E. Lesnik, V. Mohan, V. Tereshko, M. Egli, M. Manoharan, *Org. Lett.* **2004**, *6*, 1971.

[21] A. Mayer, A. Häberli, C. Leumann, *Org. Biomol. Chem.* **2005**, *3*, 1653.

[22] C. H. Gotfredsen, P. Schultze, J. Feigon, *J. Am. Chem. Soc.* **1998**, *120*, 4281.

[23] Rules for designing BNA–TFOs for optimum triplex-forming ability were described, see: B. W. Sun, B. R. Babu, M. D. Sørensen, K. Zakrzewska, J. Wengel, *Biochemistry* **2004**, *43*, 4160.

