Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1001–1011

BIOORGANIC & MEDICINAL CHEMISTRY

2′-*O*,4′-*C*-Methylene Bridged Nucleic Acid (2′,4′-BNA): Synthesis and Triplex-Forming Properties[1]

Satoshi Obika, Takeshi Uneda, Tomomi Sugimoto, Daishu Nanbu, Takashi Minami, Takefumi Doi and Takeshi Imanishi*

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

Received 3 October 2000; accepted 21 November 2000

Abstract—For development of ideal antisense and antigene molecules, various chemical modifications of oligonucleotides have been studied. However, despite their importance, there is only limited information available on the triplex-forming ability of the conformationally restricted or locked oligonucleotides. We report herein that 2′-*O*,4′-*C*-methylene bridged nucleic acid (2′,4′-BNA) modification of triplex-forming oligonucleotide (TFO) significantly enhances the binding affinity towards target dsDNA. On T_m measurements, the triplex with the 2′,4′-BNA oligonucleotides were found to be stabilized with ΔT_m/modification of +4.3 to +5 °C at pH 6.6 compared to the triplexes with the unmodified oligonucleotide. By means of gel-retardation assay, the binding constant of the 2′,4′-BNA oligonucleotide at pH 7.0 was at least 300-fold higher than that of the natural oligonucleotide. In addition, the 2′,4′-BNA oligonucleotide clearly showed the inhibition of the NF-κB transcription factor (p50)-target dsDNA binding by forming a stable triplex at pH 7.0.

Introduction

Homopyrimidine oligodeoxyribonucleotides can bind to homopurine sequences in dsDNA via Hoogsteen hydrogen bonding to form triplex DNA.[2] In recent years, interest in the possibility of regulation of gene expression by triplex-forming oligonucleotides (TFOs) has grown because of their widely usable application as molecular biological tools and as a direct way to treat serious diseases such as cancer and genetic disorders. In addition, the structure of these triplexes has also been attracting widespread attention. Since the binding affinity of the natural pyrimidine oligonucleotides towards dsDNA is relatively low under physiological conditions, extensive efforts have been directed towards developing novel DNA and RNA analogues for the practical use of the antigene technologies and for clarification of the triplex structure.[3] However, only a few modified TFOs[3–5] with sufficient binding affinity under physiological conditions have been reported, and the overall conformations of triplexes and the structural requirements for ideal TFOs are not well understood.[6] It occurred to us that restriction of the oligonucleotide conformation in a suitable form would be quite effective for triplex-formation, as well as binding with complementary ssDNA or ssRNA.[7] Recently, we have achieved the first synthesis and development of a novel class of modifications of nucleic acids, bridged nucleic acid (BNA), such as 2′-*O*,4′-*C*-methylene BNA (2′,4′-BNA; Fig. 1)[1,8–15] and 3′-*O*,4′-*C*-methylene BNA (3′,4′-BNA).[15–18] The 2′,4′-BNA oligonucleotides were found to have a potent hybridizing ability towards complementary nucleic acids, especially towards complementary RNA.[1,9,19] Furthermore, it was also found that only one 2′,4′-BNA modification of TFO promoted the marked triplex stabilization in a highly sequence-selective manner.[13] Now, we describe the triplex-forming ability of the fully or partially 2′,4′-BNA modified homopyrimidine TFO and inhibition of NF-κB (p50)–DNA binding by the triplex formation.

Results

Synthesis of 2′,4′-BNA monomers and their oligonucleotide derivatives

As shown in Scheme 1, 2′,4′-BNA monomers, 2′-*O*,4′-*C*-methyleneuridine ($\mathbf{1_U}$) and its 5-methyl congener $\mathbf{1_T}$, were synthesized effectively from 1,2-*O*-cyclohexylidene-

*Corresponding author. Tel.: +81-6-6879-8299; fax: +81-6-6879-8204; e-mail: imanishi@phs.osaka-u.ac.jp

0968-0896/01/$ - see front matter
PII: S0968-0896(00)00325-4

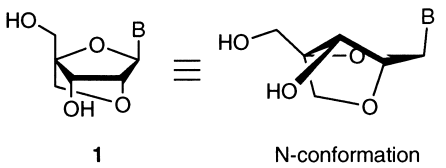
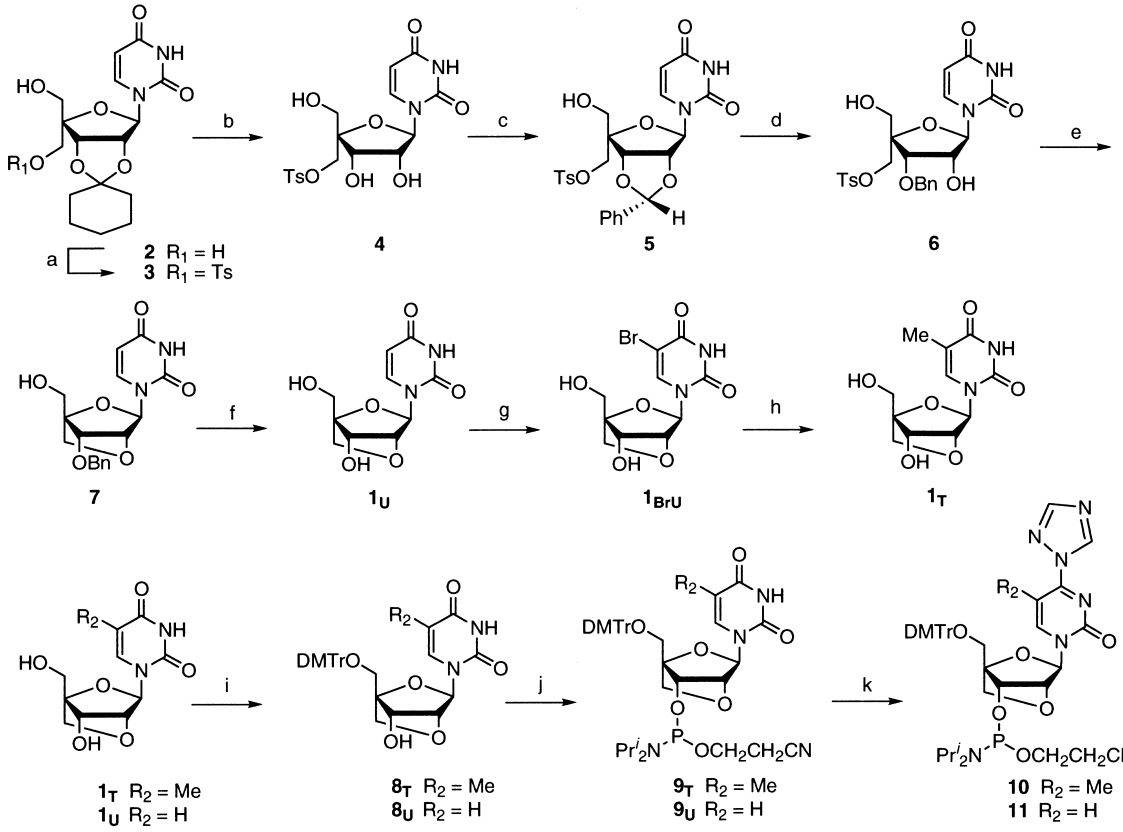


Figure 1. Structure of 2′,4′-BNA monomers, 2′-*O*,4′-*C*-methyleneribonucleosides (**1**).

4-hydroxymethyluridine (**2**).[20,21] Treatment of **2** with *p*-toluenesulfonyl chloride in pyridine gave the monotosylated compound **3**, which was in turn deprotected under acid conditions to give the triol **4**. Without the protection of the 3′-hydroxy group, the oxetane compound was obtained by the ring-closure reaction under alkaline conditions.[16] Therefore, the triol **4** was initially treated with benzaldehyde in the presence of zinc chloride to give 2′,3′-*O*-benzylidene derivative **5** as an *endo*-isomer,[22] and then **5** was reduced with sodium cyanoborohydride and titanium tetrachloride to afford the desired 3′-*O*-benzyl derivative **6**. Ring-closure reaction of **6** was smoothly achieved by using sodium hexamethyldisilazide in tetrahydrofuran, and the reductive debenzylation of **7** gave 2′-*O*,4′-*C*-methyleneuridine ($\mathbf{1_U}$). Furthermore, $\mathbf{1_U}$ was effectively converted to its 5-methyl congener $\mathbf{1_T}$[23] via 5-bromination and subsequent Pd-catalyzed cross-coupling reaction with trimethylaluminum.[25] The phosphoramidites $\mathbf{9_T}$ and $\mathbf{9_U}$, the suitable building block for DNA synthesis, were obtained by dimethoxytritylation and subsequent phosphitylation of $\mathbf{1_T}$ and $\mathbf{1_U}$, respectively. According to Xu's procedure,[26] the amidites $\mathbf{9_T}$ and $\mathbf{9_U}$ were transformed into 4-triazolo derivatives **10** and **11**, which were easily converted into the cytidine derivatives by treatment with concd ammonia after oligonucleotide synthesis. Oligonucleotide analogues **12**–**20** containing 2′-*O*,4′-*C*-methyleneribonucleosides were effectively prepared by standard phosphoramidite protocol on a DNA synthesizer. Crude oligonucleotides were purified by RP-HPLC and the structure of the prepared oligonucleotides was confirmed by MALDI-TOF MS analysis.

Triplex formation of 2′,4′-BNA oligonucleotides

At first, the triplex-forming property of the 14-mer 2′,4′-BNA oligonucleotides **12**–**20** towards the 30 bp target duplex was studied by an analysis of the UV melting curve, as shown in Table 1. The target 30 bp DNA is an upstream region of a mouse inducible NO synthase (miNOS) gene,[27] containing a part of the NF-κB binding sequence (Fig. 2). Absorbance at 260 nm was measured versus temperature. The triplex was formed by mixing both strands of the duplex (2 μM) with 2 μM TFO in a 10 mM sodium phosphate buffer (pH 6.6 or 7.2) containing 100 mM NaCl. The natural TFO **21** was not able to bind to the target duplex at both pH 6.6 and 7.2. The TFO **22** in which the cytosine base is replaced by 5-methylcytosine that is well known to enhance the



Scheme 1. (a) TsCl, pyridine, 110 °C, 69%; (b) CF_3CO_2H–H_2O (98:2), 20 °C, 94%; (c) PhCHO, $ZnCl_2$, 20 °C, 80%; (d) $NaBH_3CN$, $TiCl_4$, MeCN, 20 °C, 75%; (e) sodium bis(trimethylsilyl)amide, THF, 20 °C, 61%; (f) H_2 (1 atm), 10%Pd-C, MeOH, 20 °C, quant; (g) NBS, NaN_3, 1,2-dimethoxyethane, 20 °C, 84%; (h) (i) $(NH_4)_2SO_4$, 1,1,1,3,3,3-hexamethyldisilazane, reflux; (ii) $PdCl_2$, Ph_3P, Me_3Al, THF, reflux; (iii) NH_4Cl, MeOH–H_2O, reflux, 57%; (i) DMTrCl, DMAP, pyridine, 20 °C, 95% ($\mathbf{8_T}$), 94% ($\mathbf{8_U}$); (j) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, diisopropylammonium tetrazolide, MeCN-THF, 20 °C, 99% ($\mathbf{9_T}$), 81% ($\mathbf{9_U}$); (k) 1,2,4-triazole, $POCl_3$, Et_3N, MeCN, 0 °C, 91% (**10**), 89% (**11**).

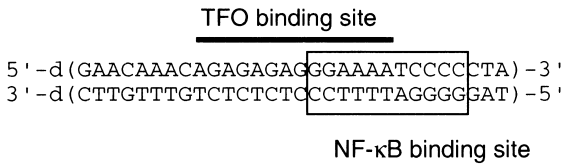


Figure 2. DNA sequence of the target 30 bp duplex used in this study. The bold line indicates the site of triplex formation. The boxed sequence is the NF-κB binding site.

triplex stability under neutral conditions,[28] was able to form the triplex at pH 6.6. On the other hand, the partially 2′,4′-BNA modified TFOs **12**–**18** were shown to have significantly enhanced triplex-forming ability. In particular, the 2′,4′-BNA modified TFOs **13**, **15** and **17** containing a 5-methylcytosine moiety, showed remarkable stabilizing effect of the triplex with ΔT_m/modification ranging from +4.3 to +5 °C compared to **22** at pH 6.6. Unexpectedly, the fully 2′,4′-BNA modified (except for the 3′-end) TFOs **19** and **20** exhibited no triplex-forming ability at both pH 6.6 and 7.2.

Because of the overlapping of the transition curves of duplex and triplex dissociation, it was hard to evaluate the triplex-forming ability accurately at lower pH by T_m measurements. Thus, DNase I footprinting was carried out on the 521 bp duplex containing the target 30 bp sequence (Fig. 3). At both pH 5.5 and 6.0, DNA cleavage by DNase I was perfectly inhibited in a sequence-dependent manner by addition of the natural TFOs **21**, **22** or the partially 2′,4′-BNA modified TFOs **12**–**18**. However, it was not sufficiently inhibited by addition of the fully 2′,4′-BNA modified TFO **20**. At pH 6.6, the target sequence was also protected from DNase I cleavage by addition of **22** or **12**–**18**, while the natural TFO **21** and the fully 2′,4′-BNA modified TFO **20** was not able to inhibit the cleavage. Furthermore, the partially 2′,4′-BNA modified TFOs **13**, **15** and **17** formed a triplex towards the target site and protected from DNase I digestion even at pH 7.2. These results from DNase I footprinting experiments coincide with those of T_m measurements.

Next, the binding affinity of the partially 2′,4′-BNA modified TFO **17** towards the target duplex was also elucidated by using gel-retardation assay (Fig. 4). Both strands of the target duplex were 5′-end labeled and incubated with the indicated concentration of **17** or **22**

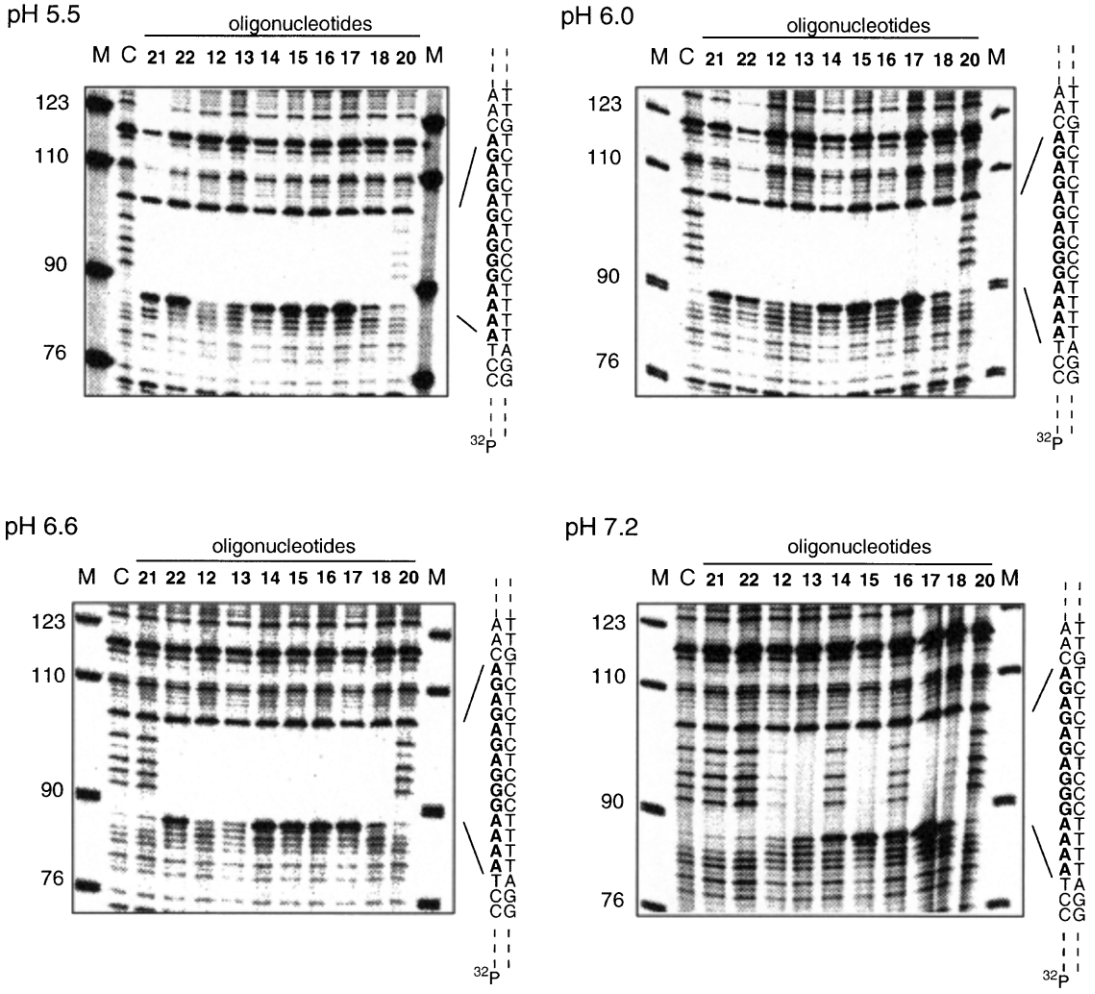


Figure 3. DNase I footprinting assay demonstrating the binding of the 2′,4′-BNA modified (**12**–**18**, **20**) and natural (**21**, **22**) TFOs to the oligopurine target site AGAGAGAGGGAAAA. A 521 bp ^{32}P-labeled fragment (0.2 nM) was treated with indicated oligonucleotides (2 μM) at 18 °C for 48 h in a 50 mM sodium acetate buffer (pH 5.5) or in a 50 mM sodium phosphate buffer (pH 6.0, 6.6 or 7.2) containing 10 mM magnesium chloride, and then digested by DNase I. Lane C (control) lacking an oligonucleotide is also shown. Lane M contains markers of the indicated sizes in bases.

in 50 mM HEPES buffer (pH 7.0) containing 20 mM $MgCl_2$ and then loaded onto a non-denaturing gel. Upon addition of **17** to a 2×10^{-10} M solution of the 30 bp target duplex, triplex formation was detected with a midpoint near 3×10^{-8} M at pH 7.0 (Fig. 4a). The addition of **17** at 1×10^{-7} M final concentration led to the complete shift of the target duplex (*DS*) to a triplex band (*TS*), and no other shifted bands were detected in these conditions. On the other hand, addition of TFO 22, a 5-methylcytosine analogue of **21**, showed no triplex-formation even at 3×10^{-6} M (Fig. 4b). The data in Fig. 4a have been quantified and are plotted against the concentration of the triplex-forming oligonucleotides (Fig. 4c), where the dissociation constant (K_d) of **17** was estimated to be 2.5×10^{-8} M at pH 7.0,[29] which was at least 300-fold lower than that of **22**. Considering the relatively high G•C base pair contents of the target sequence and binding conditions (without a polyamine such as spermine), the 2′,4′-BNA oligonucleotides appears to be one of the most potent triplex-forming oligonucleotides via Hoogsteen base pairing.

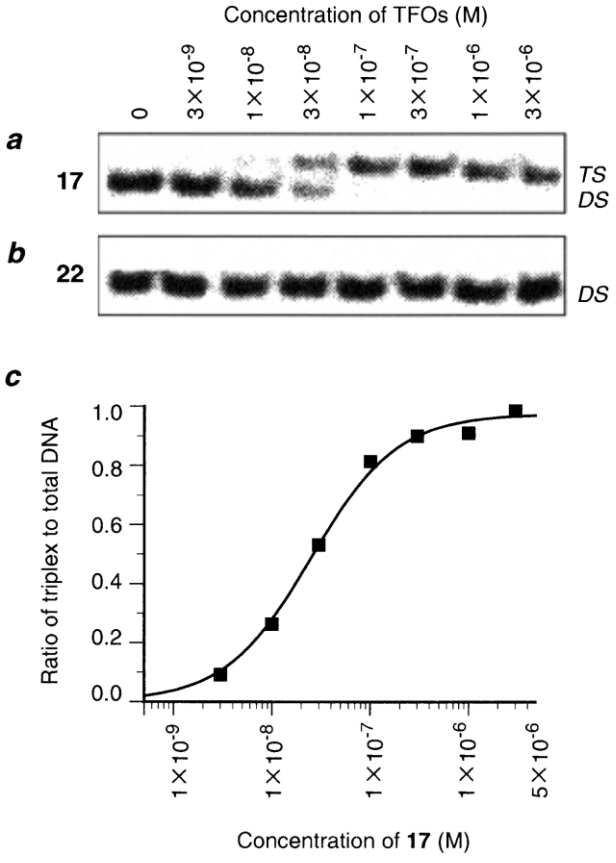
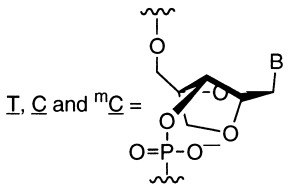


Figure 4. Gel-retardation experiments showing the binding of the TFOs to the 30-bp target duplex. The indicated concentration (M) of the TFOs **17** (panel a) and **22** (panel b) was incubated with a ^{32}P-labeled target fragment (~0.2 nM), 5′-d(GAACAAACAGAGAGAGGGAAAATCCCCCTA)/3′-d(CTTGTTTGTCTCTCTCCCTTTTAGGGGGAT), at 4 °C for 48 h in 50 mM HEPES buffer (pH 7.0) containing 20 mM magnesium chloride. *TS* and *DS* indicate mobility of the triplex and duplex, respectively. Panel c shows the titration curve of TFO **17**. The ratio of triplex band (*TS*) relative to total DNA (*TS* + *DS*) is plotted against the concentration of TFO **17**.

Table 1. T_m values (°C) of the triplexes consisting of the target 30-bp duplex and TFOs **10**–**20**[a]



Oligonucleotides[b]		pH 6.6	pH 7.2
5′-d(TCTCTCTCCCTTT)-3′	(**21**)	—[c] (64)	—[c] (64)
5′-d(T^{m}CT^{m}CT^{m}CT^{m}C^{m}C^{m}CTTT)-3′	(**22**)	28 (63)	—[c] (63)
5-′d(TCTCTCTCCCTTTT)-3′	(**12**)	39 (64)	23 (63)
5′-d(T^{m}CT^{m}CT^{m}CT^{m}C^{m}C^{m}CTTTT)-3′	(**13**)	53 (64)	36 (65)
5′-d(TCTCTCTCCCTTTT)-3′	(**14**)	41 (64)	—[c] (64)
5′-d(T^{m}CT^{m}CT^{m}CT^{m}C^{m}C^{m}CTTTT)-3′	(**15**)	54 (64)	37 (64)
5′-d(TCTCTCTCCCTTTT)-3′	(**16**)	41 (64)	—[c] (63)
5′-d(T^{m}CT^{m}CT^{m}CT^{m}C^{m}C^{m}CTTTT)-3′	(**17**)	55 (64)	33 (64)
5′-d(TCTCTCTCCCTTTT)-3′	(**18**)	48 (63)	28 (63)
5′-d(TCTCTCTCCCTTTT)-3′	(**19**)	—[c] (64)	—[c] (63)
5′-d(T^{m}CT^{m}CT^{m}CT^{m}C^{m}C^{m}CTTT)-3′	(**20**)	—[c] (64)	—[c] (63)

[a]The melting temperatures (T_m values) were obtained as the maxima of the first derivatives of the melting curves (A_{260} versus temperature) using 2 μM concentration of the triplex in a buffer containing 10 mM sodium phosphate (pH 6.6 or 7.2) and 100 mM sodium chloride. The values in the parentheses are T_m of the target duplex.
[b]T, thymidine, C, 2′-deoxycytidine, ^{m}C, 2′-deoxy-5-methylcytidine.
[c]Obvious thermal hypochromicity was not observed.

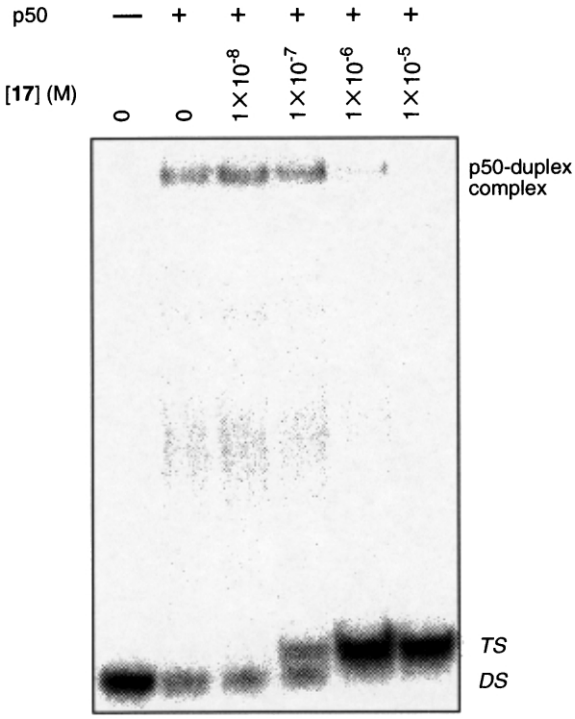


Figure 5. Gel-retardation experiments showing the inhibition of the NF-κB (p50) binding toward the 30-bp target duplex by addition of TFO **17**. The indicated concentration of **17** was incubated with a ^{32}P-labeled target fragment (~1 nM), 5′-d(GAACAAACAGAGAGAGGGAAAATCCCCCTA)/3′-d(CTTGTTTGTCTCTCTCCCTTTTAGGGGGAT), and p50 (1.2 gel shift units) at 4 °C for 48 h in 9 mM HEPES buffer (pH 7.0) containing 45 mM KCl, 0.2 mM EDTA, 9 mM $MgCl_2$, 2 mM DTT, 9% glycerol, 0.05% NP40, 0.05 μg/μL poly (dI-dC)•poly (dI-dC).

Inhibition of NF-κB (p50) binding to target sequence by triplex formation

The 30 bp target duplex used in the above experiments contains an NF-κB binding sequence, and the NF-κB binding site overlaps the TFO binding site in part (Fig. 2). To elucidate the inhibition of NF-κB-DNA association by the 2′,4′-BNA modified TFO **17**, gel retardation experiments were examined in the presence of a subunit of NF-κB, p50, which contains a DNA binding motif and can bind to DNA as a p50 dimer.[30] The labeled target duplex (1 nM) and p50 were incubated with the indicated concentration of **17** at pH 7.0. In the absence of **17**, the shifted band coming from the p50-target duplex complex was observed (Fig. 5). No change was detected in the gel-shift pattern by the addition of **17** at 1×10^{-8} M final concentration, while 1×10^{-7} M of **17** led to the formation of a triplex. Further addition of **17** showed inhibition of the p50 binding to the target duplex along with the formation of a triplex. At 1×10^{-6} M concentration, the formation of a p50–duplex complex was prevented for the most part, and the complete inhibition was observed at 1×10^{-5} M.

Discussion

The ideal sugar conformation of TFOs has been discussed by some groups to date.[4,31,32] Gryaznov and his co-workers reported that the N3′–P5′ phosphoramidate oligonucleotides significantly enhance triplex formation.[4] By means of X-ray crystallography of the duplex consisting of the self-complementary phosphoramidate oligonucleotide, the phosphoramidate oligonucleotides were found to prefer existing in A-form (N-form sugar puckering).[33] Recently, Asensio also reported that the 2′-OMe oligonucleotide was able to form a stable triplex with dsDNA where the 2′-OMe ribonucleoside derivatives exist in *N*-conformation predominantly.[31] Although these data strongly suggested that the *N*-form sugar puckering is suitable for triplex formation, the investigation by using the oligonucleotides with dis-

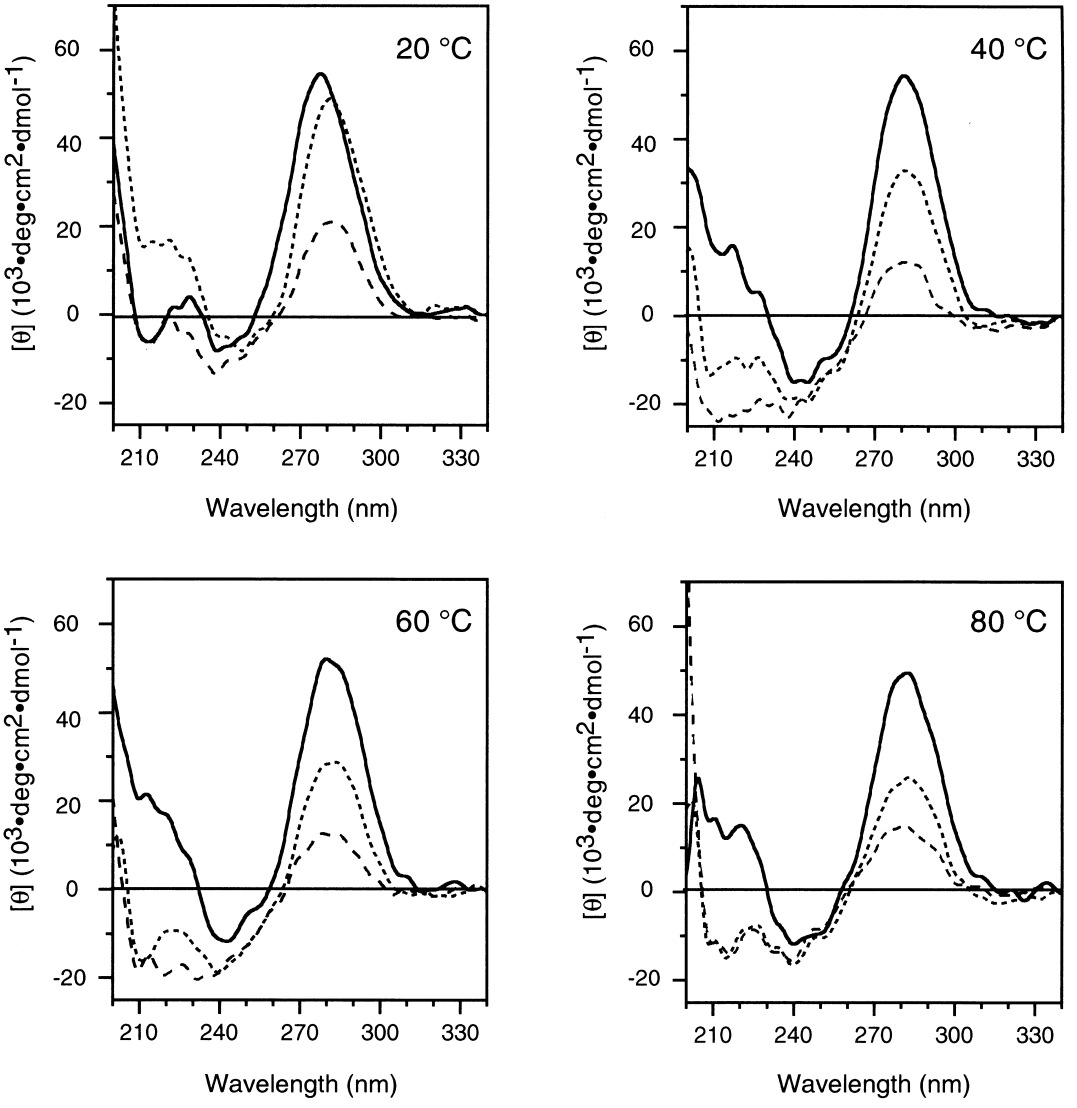


Figure 6. Comparison of the CD spectra of the fully 2′,4′-BNA modified oligonucleotide **20** (solid line), partially modified oligonucleotide **17** (dotted line) and natural oligonucleotide **22** (dashed line) in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl.

tinctly locked sugar conformation was of great importance to clarify the relationship between the triplex stability and the sugar conformation in TFO. The triplex formation of the conformationally restricted oligonucleotides, in which the sugar conformation was restricted in S-form, being in opposite conformation to our oligonucleotides, was reported by Leumann et al.[34-36] Their oligonucleotide analogues with S-form sugar puckering, 'bicyclo-DNA' and 'tricyclo-DNA', can effectively bind to complementary RNA;[34–36] however, the stabilization effect of triplex formation by these oligonucleotide analogues was moderate (the maximum ΔT_m/modification = +0.9 °C).[35] On the contrary, our data represented here clearly reveal that the 2′,4′-BNA oligonucleotides with N-form sugar puckering have significantly high binding affinity towards the target duplex. On the DNA melting experiments, the partailly 2′,4′-BNA modified TFOs showed remarkable increase in T_m value (the maximum ΔT_m/modification = +5 °C), and on the gel retardation experiments, the association constant of **17** in triplex formation was more than 300-fold higher comparing to that of natural TFO **22**. From the overall viewpoint, it is suggested that the N-form sugar puckering is suitable for the pyrimidine motif triplex formation.

Unexpectedly, lack of triplex formation by the fully 2′,4′-BNA modified oligonucleotides **19** and **20** was observed (Table 1 and Fig. 3). To evaluate these unexpected results in detail, we recorded the CD spectra of natural oligonucleotide **22**, partially 2′,4′-BNA modified oligonucleotide **17** and fully modified oligonucleotide **20** at several temperatures (Fig. 6). At 20 °C, the 2′,4′-BNA oligonucleotides **17** and **20** showed a strong positive band at around 280 nm, compared to the natural oligonucleotide **22**, indicating that the modified oligonucleotides have a relatively ordered structure by the conformational restriction. On raising the temperature to 80 °C, the partially modified oligonucleotides exhibited a remarkable decrease in intensity at around 280 nm. However, the fully modified oligonucleotide **20** preserved the strong positive band at around 280 nm even at 80 °C. These results suggest that the fully modified oligonucleotide does not cause the conformational change not only in the sugar puckering but also in the phosphodiester backbone. Although other evaluations, such as NMR experiments, are of course needed, we think the lack of triplex formation by the fully 2′,4′-BNA modified oligonucleotide **22** would appear to come from its too rigid structure to form a triplex for the present.

Although the overlapping of the TFO binding site and the NF-κB consensus sequence is only 6 bp, the 2′,4′-BNA modified TFO **17** clearly showed the inhibition of p50 binding to the target duplex at 1×10^{-6} M concentration (Fig. 5). Natural TFO was reported to inhibit the NF-κB specific transcriptional activation in living cells by triplex-formation in spite of its low affinity towards the target duplex ($K_d > 1\times10^{-5}$ M).[37] Considering the high stability of the triplex consisting of the target duplex and **17**, it is thought that the 2′,4′-BNA oligonucleotide **17** would be a promising inhibitor for the transcriptional activation mediated by NF-κB in living cells.

Conclusion

The partailly 2′,4′-BNA modified TFOs form a very stable triplex with the target dsDNA in a sequence-specific manner. These hybridization characteristics are dependent on pH; however, the partially modified TFOs having 5-methylcytosine moieties show triplex–formation even under neutral conditions. Furthermore, the 2′,4′-BNA modified TFO effectively prevented the NF-κB (p50)-dsDNA binding by the triplex–formation at pH 7.0. On the other hand, the fully modified TFOs lost their binding affinity towards the target dsDNA. Although further evaluation of the triplex-forming ability of the fully modified TFO is needed, these results reveal that the preorganization of oligonucleotides is quite effective for stable triplex–formation and that the 2′,4′-BNA oligonucleotide is a promising candidate for a novel and practical antigene molecule, as well as an antisense molecule.[9,19,38]

Experimental

General considerations

All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Varian VXR-200 (^{1}H, 200 MHz; ^{31}P, 86.4 MHz), JEOL EX-270 (^{1}H, 270 MHz; ^{13}C, 67.5 MHz) or JEOL GX-500 (^{1}H, 500 MHz) spectrometer. IR spectra were recorded on a JASCO FT/IR-200 spectrometer. Mass spectra were measured on JEOL JMS-D300 or JMS-600 mass spectrometer. Optical rotations were recorded on a JASCO DIP-370 instrument. For column chromatography, Merck Kieselgel 60 (70–200 mesh) or Fuji Silysia BW-127ZH (100–200 mesh) was used.

2′,3′-*O*-Cyclohexylidene-4′-(*p*-toluenesulfonyloxymethyl)-uridine (3). Under N_2 atmosphere, *p*-toluenesulfonyl chloride (137 mg, 0.721 mmol) was added to a stirred solution of **2**[20,21] (150 mg, 0.424 mmol) in pyridine (3 mL) at 110 °C. After having been stirred for 3 h at the same temperature, the reaction mixture was partitioned with saturated $NaHCO_3$ and the mixture was extracted with $CHCl_3$. Usual work up and purification by SiO_2 column chromatography ($CHCl_3$:MeOH, 15:1) afforded **3** (149 mg, 0.293 mmol, 69%) as a white powder, mp 104–106 °C (benzene/hexane), $[\alpha]_D^{19}$ −18.5 (*c* 0.98, acetone). IR ν (KBr): 3326, 2929, 2850, 1628, 1577, 1544, 1437, 1311, 1244 cm^{-1}. ^{1}H NMR (acetone-d_6): δ 1.45–1.67 (10H, m), 2.45 (3H, s), 3.71 (2H, ABq, J = 12 Hz), 4.20 (2H, ABq, J = 11 Hz), 4.92 (1H, d, J = 6 Hz), 5.05, 5.06 (1H, dd, J = 4, 6 Hz), 5.60 (1H, d, J = 7 Hz), 5.75 (1H, d, J = 4 Hz), 7.48 (2H, d, J = 8 Hz), 7.77 (1H, d, J = 8 Hz), 7.81 (2H, d, J = 8 Hz), 10.10 (1H, s). ^{13}C NMR (acetone-d_6): δ 21.5, 24.1, 24.5, 25.5, 34.8, 36.9, 63.5, 69.7, 82.5, 84.7, 87.8, 92.9, 102.9, 115.4, 128.8, 130.8, 133.9, 142.7, 145.9, 151.3, 163.5. MS (EI): *m*/*z*

508 (M^+, 81.4), 465 (100). Anal. calcd for $C_{23}H_{28}N_2O_9S\cdot 1/3\ H_2O$: C, 53.69; H, 5.61; N, 5.44; S, 6.22. Found: C, 53.99; H, 5.48; N, 5.42; S, 6.10.

4′-(*p*-Toluenesulfonyloxymethyl)uridine (4). Compound **3** (107 mg, 0.21 mmol) was treated with TFA/H_2O (98:2, 1 mL) for 10 min at 20 °C. The reaction mixture was rapidly evaporated in vacuo and the residue was coevaporated several times with EtOH. Purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 10:1) afforded **4** (85.0 mg, 0.20 mmol, 94%) as a white powder, mp 119–120 °C, $[\alpha]_D^{20}$ −24.4 (*c* 0.50, acetone). IR ν (KBr): 3227, 3060, 2932, 2837, 1709, 1508, 1464, 1252 cm^{-1}. ^1H NMR (acetone-d_6): δ 2.31 (3H, s), 2.84 (3H, s), 3.71 (2H, s), 4.13, 4.20 (2H, ABq, $J=11$ Hz), 4.28, 4.31 (1H, dd, $J=9$, 6 Hz), 4.36 (1H, d, $J=6$ Hz), 5.54 (1H, d, $J=8$ Hz), 5.75 (1H, d, $J=7$ Hz), 7.32 (2H, d, $J=8$ Hz), 7.67 (2H, d, $J=8$ Hz), 7.70 (1H, d, $J=8$ Hz), 10.14 (1H, s). ^{13}C NMR (acetone-d_6): δ 21.5, 63.7, 70.8, 72.7, 74.6, 86.8, 88.8, 103.1, 128.8, 130.7, 133.9, 141.7, 145.8, 151.8, 163.9. MS (EI): *m*/*z* 256 (M^+−TsOH, 9.8), 172 (100). Anal. calcd for $C_{17}H_{20}N_2O_9S\cdot 4/5\ H_2O$: C, 46.11; H, 4.92; N, 6.33; S, 7.49. Found: C, 46.19; H, 4.89; N, 6.11; S, 7.20.

2′,3′-*O*-Benzylidene-4′-(*p*-toluenesulfonyloxymethyl)uridine (5). Under N_2 atmosphere, a mixture of **4** (400 mg, 0.93 mmol), $ZnCl_2$ (670 mg, 5.0 mmol) and benzaldehyde (2.4 mL) was stirred at room temperature for 5 h. The reaction mixture was partitioned with saturated $NaHCO_3$ and the mixture was extracted with $CHCl_3$. Usual work up and purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 40:1) afforded **5** (380 mg, 0.74 mmol, 80%) as a white powder, mp 99–102 °C (CH_2Cl_2/hexane). $[\alpha]_D^{23}$ −26.7 (*c* 1.0, $CHCl_3$). IR ν (KBr): 3059, 1691, 1460, 1362, 1269, 1218, 1177 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.41 (3H, s), 3.25 (1H, br), 3.79 (2H, m), 4.19 (2H, s), 5.09 (1H, d, $J=7$ Hz), 5.28 (1H, dd, $J=4$, 7 Hz), 5.60 (1H, d, $J=4$ Hz), 5.73 (1H, d, $J=8$ Hz), 5.94 (1H, s), 7.24 (1H, d, $J=8$ Hz), 7.38 (2H, d, $J=9$ Hz), 7.42 (5H, br), 7.69 (2H, d, $J=9$ Hz), 9.11 (1H, br). ^{13}C NMR ($CDCl_3$): δ 21.6, 63.5, 68.3, 77.2, 82.8, 84.2, 87.7, 94.9, 102.6, 107.5, 126.5, 127.9, 128.5, 129.7, 132.2, 135.0, 143.0, 145.0, 150.4, 163.5. MS (EI): *m*/*z* 516 (M^+, 28.7), 91(100). Anal. calcd for $C_{24}H_{24}N_2O_9S\cdot 1/3\ H_2O$: C, 55.17; H, 4.76; N, 5.36; S, 6.14. Found: C, 55.19;H, 4.66; N, 5.29; S, 5.98.

3′-*O*-Benzyl-4′-(*p*-toluenesulfonyloxymethyl)uridine (6). Under N_2 atmosphere, $NaBH_3CN$ (92 mg, 1.5 mmol) was added to a stirred solution of **5** (150 mg, 0.29 mmol) in MeCN (3 mL) at room temperature, then $TiCl_4$ (0.16 mL, 1.5 mmol) was added dropwise to the mixture at 0 °C. After having been stirred for 15 h, the reaction mixture was partitioned with saturated $NaHCO_3$ and the mixture was extracted with $CHCl_3$. Usual work up and purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 25:1) afforded **6** (112 mg, 0.22 mmol, 75%) as colorless crystals, mp 195–197 °C (AcOEt-hexane). $[\alpha]_D^{23}$ −14.6 (*c* 1.0, $CHCl_3$). IR ν (KBr): 3033, 2885, 2820, 1726, 1470, 1361, 1274, 1175, 1119 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.40 (3H, s), 3.59–3.77 (3H, m), 4.10,4.24 (2H, AB, $J=11$ Hz), 4.32 (1H, d, $J=6$ Hz), 4.56 (2H, m), 4.69 (1H, d, $J=11$ Hz), 5.52 (1H, d, $J=6$ Hz), 5.67 (1H, d, $J=8$ Hz), 7.24–7.29 (7H, m), 7.48 (1H, d, $J=8$ Hz), 7.70 (2H, d, $J=9$ Hz), 9.91 (1H, s). ^{13}C NMR ($CDCl_3$): δ 21.6, 63.2, 69.2, 73.6, 74.6, 78.1, 86.6, 92.9, 102.5, 127.9, 128.2, 128.3, 128.6, 129.9, 132.3, 136.9, 142.4, 145.2, 150.7, 163.8. MS (FAB): *m*/*z* 519 ($M^+ + H$). Anal. calcd for $C_{24}H_{26}N_2O_9S$: C, 55.59; H, 5.05; N, 5.40; S, 6.18. Found: C, 55.41; H, 5.02; N, 5.32; S, 6.15.

3′-*O*-Benzyl-2′-*O*,4′-*C*-methyleneuridine (7). Under N_2 atmosphere, sodium bis(trimethylsilyl)amide (1 M in THF, 6.38 mL, 6.38 mmol) was added to a solution of **6** (1.00 g, 1.93 mmol) in THF (10 mL) at 20 °C. After having been stirred for 3 h at the same temperature, the reaction mixture was partitioned with saturated $NaHCO_3$ and the mixture was extracted with $CHCl_3$. Usual work-up, purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 10:1) and recrystallization from MeOH afforded **7** (411 mg, 1.1 mmol, 61%) as colorless crystals, mp 217–219 °C (MeOH), $[\alpha]_D^{23}$ +108.4 (*c* 0.3, MeOH). IR ν (KBr): 3059, 2951, 1688, 1459, 1271, 1053 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.75, 3.85 (2H, AB, $J=8$ Hz), 3.77 (2H, d, $J=5$ Hz), 3.92 (1H, s), 4.44 (1H, s), 4.60 (2H, s), 5.39 (1H, t, $J=5$ Hz), 5.48 (1H, s), 7.31 (5H, m), 7.72 (1H, d, $J=8$ Hz), 11.37 (1H, s). ^{13}C NMR (DMSO-d_6): δ 56.0, 71.1, 71.6, 75.8, 76.5, 86.5, 88.3, 100.9, 127.4, 127.6, 128.2, 137.9, 139.0, 150.0, 163.3. MS (EI): *m*/*z* 346 (M^+, 1.1), 91 (100). Anal. calcd for $C_{17}H_{18}N_2O_6$: C, 58.96; H, 5.24; N, 8.09. Found: C, 58.67; H, 5.23; N, 8.05.

2′-*O*,4′-*C*-Methyleneuridine ($\mathbf{1_U}$). Under H_2 atmosphere, 10% Pd-C (25 mg) was added to a stirred solution of **7** (25 mg, 0.072 mmol) in MeOH (2.5 mL) at 20 °C. After having been stirred for 15 h, the reaction mixture was filtered, and the filtrate was concentrated. Purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 10:1 to 5:1) afforded $\mathbf{1_U}$ (18.3 mg, quant.) as colorless crystals, mp 239–243 °C (MeOH). $[\alpha]_D^{23}$ +92.2 (*c* 0.3, MeOH). IR ν (KBr): 3331, 3091, 3059, 2961, 1689, 1463, 1272, 1049 cm^{-1}. ^1H NMR (CD_3OD) δ: 3.76, 3.96 (2H, AB, $J=8$ Hz), 3.90 (2H, s), 4.04 (1H, s), 4.28 (1H, s), 5.55 (1H, s), 5.69 (1H, d, $J=8$ Hz), 7.88 (1H, d, $J=8$ Hz). ^{13}C NMR (DMSO-d_6): δ 56.0, 68.7, 71.1, 78.9, 86.4, 89.0, 100.9, 139.2, 150.1, 163.4. MS (EI): *m*/*z* 256 (M^+, 27.2), 69 (100). Anal. calcd for $C_{10}H_{12}N_2O_6$: C, 46.88; H, 4.72; N,10.93. Found: C, 46.74; H, 4.70; N, 10.84.

5-Bromo-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{1_{BrU}}$). A solution of sodium azide (183 mg, 2.82 mmol) in H_2O (0.55 mL) and *N*-bromosuccinimide (136 mg, 0.764 mmol) were added to a stirred solution of 2′-*O*,4′-*C*-methyleneuridine (150 mg, 0.585 mmol) in 1,2-dimethoxyethane (88 mL) at 20 °C. After having been stirred for 27 h at the same temperature, the reaction mixture was concentrated under reduced pressure and the residue was purified by SiO_2 column chromatography ($CHCl_3$/MeOH, 9:1 to 3:1) to afford $\mathbf{1_{BrU}}$ (165 mg, 84%) as colorless crystals, mp 255–256.5 °C (MeOH), $[\alpha]_D^{23}$ +24.0° (*c* 0.30, MeOH). IR ν_{max} (KBr): 3557, 3330, 3178, 3059, 2958, 2831, 1697, 1615, 1441, 1271, 1126, 1059 cm^{-1}. ^1H

NMR (CD_3OD): δ 3.73, 3.94 (2H, AB, $J=8$ Hz), 3.90 (2H, s), 4.06 (1H, s), 4.29 (1H, s), 5.53 (1H, s), 8.21 (1H, s). ^{13}C NMR (DMSO): δ 55.3, 68.2, 70.9, 78.6, 86.5, 89.0, 95.3, 138.8, 149.3, 159.3. Anal. calcd for $C_{10}H_{11}N_2O_6Br$: C, 35.84; H, 3.31; N, 8.36; Br, 23.84. Found: C, 35.72; H, 3.29; N, 8.32; Br, 23.85.

5-Methyl-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{1_T}$). A mixture of $\mathbf{1_{BrU}}$ (100 mg, 0.298 mmol), ammonium sulfate (1.5 mg, 0.011 mmol) and 1,1,1,3,3,3-hexamethyldisilazane (10 mL) was refluxed under N_2 atmosphere for 24 h. The reaction mixture was concentrated under reduced pressure. To the residue were added palladium chloride (5 mg, 0.029 mmol), triphenylphosphine (16 mg, 0.061 mmol) and THF (12 mL), and the mixture was refluxed under N_2 atmosphere for 10 min. Then, trimethylaluminium (2 M in hexane, 0.45 mL, 0.90 mmol) was added dropwise and the mixture was refluxed for 67 h. The solvent was removed under reduced pressure, water was added to the residue, and the mixture was extracted with $CHCl_3$. The extract was dried and concentrated under reduced pressure. To the residue was added a solution of ammonium chloride (95 mg, 1.78 mmol) in water (1.5 mL) and MeOH (30 mL) and the mixture was refluxed for 5 h. After removing the solvent, the residue was purified by SiO_2 column chromatography (H_2O/MeOH, 1:0 to 4:1) to afford $\mathbf{1_T}$ (46 mg, 57%) as colorless crystals, mp 204–205 °C (MeOH), $[\alpha]_D^{23}$ +58.1° (*c* 1.01, EtOH). IR ν_{max} (KBr): 3323, 3163, 3027, 2889, 2826, 1689, 1471, 1276, 1057 cm^{-1}. ^1H NMR (CD_3OD): δ 1.89 (3H, q, $J=1$ Hz), 3.74, 3.95 (2H, AB, $J=8$ Hz), 3.90 (2H, s), 4.07 (1H, s), 4.26 (1H, s), 5.53 (1H, s), 7.74 (1H, d, $J=1$ Hz). ^{13}C NMR (CD_3OD): δ 12.6, 57.6, 70.3, 72.4, 80.8, 88.3, 90.4, 110.7, 136.8, 151.8, 166.5. MS (EI): *m*/*z* 270 (M^+, 47.7), 69 (100). Anal. calcd for $C_{11}H_{14}N_2O_6 \cdot 3/4H_2O$: C, 46.42; H, 5.38; H, 9.61. Found: C, 46.56; H, 5.51; N, 9.87.

5′-*O*-(4-4′-Dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{8_T}$). Under N_2 atmosphere, 4,4′-dimethoxytrityl chloride (477 mg, 1.41 mmol) and 4-dimethylaminopyridine (14 mg, 0.114 mmol) were added to a stirred solution of $\mathbf{1_T}$ (318 mg, 1.18 mmol) in pyridine (2.6 mL) at 20 °C. After having been stirred for 4 h at the same temperature, the reaction mixture was partitioned with saturated Na_2CO_3 solution, and extracted with AcOEt. Usual work up and purification by SiO_2 column chromatography (AcOEt *n*-hexane:Et_3N, 74:25:1 to AcOEt/MeOH/Et_3N, 94:5:1) afforded $\mathbf{8_T}$ (640 mg, 95%) as a white solid, mp 115–116 °C, $[\alpha]_D^{26}$ +4.16° (*c* 1.01, $CHCl_3$). IR ν_{max} (KBr): 4401, 4069, 3156, 3048, 2952, 2835, 2552, 2249, 2050, 1710, 1606, 1508, 1463, 1252, 1179, 1054 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.70 (3H, d, $J=1$ Hz), 3.46, 3.55 (2H, AB, $J=11$ Hz), 3.80 (6H, s), 3.81, 3.87 (2H, AB, $J=8$ Hz), 4.27 (1H, s), 4.42 (1H, s), 5.63 (1H, s), 6.85 (4H, dd, $J=1$, 9 Hz), 7.21–7.47 (9H, m), 7.64 (1H, d, $J=1$ Hz), 8.32 (1H, br). ^{13}C NMR ($CDCl_3$): δ 12.6, 55.2, 58.2, 70.3, 71.7, 79.3, 86.7, 87.0, 88.1, 110.4, 113.3, 127.1, 128.0, 128.0, 130.0, 134.6, 135.3, 144.4, 149.9, 158.7, 164.1. MS (FAB): *m*/*z* 573 ($M^+ + H$). Anal. calcd for $C_{32}H_{32}N_2O_8 \cdot 3/4H_2O$: C, 65.57; H, 5.76; N, 4.78. Found: C, 65.46; H, 5.74; N, 4.63.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{9_T}$). Under N_2 atmosphere, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (142 mg, 0.472 mmol) was added to a solution of $\mathbf{8_T}$ (221 mg, 0.39 mmol) and diisopropylammonium tetrazolide (50 mg, 0.271 mmol) in MeCN-THF (5 mL, 3:1) at 20 °C. The stirring was continued for 3 h and the reaction mixture was concentrated under reduced pressure. The residue was purified by SiO_2 column chromatography (AcOEt/*n*-hexane/Et_3N, 66:33:1) to afford $\mathbf{9_T}$ (295 mg, 99%) as a white solid. ^{31}P NMR ($CDCl_3$): δ 149.1, 149.2.

1-[3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl]-5-methyl-4-(1,2,4-triazol-1-yl)pyrimidinone (10). To a stirred suspension of 1,2,4-triazole (2.14 g, 31.0 mmol) in MeCN (38 mL) was added slowly $POCl_3$ (1.00 g, 6.54 mmol) followed by Et_3N (3.34 g, 33.0 mmol) at 0 °C. After having been stirred for 30 min at 0 °C, a solution of $\mathbf{9_T}$ (400 mg, 0.519 mmol) in MeCN (7.7 mL) was added over a period of 15 min. The stirring was continued for 100 min at the same temperature. The reaction was quenched by addition of saturated $NaHCO_3$ solution, and then the mixture was extracted with CH_2Cl_2. Usual work up and purification by SiO_2 column chromatography (AcOEt/*n*-hexane, 3:2 to 1:0) afforded **10** (391 mg, 91%) as a white solid. ^{31}P NMR ($CDCl_3$): δ 149.0, 149.6.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{8_U}$). Under N_2 atmosphere, 4,4′-dimethoxytrityl chloride (292 mg, 0.86 mmol) and 4-dimethylaminopyridine (8.8 mg, 0.072 mmol) were added to a stirred solution of $\mathbf{1_U}$ (184 mg, 0.72 mmol) in pyridine (1.7 mL) at 20 °C. After having been stirred for 5 h at the same temperature, the reaction mixture was partitioned with saturated Na_2CO_3 solution, and extracted with AcOEt. Usual work up and purification by SiO_2 column chromatography ($CHCl_3$/MeOH, 40:1) afforded $\mathbf{8_U}$ (438 mg, 94%) as a white solid, mp 117–120 °C ($CHCl_3$), $[\alpha]_D^{23}$ +17.2° (*c* 1.0, $CHCl_3$). IR ν_{max} (KBr): 3393, 3101, 2885, 1689, 1464, 1272, 1047 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.59 (1H, br), 3.49, 3.57 (2H, AB, $J=11$ Hz), 3.78 (7H, m), 3.87 (1H, d, $J=7$ Hz), 4.26 (1H, s), 4.47 (1H, s), 5.60 (1H, d, $J=9$ Hz), 5.63 (1H, s), 5.84 (4H, d, $J=9$ Hz), 7.22–7.45 (9H, m), 7.93 (1H, d, $J=9$ Hz). ^{13}C NMR ($CDCl_3$): δ 50.1, 53.2, 64.8, 66.9, 72.2, 74.1, 81.6, 82.1, 83.1, 96.8, 108.4, 122.0, 122.9, 125.1, 130.1, 134.4, 139.7, 145.3, 153.6, 159.3. MS (EI): *m*/*z* 558 (M^+, 5.2), 69 (100). Anal. calcd for $C_{31}H_{30}N_2O_8 \cdot 1/4H_2O$: C, 66.12; H, 5.46; N, 4.97. Found: C, 65.91; H, 5.50; N, 4.91.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-methyleneuridine ($\mathbf{9_U}$). Under N_2 atmosphere, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (114 mg, 0.37 mmol) was added to a solution of $\mathbf{8_U}$ (173 mg, 0.31 mmol) and diisopropylammonium tetrazolide (40 mg, 0.23 mmol) in MeCN-THF (4 mL, 3:1) at 20 °C. The stirring was continued for 1.5 h and the reaction mixture was concentrated under reduced pressure. The residue was

purified by SiO_2 column chromatography (AcOEt/*n*-hexane/Et_3N, 75:25:1) to afford $\mathbf{9_U}$ (181 mg, 81%) as a white solid. ^{31}P NMR ($CDCl_3$): δ 149.9.

1-[3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl]-4-(1,2,4-triazol-1-yl)pyrimidinone (11). To a stirred suspension of 1,2,4-triazole (1.99 g, 28.8 mmol) in MeCN (45 mL) was added slowly $POCl_3$ (1.00 g, 6.54 mmol) followed by Et_3N (3.34 g, 33.0 mmol) at 0 °C. After having been stirred for 40 min at 0 °C, a solution of $\mathbf{9_U}$ (632 mg, 0.847 mmol) in MeCN (3.6 mL) was added over a period of 15 min. The stirring was continued for 4 h at the same temperature and for 2.5 h at 20 °C. The reaction was quenched by addition of saturated $NaHCO_3$ solution, and then the mixture was extracted with $CHCl_3$. Usual work up afforded **11** (605 mg, 89%) as a white solid. ^{31}P NMR ($CDCl_3$): δ 150.0.

Synthesis of the 2′,4′-BNA modified oligonucleotides

Synthesis of the modified oligonucleotides was performed with solid-phase phosphoramidite methodology on the 0.2 μmol scale using a Pharmacia LKB Gene Assembler™ Plus instrument. Reagent solutions were purchased from Amersham Pharmacia Biotech. The assembly of modified nucleotides was performed according to the standard synthesis cycles, with the exception of a prolonged coupling time (4 min). All syntheses were performed in the trityl on mode, ending with 5′-tritylated oligonucleotides. After synthesis, the solid support was suspended in concentrated NH_3 solution (ca. 1 mL) and left for 3 h at 70 °C. The crude product was detritylated and purified by NENSORB™ PREP cartridge (NEN Life Science Products), and then purified by RP-HPLC on a CHEMCOBOND 5-ODS-H (4.6×250 mm) column (Chemco). The MALDI-TOF-MS data of the isolated oligonucleotides were obtained by using a Voyager DE instrument (PerSeptive Biosystems).

MALDI-TOF-MS data $[M+H]^+$ for the modified oligonucleotides 12–20

Oligonucleotides	Calculated	Found
12	4246.7	4247.9
13	4330.8	4332.6
14	4274.7	4276.6
15	4358.9	4358.0
16	4274.7	4275.9
17	4358.9	4359.7
18	4303.7	4305.9
19	4471.7	4473.9
20	4555.0	4556.3

DNA melting experiments

DNA melting experiments were carried out on a Beckman DU650 spectrophotometer equipped with T_m analysis accessory using quartz cuvettes of 1 cm optical path length. The temperature of the cell holder was increased from 5 to 95 °C at a rate of 0.2 °C/min, and the absorbance at 260 nm was recorded every 2.5 min. Samples were dissolved at 2 μM strand concentration in 10 mM sodium phosphate buffer (pH 6.6 or 7.2) containing 100 mM NaCl. The mixture was incubated at 85 °C for 5 min and then cooled back slowly to 4 °C before the T_m mesurements. The melting temperature (T_m) was evaluated as the temperature of half-dissociation of the formed triplexes determined by the first derivative of the melting curve.

Gel retardation experiments

Target oligonucleotides were labeled at their 5′-end with T4 polynucleotide kinase and [^{32}P]ATP. Samples were prepared by mixing ~0.2 nM labeled strands and the appropriate concentration of the third strand in a 50 mM HEPES buffer (pH 7.0) containing 20 mM $MgCl_2$. The mixture was incubated at 95 °C for 5 min and then cooled back slowly to room temperature before storage at 4 °C for 48 h. Gel electrophoresis was run on a 10% polyacrylamide/bisacrylamide (40:1) nondenaturing gel in a 100 mM HEPES buffer (pH 7.0) containing 40 mM $MgCl_2$ with 3 W power at 4 °C. The gel was dried and visualized by autoradiography.

Preparation of 521 bp DNA fragment for DNase I footprinting experiments

A 36 bp DNA fragment containing target sequence was synthesized and inserted into *Hin*d III/*Bam* HI site of pcDNA3. The ^{32}P-labeled 521 bp DNA fragment used in DNase I footprinting experiments was prepared by digestion of the plasmid construct with *Nde* I and *Xba* I followed by 3′-end labeling with [^{32}P]dCTP using Klenow fragment of DNA polymerase. The labeled DNA fragment was purified by Sephadex G-50.

DNase I footprinting experiments

The ^{32}P-labeled DNA fragment (~0.2 nM) was incubated with 2.2 μM third strand for 48 h at 18 °C in a 50 mM sodium acetate buffer (pH 5.5) or in a 50 mM sodium phosphate buffer (pH 6.0, 6.6 or 7.2) containing 10 mM $MgCl_2$. The mixture was digested with DNase I for 1 min, and the reaction was terminated by the addition of 0.2 M NaCl, 20 mM EDTA and 0.05 μg/μL glycogen. The digested DNA fragment was precipitated with ethanol and resuspended in 98% formamide containing bromophenol blue and xylene cyanol and denatured by heating at 90 °C for 2 min. The sample was quickly cooled on ice and then analyzed on a 6% polyacrylamide/bisacrylamide (19:1) sequence gel in the presence of 7 M urea. The gel was dried and visualized by autoradiography.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a JASCO J-720W spectropolarimeter equipped with a temperature controller PTC-348W. Samples were dissolved at 2 μM strand concentration in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl.

The concentration used to calculate the CD spectra was that of the nucleotide unit.

Inhibition of NF-κB (p50) binding

Target oligonucleotides were labeled at their 5′-end with T4 polynucleotide kinase and [^{32}P]ATP. Samples were prepared by mixing ~1 nM labeled strands in a 9 mM HEPES buffer (pH 7.0) containing 9 mM $MgCl_2$, 45 mM KCl and 0.2 mM EDTA. The mixture was incubated at 95 °C for 5 min and then cooled back slowly to room temperature before addition of 2 mM DTT, 9% glycerol, 0.05% NP40, 0.05 μg/μL poly (dI-dC), 1.2 gel shift units of p50 (Promega) and the appropriate concentration of **15**. After incubation at 18 °C for 48 h, gel electrophoresis was run on a 10% polyacrylamide/bisacrylamide (40:1) nondenaturing gel in a 10 mM HEPES buffer (pH 7.0) with 3 W power at 4 °C. The gel was dried and visualized by autoradiography.

Acknowledgements

A part of this work was supported by a Grant-in Aid for Scientific Research (B), Nos 11470469 and 12557201, from Japan Society for the Promotion of Science. We are also grateful to Takeda Science Foundation for financial support.

References and Notes

1. A part of this work appeared in a preliminary report: Imanishi, T.; Obika, S. *J. Synth. Org. Chem., Jpn* **1999**, *57*, 969.
2. Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645. Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J. L.; Thuong, N. T.; Lhomme, J.; Hélène, C. *Nucleic Acids Res.* **1987**, *15*, 7749. Lyamichev, V. I.; Mirkin, S. M.; Frank-Kamenetskii, M. D.; Cantor, C. R. *Nucleic Acids Res.* **1988**, *16*, 2165. Fox, K. R. *Curr. Med. Chem.* **2000**, *7*, 17.
3. Thuong, N. T.; Hélène, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666. Luyten, I.; Herdewijn, P. *Eur. J. Med. Chem.* **1998**, *33*, 515. Gowers, D. M.; Fox, K. R. *Nucleic Acids Res.* **1999**, *27*, 1569.
4. N3′–P5′ Phosphoramidate oligonucleotides are known to form a stable triplex with a homopurine•homopyrimidine tract in dsDNA. See: Gryaznov, S. M.; Chen, J.-K. *J. Am. Chem. Soc.* **1994**, *116*, 3143. Escudé, C.; Giovannangeli, C.; Sun, J.-S.; Lloyd, D. H.; Chen, J.-K.; Gryaznov, S. M.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4365. Gryaznov, S. M.; Winter, H. *Nucleic Acids Res.* **1998**, *26*, 4160.
5. Only C-rich homopyrimidine sequences of peptide nucleic acids (PNA) can bind to dsDNA to form PNA•DNA•DNA triplex. See: Wittung, P.; Nielsen, P.; Nordén, B. *Biochemistry* **1997**, *36*, 7973. Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D. W. *Angew. Chem. Int. Ed.* **1998**, *37*, 2796. However, except for the C-rich sequences, the strand invasion (PNA•DNA•PNA triplex formation) was favored. See: Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.
6. Although the conformation of triplexes was believed to be A-form-like structures (N-form sugar puckering) from the X-ray fiber analysis of poly(dT)•poly(dA)•poly(dT), recent studies have shown that some triplex structures differ from the A form. See: Soyfer, V. N.; Potaman, V. N. *Triple-Helical Nucleic Acids*; Springer-Verlag: New York, 1996 pp 100–150.
7. Recent reviews for conformationally restricted or locked oligonucleotides. See: Herdewijn, P. *Liebigs Ann. Chem.* **1996**, 1337. Kool, E. T. *Chem. Rev.* **1997**, *97*, 1473. Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301.
8. Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735.
9. Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401.
10. Obika, S.; Andoh, J.; Sugimoto, T.; Miyashita, K.; Imanishi, T. *Tetrahedron Lett.* **1999**, *40*, 6465.
11. Obika, S.; Hari, Y.; Morio, K.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 215.
12. Obika, S.; Hari, Y.; Morio, K.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 221.
13. Obika, S.; Hari, Y.; Sugimoto, T.; Sekiguchi, M.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 8923.
14. Torigoe, H.; Hari, Y.; Sekiguchi, M.; Obika, S.; Imanishi, T. *J. Biol. Chem.*, in press.
15. Obika, S. *Yakuga Kuzasshi* **2000**, *120*, 147.
16. Obika, S.; Morio, K.; Nanbu, D.; Imanishi, T. *Chem. Commun.* **1997**, 1643.
17. Obika, S.; Morio, K.; Hari, Y.; Imanishi, T. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 515.
18. Obika, S.; Morio, K.; Hari, Y.; Imanishi, T. *Chem. Commun.* **1999**, 2423.
19. Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607.
20. Cook, S. L.; Secrist, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 1554.
21. Jones, G. H.; Taniguchi, M.; Tegg, D.; Moffatt, J. G. *J. Org. Chem.* **1979**, *44*, 1309.
22. The stereochemistry of **5** was confirmed by the observation of NOE between the benzyliden hydrogen and 2′- or 3′-hydrogen. It is thought that the *endo*-isomer was selectively obtained thermodynamically. See: Li, C.; Vasella, A. *Helv. Chim. Acta* **1993**, *76*, 211.
23. Recently, Wengel et al. reported the synthesis of **1** from 4-*C*-hydroxymethylribofuranose derivative.[19,24] Independently, we also achieved the effective synthesis of **1** in a similar manner.[15]
24. Koshkin, A. A.; Rajwanshi, V. K.; Wengel, J. *Tetrahedron Lett.* **1998**, *39*, 4381.
25. Kumar, R.; Wiebe, L. I.; Knaus, E. E. *Can. J. Chem.* **1994**, *72*, 2005. Hirota, K.; Kitade, Y.; Kanbe, Y.; Isobe, Y.; Maki, Y. *Synthesis* **1993**, 213.
26. Xu, Y.-Z.; Zheng, Q.; Swann, P. F. *J. Org. Chem.* **1992**, *57*, 3839.
27. Xie, Q.; Nathan, C. *J. Leukoc. Biol.* **1994**, *56*, 576.
28. Lee, J. S.; Woodsworth, M. L.; Latimer, L. J.; Morgan, A. R. *Nucleic Acids Res.* **1984**, *12*, 6603.
29. The association constants were calculated according to the literature. See: Akiyama, T.; Hogan, M. E. *J. Biol. Chem.* **1996**, *271*, 29126.
30. Baeuerle, P. A. *Biochim. Biophys. Acta* **1991**, *1072*, 63.
31. Asensio, J. L.; Carr, R.; Brown, T.; Lane, A. N. *J. Am. Chem. Soc.* **1999**, *121*, 11063.
32. Hendrix, C.; Rosemeyer, H.; Verheggen, I.; Seela, F.; Van Aershot, A.; Herdewijn, P. *Chem. Eur. J.* **1997**, *3*, 110.
33. Tereshko, V.; Gryaznov, S.; Egli, M. *J. Am. Chem. Soc.* **1998**, *120*, 269.
34. Bolli, M.; Leumann, C. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 694.
35. Steffens, R.; Leumann, C. *J. Am. Chem. Soc.* **1999**, *121*, 3249.

36. Tarköy, M.; Leumann, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1432. Tarköy, M.; Bolli, M.; Leumann, C. *Helv. Chim. Acta* **1994**, *77*, 716.
37. Kochetkova, M.; Shannon, M. F. *J. Biol. Chem.* **1996**, *271*, 14438.
38. Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K.; Ossipov, M.; Koshkin, A.; Jakobsen, N.; Skouv, J.; Oerum, H.; Jacobsen, M. H.; Wengel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5633.