CHEMBIOCHEM

DOI: 10.1002/cbic.200700580

Oligodeoxynucleotides Containing 3-Bromo-3-deazaadenine and 7-Bromo-7-deazaadenine 2′-Deoxynucleosides as Chemical Probes to Investigate DNA–Protein Interactions

Noriaki Minakawa,* Yuri Kawano, Shunpei Murata, Naonori Inoue, and Akira Matsuda[a]

We describe the design and proof of concept of a pair of chemical probes for investigating DNA–protein interactions—specifically, the incorporation of 7-bromo-7-deazaadenine and 3-bromo-3-deazaadenine 2′-deoxynucleosides (Br^7C^7dA and Br^3C^3dA) into oligodeoxynucleotides (ODNs)—and their utility. Whereas the bromo substituent of the Br^7C^7dA unit in an ODN duplex acts sterically to inhibit binding with NF-κB, which interacts with the duplex in its major groove, the bromo substituent of the Br^3C^3dA unit acts sterically to inhibit binding with RNase H, which interacts with the duplex in its minor groove. In addition, the utilization of ODNs containing 7-deazaadenine and 3-deazaadenine 2′-deoxynucleosides (C^7dA and C^3dA), together with the pair of chemical probes, afforded valuable information on the requirement for nitrogen atoms located in either the major or minor grooves. Accordingly, we were able to show the utility of ODNs containing Br^7C^7dA, Br^3C^3dA, C^7dA, and C^3dA for the investigation of DNA–protein interactions.

Introduction

The interaction of DNA with proteins such as enzymes and transcription factors plays a central role in numerous biological processes, including the regulation of gene expression.[1] Since this interaction occurs in a strict and specific manner, an understanding of how proteins bind structurally to DNA is critical in determining the mechanisms of the biological process. In addition, this information is expected to provide clues for the development of nucleic acid-based therapeutics, such as decoy molecules and aptamers.

DNA, as is well known, generally forms a right-handed B-form helix, based on the Watson–Crick base pairs. In this helical structure, two grooves exist, a major and a minor, and the Watson–Crick base pairs face these grooves [i.e., the N6 (or O6) and N7 positions of the purine bases and the O4 (or N4) position of the pyrimidine bases face the major groove, while the N3 position of the purine bases and the O2 position of the pyrimidine bases face the minor groove (Figure 1 A)]. In molecular recognition, proteins are thought to recognize DNA by the shape of its groove(s) and the sequence of the nucleobases facing each groove. Thus far, a number of analyses of structural interactions between DNA and proteins have been reported. For example, NF-κB,[2] a transcription factor, and *Eco*RI,[3] a restriction enzyme, are known to bind to the major groove. Recognition of the nucleobases facing the major groove is suggested in both these proteins, while RNase H,[4] an endonuclease, and DNA polymerase[5] are known as proteins that bind to the minor groove without sequence specificity. Proteins that bind to DNA in both the major and the minor grooves are also known.[6] In general, such interactions between DNA and proteins have been analyzed mainly by crystallographic studies and, in some cases, by NMR studies.[7] However, the difficulty of cocrystallization and the huge sizes of the proteins tend to limit the utility of these methods. Accordingly, in order to investigate DNA–protein interactions, the development of logical chemical probes is required. Based on these considerations, we

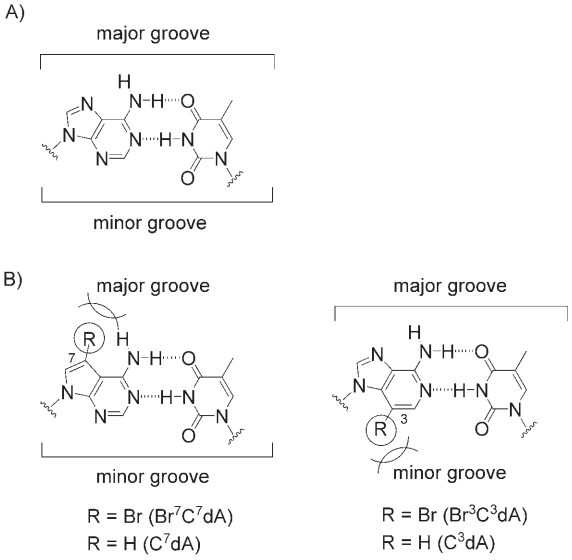

Figure 1. A) Structure of A:T base pair. B) Structures of modified base pairs. Major groove modified probes (left) and minor groove modified probes (right).

[a] *Prof. N. Minakawa, Y. Kawano, S. Murata, Dr. N. Inoue, Prof. A. Matsuda*
Graduate School of Pharmaceutical Sciences, Hokkaido University
Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812 (Japan)
Fax: (+81) 11-706-4980
E-mail: noriaki@pharm.hokudai.ac.jp

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envisioned introducing a certain substituent, which would sterically hinder DNA–protein interactions, into the nucleobase in such a manner as to face into each groove. Specifically, incorporation of the 7-bromo-7-deazaadenine and 3-bromo-3-deazaadenine 2′-deoxynucleosides (Br^7C^7dA and Br^3C^3dA) into the oligodeoxynucleotides (ODNs) was expected to provide information on their use as chemical probes.[8] As shown in Figure 1 B, each nucleobase forms a base pair with thymine in the usual manner of the Watson–Crick base pair. The incorporated bromo groups are expected to be located in the major and minor grooves, respectively, without affecting the overall structure of the helix. In addition, the presences of these bromo groups at each of the deaza positions should minimize the alteration in the pK_a values of the nucleobases arising from the lack of nitrogen atoms at the 3- and 7-positions.[9] Therefore, a combination of Br^7C^7dA and Br^3C^3dA should be a versatile chemical probe system to determine which groove is critical for DNA–protein interactions.

Thus far, several studies using modified nucleoside units to investigate DNA–protein interactions focusing either on the major or the minor grooves have been conducted,[10] but only a few have been reported for both grooves. Moreover, each substituent was introduced on a different nucleoside component.[11] For example, the use of 5-phenyl-2′-deoxyuridine and N^2-methyl-2′-deoxyguanosine as chemical probes has been reported by Uchiyama et al.,[11a] with the two substituents, namely the phenyl and the methyl groups, being introduced on the uracil and the guanine bases, respectively. Conversely, in our concept the same type of substituent (a bromo group) can be introduced on the same nucleobase (a deazaadenine derivative instead of an adenine base). Furthermore, the use of the 7-deazaadenine and 3-deazaadenine 2′-deoxynucleosides (C^7dA and C^3dA)[8] in conjunction with Br^7C^7dA and Br^3C^3dA should also afford information relating to the effects of nitrogen at the 7- and 3-positions for DNA–protein interactions,[12] which would confirm their advantage as chemical probes.

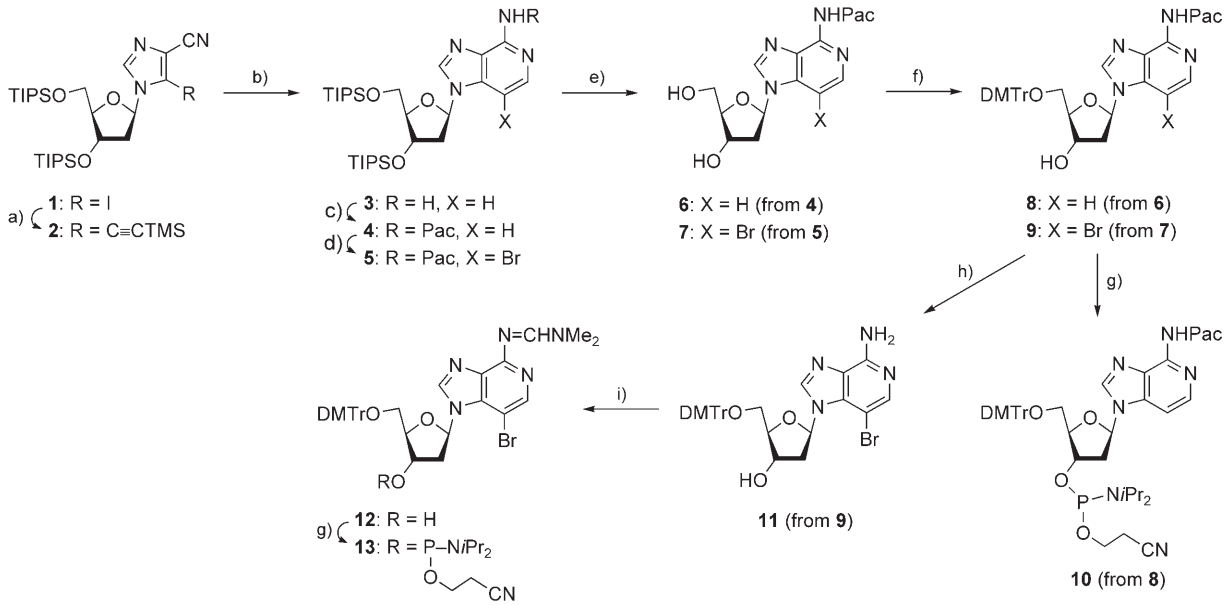
In this paper we describe the synthesis of ODNs containing Br^7C^7dA, Br^3C^3dA, C^7dA, and C^3dA and their utility as chemical probes to investigate DNA–protein interactions with the aid of NF-κB and RNase H as model proteins.

Results and Discussion

Chemistry

We and other groups have previously reported the practical syntheses of 3-deazaadenine and 7-deazaadenine nucleosides.[13,14] Additionally, the syntheses of ODNs containing Br^7C^7dA, C^3dA, and C^7dA, though not those containing Br^3C^3dA, have also been reported.[12,15] With these previous literature references as examples, we first examined the synthesis of the phosphoroamidite units of C^3dA and Br^3C^3dA (Scheme 1). Starting from **1**,[16] the Stille cross-coupling reaction of the stannic compound in the presence of $(PhCN)_2PdCl_2$ afforded the trimethylsilylethynyl derivative **2**. Treatment of the resulting **2** with NH_3/MeOH in a steel container at 120 °C gave the 3-deazaadenine derivative **3**. After protection of the exocyclic amino group of **3** with phenoxyacetyl chloride (PacCl), the resulting **4** was treated with tetrabutylammonium fluoride (TBAF) to give **6**. According to the literature,[15c] **6** was converted into the phosphoramidite unit **10** by treatment with dimethoxytrityl chloride (DMTrCl), followed by phosphitylation.

For the synthesis of the 3-bromo-3-deazaadenine derivative, the introduction of a bromo group was achieved by treatment of **4** with *N*-bromosuccinimide (NBS) in CH_2Cl_2 to give the 3-bromo-3-deazaadenine derivative **5**. Compound **5** was then converted into **9** by removal of the silyl groups, followed by treatment with DMTrCl.



Scheme 1. Reagents: a) TMSC≡C*n*Bu$_3$, $(PhCN)_2PdCl_2$, CH_3CN, 100 °C; b) NH_3/MeOH, 120 °C; c) PacCl, Et_3N, CH_3CN, then NaOMe in MeOH; d) NBS, CH_2Cl_2; e) TBAF, THF; f) DMTrCl, pyridine; g) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *i*Pr$_2$NEt, DMAP, CH_2Cl_2; h) NH_3/MeOH; i) DMF dimethyl acetal, DMF.

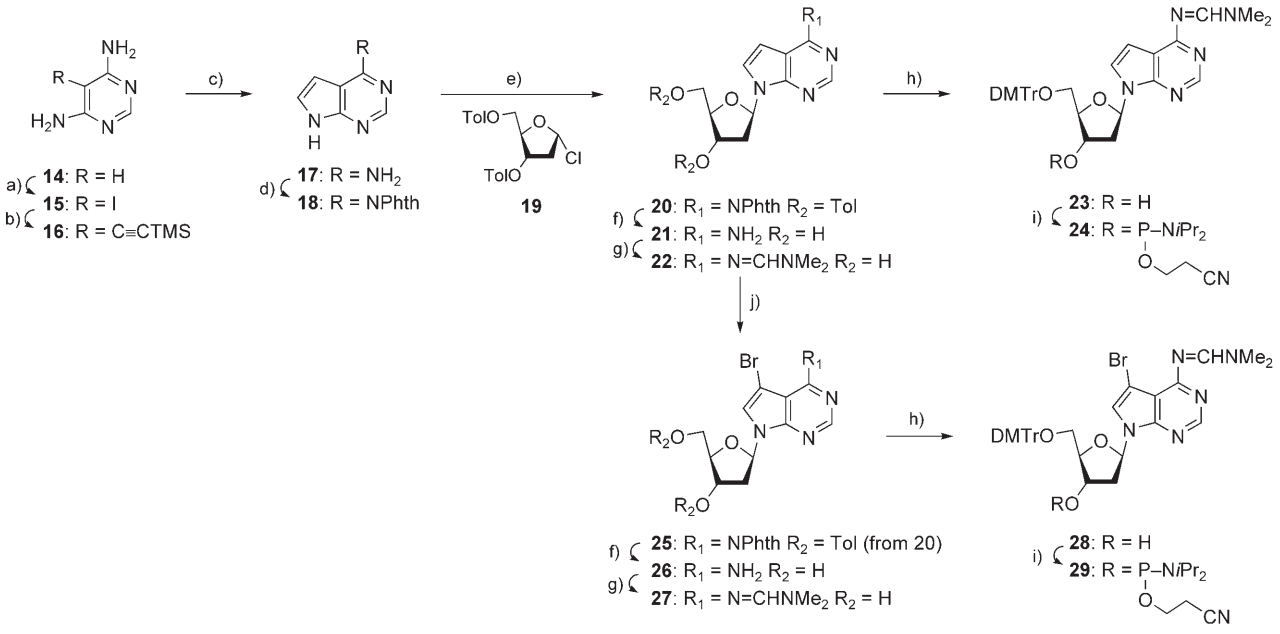
Since ODN synthesis under the standard phosphoramidite method requires acidic conditions (3% trichloroacetic acid in CH_2Cl_2), stability against depurination was examined with **5**. As a result, it was revealed that cleavage of the glycosidic linkage of **5** under acidic conditions occurred more rapidly than that in the corresponding *N*-benzoyladenosine derivative ($t_{1/2}$ values were estimated to be 60 and 125 s, respectively). Therefore, the protecting group of the exocyclic amino group was changed. The Pac group of **9** was removed by treatment with NH_3/MeOH to give **11**. The resulting **11** was treated with dimethylformamide dimethyl acetal to give **12**. The protecting group of **12** stabilized the glycosidic linkage better than the Pac group ($t_{1/2}$ was estimated to be 250 s) and was easily removed under basic conditions. Conversion of **12** into the desired phosphoramidite unit **13** was carried out under the usual phosphitylation conditions.

Studies on the use of the 7-deazaadenine derivatives, including their synthesis and application, have been investigated more deeply than those on the use of 3-deazaadenine derivatives.[14,15] Thus far, several synthetic methods are known.[14] However, most of these involve harsh conditions such as treatment with hydrogen chloride (gas) and sulfuric acid. Therefore, an alternative method was developed as shown in Scheme 2. Starting from commercially available **14**, iodination at the 5-position was performed by treatment with iodine in DMF to give **15**.[17] The resulting **15** was converted into the trimethylsilylethynyl derivative **16** by a cross-coupling reaction. When **16** was heated with KO*t*Bu in *N*-methylpyrrolidinone (NMP) at 80 °C according to the literature,[18] the 7-deazaadenine (**17**) was obtained quantitatively. Accordingly, the synthesis of **17** was readily achieved in three steps from **14**. After conversion of **17** into **18**, glycosylation with the α-chlorosugar **19**[19] afforded the 7-deaza-2′-deoxyadenosine derivative **20** with the preferred β-selectivity. When the resulting **20** was treated with K_2CO_3/MeOH, partial removal of the phthaloyl group along with removal of the toluoyl groups was observed. Thus, the resulting mixture was successively treated with $MeNH_2$ to give 7-deaza-2′-deoxyadenosine (**21**). Compound **21** was converted into the phosphoramidite unit **24** by a known method[15a] in three steps.

The synthesis of the corresponding 7-bromo derivative required a regioselective 7-bromination on the 7-deazapurine ring. Since it is known that an electron-donating substituent such as a free amino group on the pyrimidine portion promotes dibromination at the 7- and 8-positions of 7-deazapurine,[20] **20** was used as a substrate for the bromination. When **20** was treated with NBS in CH_2Cl_2, the monobromide **25** was obtained in quantitative yield. The position of the bromination was confirmed after deprotection of **25** to give **26**, the spectral data for which were identical with those for the known compound.[9a] Likewise, for the synthesis of **24**, **26** was converted into the phosphoramidite unit **29** in three steps. The experimental details are presented in the Supporting Information.

Investigation of the interaction with NF-κB

With the desired phosphoramidite units to hand, we evaluated the modified ODNs containing these units as chemical probes. For a first attempt, we chose the NF-κB (p50 homodimer). This protein is known to bind with the 10-base-pair κB recognition site in a double-stranded DNA (dsDNA) sequence through its major groove.[2] Accordingly, sequences of 18-mer ODNs (a series of NF1s and NF2s) were designed for the investigation. As shown in Table 1, each sequence is complementary to form dsODNs (a series of dsNFs), and the resulting dsODN includes the 10-base-pair κB recognition site (a palindromic part; bold



Scheme 2. Reagents: a) I_2, K_2CO_3, H_2O/DMF; b) TMSC≡CH, $(PhCN)_2PdCl_2$, Et_3N, CuI, DMF, 60 °C; c) potassium *tert*-butoxide, NMP, 90 °C; d) phthalic anhydride, DMF, 100 °C; e) **19**, NaH, CH_3CN; f) K_2CO_3, MeOH, then $MeNH_2$, MeOH; g) DMF dimethyl acetal, DMF; h) DMTrCl, pyridine; i) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *i*Pr_2NEt, DMAP, CH_2Cl_2; j) NBS, CH_2Cl_2.

Table 1. Sequences of dsNFs and their hybridization data.[a]

5′-GGTT**GGGAATTCCC**TTGG-3′ (**NF1s**)
3′-CCAA**CCCTTAAGGG**AACC-5′ (**NF2s**)

A	dsNFs	T_m [°C]	ΔT_m [°C][b]
dA	dsNF-A	60.1 ± 0.1	–
C^3dA	dsNF-C^3A	55.3 ± 0.4	−5.7
C^7dA	dsNF-C^7A	58.6 ± 0.5	−2.4
Br^3C^3dA	dsNF-Br^3C^3A	56.3 ± 0.5	−4.7
Br^7C^7dA	dsNF-Br^7C^7A	60.6 ± 0.5	−0.4

[a] Recognition sites of NF-kB are indicated in bold letters, and modifications by deazaadenosine derivatives are underlined. Experimental conditions are described in the Experimental Section. The data presented are averages of triplicates. [b] The ΔT_m values were obtained by subtracting the value for the T_m of dsNF-A from that for each duplex.

letter). The modified nucleoside units—that is, C^3dA, C^7dA, Br^3C^3dA, and Br^7C^7dA—were incorporated at the underlined positions.

The N7 position of this adenine residue is known to be recognized by NF-κB in their interaction. We prepared the requisite ODNs by the standard phosphoramidite method with a DNA/RNA synthesizer. Each ODN was completely hydrolyzed to its corresponding nucleosides with a mixture of nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase, and the nucleoside composition was analyzed by HPLC. The peaks corresponding to C^3dA, C^7dA, Br^3C^3dA, and Br^7C^7dA, confirmed by coelution with authentic samples, were clearly observed, and the nucleoside composition calculated from the areas of the peaks supported the structure of each ODN (Figure S1 and Table S1).

Prior to investigation of the DNA–protein interactions, measurements of melting temperature (T_m) and CD spectra of each dsNF were carried out to check the effect of chemical modification. The T_m measurements by UV melting experiments were performed in sodium cacodylate buffer (10 mM, pH 7.0, 100 mM NaCl), and the resulting T_m and ΔT_m values calculated on the basis of the T_m value of dsNF-A are listed in Table 1. As can be seen, the ODNs containing C^3dA and C^7dA showed T_m values lower than that of dsNF-A. The incorporation of the bromo substituents on their deazaadenine nucleobases enhanced the duplex stability, but these T_m values were still lower than that of dsNF-A. Although it has been reported that the 7-halogeno substituents on the 7-deazaadenine enhanced dsDNA stability relative to the natural A:T pair,[15d] no such improvement was observed in our sequence. Nevertheless, these alterations in T_m values were considered to be negligible for further investigation. With regard to the structural aspects of dsNFs (based on the CD spectra), neither dsNF-A nor any modified dsDNAs showed the typical B-form spectra;[21] however, each spectrum was considered to be similar to the others (Figure S2). Accordingly, investigation of the interaction between dsNFs and NF-κB was carried out.

To check their potential as chemical probes, we next examined the binding affinities of the dsNFs to NF-κB in a competition assay. The experiments were performed in a buffer solution with increasing concentrations of each unlabeled dsNF together with a fixed amount of the labeled natural dsDNA (dsNF-A). As a positive control, self-competition by unlabeled dsNF-A was also examined. Results of the gel mobility shift assays are shown in Figure 2. A progressive reduction in the radiographic signal corresponding to the complex of NF-κB and labeled dsNF-A was observed upon addition of dsNF-C^3A and dsNF-C^7A, as well as dsNF-A. The IC_{50} value for each competitor was calculated from the quantification of titration experiments by fitting data to a dose-response curve (data not shown). The calculated IC_{50} values for dsNF-C^3A and dsNF-C^7A were 17.9 and 15.0 nM, respectively. These values were almost equal to that of dsNF-A (10.7 nM). When dsNF-Br^3C^3A was used, although a higher concentration of dsNF-Br^3C^3A was required, this dsDNA acted as a competitor, with an IC_{50} value of 136.8 nM . On the other hand, dsNF-Br^7C^7A did not compete with dsNF-A at all.

These data indicate that neither the nitrogen atom at the 3-position of the A residue nor that at the 7-position of the A residue had any affect on the binding affinity between dsDNA and NF-κB. In the X-ray structural analysis, the formation of hydrogen bonding (H-bonding) between NF-κB (R305-NH_2 residue) and the N7 in the A residue is suggested;[2] however, it was shown that this H-bonding is negligible for the dsDNA and NF-κB interaction. Since NF-κB interacts with dsDNA through its major groove, the result with dsNF-Br^7C^7A was reasonable, and it was shown that the bromo substituent in the major groove inhibited the DNA–protein interaction, as expected. On the other hand, the increased IC_{50} value under the conditions using dsNF-Br^3C^3A was an unexpected result, since the bromo substituent here is located in the minor groove. Recently, Huang et al. reported on the structural differences between

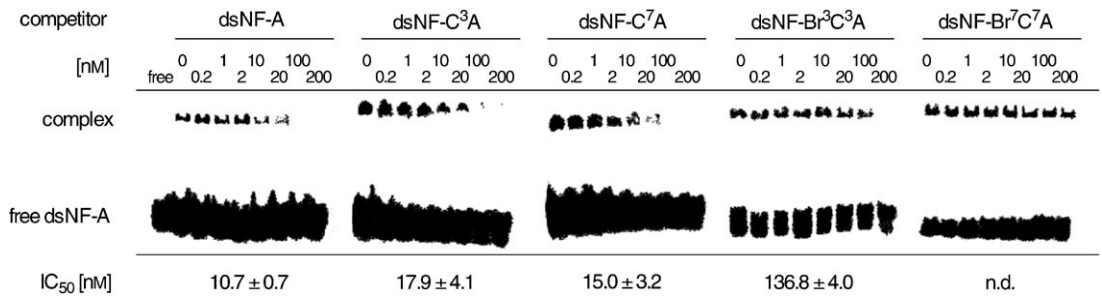


Figure 2. Competition of dsNFs for binding of NF-κB to radiolabeled dsNF-A. Labeled dsNF-A was incubated with various concentrations of unlabeled competitors in the presence of NF-κB. Experimental conditions are described in the Experimental Section. The data presented are averages of triplicates.

a free dsDNA and a dsDNA-bound NF-κB (p65).[22] According to the literature, the minor groove in the free dsDNA became significantly narrower upon NF-κB binding. Although this was observed for p65 and not for the p50 used in their experiment, it is not surprising that this phenomenon was also observed for p50, given the high sequence homology of the NF-κB family proteins. Accordingly, we conjectured that the conformational change that causes the minor groove to become narrower must have been inhibited by the existence of the bromo substituent in the minor groove and that the dsNF-Br^3C^3A thus showed an IC_{50} value higher than those of dsNF-C^3A and dsNF-C^7A.

Investigation of the interaction with RNase H

As described above, it was demonstrated that the bromo substituent in the major groove sterically hindered the DNA-NF-κB (p50) interaction. Accordingly, we next carried out the investigation using a protein that would interact with nucleic acids in the minor groove. RNase H was chosen for the investigation. This protein (enzyme) is known to interact with the DNA–RNA hybrid in its minor groove, and to cleave the RNA strand through its endonuclease activity. As described in the Introduction, interaction in the minor groove was suggested by Uchiyama et al. after the use of chemical probes.[11a] Recently, this suggestion was confirmed by a cocrystallization study of RNase H with the DNA–RNA hybrid.[4b] Therefore, RNase H was thought to be suitable protein to check the potential of our chemical probes.

In order to conduct the investigation, we designed sequences of 10-mer ODNs containing 2′-*O*-methylnucleosides (a series of GAPs; Table 2). These sequences included the deazaadenine 2′-deoxynucleosides at their A positions (underlined), and were designed to restrict the cleavage site of the complementary RNA (RNA1) in a single position when the GAPs hybridized with RNA1 in the presence of RNase H (cleavage site is indicated by an arrow).[11a] The requisite GAPs were prepared with the aid of a DNA/RNA synthesizer, and their structures were confirmed by MALDI-TOF/MS spectrometry.

Table 2. Sequences of RNA1 and GAPs and their hybridization data.[a]

5′-r(ACGACGUCUG)-3′ (RNA1)
3′-ugc**TTCA**gac-5′ (GAPs)

A	GAPs	T_m [°C][b]	ΔT_m [°C][c]
dA	dsNF-A	52.1 ± 0	–
C^3dA	dsNF-C^3A	47.7 ± 0.5	−4.4
C^7dA	dsNF-C^7A	50.1 ± 0	−2.0
Br^3C^3dA	dsNF-Br^3C^3A	53.1 ± 0.5	+1.0
Br^7C^7dA	dsNF-Br^7C^7A	52.1 ± 0	±0

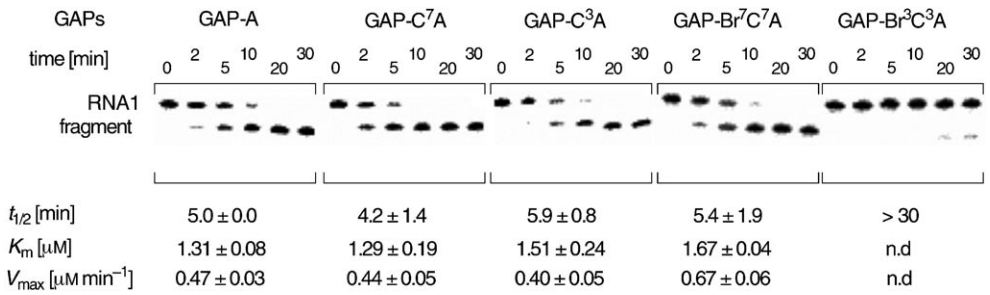
[a] GAPs consisting of 2′-OMe nucleoside units (lower case) and a cluster of 2′-deoxynucleoside units (upper case). Modifications by deazaadenosine derivatives are underlined. Cleavage site of RNA1 by RNase H is indicated by an arrow. Experimental conditions are described in the Experimental Section. The data presented are averages of triplicates. [b] The T_m values were obtained from GAPs–RNA1 hybrids. [c] The ΔT_m values were obtained by subtracting the value for the T_m of the GAP-A–RNA1 hybrid from that for each other hybrid.

Measurements of melting temperature (T_m) and CD spectra of each GAP–RNA1 hybrid were first carried out. Thus, as in the case of dsNFs, the incorporation of C^3dA and C^7dA resulted in decreases in the duplex stability, while the incorporation of Br^3C^3dA and Br^7C^7dA resulted in the restoration of thermal stability. A remarkable difference from the results for previous duplexes (Table 1) was that the GAP-Br^3C^3A-RNA1 showed the highest T_m value. One explanation for this improvement might be attributable to the anticipated structures of the GAPs–RNA1 hybrids: in CD measurements, GAP-Br^3C^3A-RNA1 showed an A-form spectrum, as did other GAPs–RNA1 hybrids (Figure S2). Since the A-form duplex is known to have a rather wide and shallow minor groove, the steric effect of the bromo substituent in the A-form duplex should be smaller than that in the B-form duplex. Thus, the bromo substituent proved to be advantageous for duplex stability, just as it had for the major groove.[15d] Although more data will be required for conclusive evidence, the variation of T_m values and the structures of each GAP–RNA1 hybrid were thought to be acceptable for the investigation with RNase H.

After conducting ^{32}P labeling at the 5′-end of RNA1, we then hybridized this strand with each unlabeled GAP strand. The resulting duplexes were incubated in the presence of *E. coli* RNase H at 30 °C, and the cleavage reaction of RNA1 was analyzed by PAGE at each time point under denaturing conditions. Figure 3 shows the results of this experiment. When RNA1 hybridized with GAP-A, RNA1 was rapidly cleaved. As expected, the sixth phosphodiester linkage from the 5′-terminal was cleaved to give a radioactive 7-mer RNA fragment (the cleavage site is indicated by an allow in Table 2). The half-life ($t_{1/2}$) was estimated from the ratio of the remaining RNA1 as 5.0 min. Similarly, the duplexes not only with GAP-C^7 and GAP-C^3, but also with GAP-Br^7C^7A, afforded the 7-mer RNA fragment, and showed $t_{1/2}$ values almost equal to that of GAP-A.

For a more accurate comparison, the kinetic parameters of each reaction were calculated from the Lineweaver–Burk plot, and the resulting K_m and V_{max} values are given in Figure 3. These resulting data also supported the conjecture that there is no remarkable difference between GAP-A and GAP-C^7, GAP-C^3, and GAP-Br^7C^7A in the reaction catalyzed by RNase H.

Contrary to the above results, the duplex with GAP-Br^3C^3A did not afford the expected RNA fragment, although a very small radioactive band corresponding to a 6-mer RNA fragment was observed instead 20 min later. Since it has been suggested that the bromo substituent in the minor groove acts sterically to hinder the interaction with RNase H, a competition assay using the duplex with GAP-Br^3C^3A was carried out for further confirmation. Thus, the enzymatic cleavage of the duplex with GAP-A was performed in the presence of the duplex with GAP-Br^3C^3A (1 μM, unlabeled). As a result, the kinetic parameters of the duplex with GAP-A were not changed at all (data not shown). Accordingly, the effectiveness of the bromo substituent in the minor groove has been corroborated. These data indicate that neither a nitrogen atom at the 7-position nor one at the 3-position of the A residue has any affect on



	GAP-A	GAP-C^7A	GAP-C^3A	GAP-Br^7C^7A	GAP-Br^3C^3A
$t_{1/2}$ [min]	5.0 ± 0.0	4.2 ± 1.4	5.9 ± 0.8	5.4 ± 1.9	> 30
K_m [μM]	1.31 ± 0.08	1.29 ± 0.19	1.51 ± 0.24	1.67 ± 0.04	n.d
V_{max} [μM min^{-1}]	0.47 ± 0.03	0.44 ± 0.05	0.40 ± 0.05	0.67 ± 0.06	n.d

Figure 3. PAGE analysis and kinetic parameters of the RNase H reaction. Experimental conditions are described in the Experimental Section. The data presented are averages of triplicates.

RNase H activity. Although formation of hydrogen bonding between the nitrogen atom at the 3-position of the A residue and RNase H was suggested in the previous study,[4a] our results showed that this interaction is negligible for RNase H activity. Each result for the duplexes with GAP-Br^7C^7A and GAP-Br^3C^3A was reasonable because RNase H interacts through the minor groove. Thus, the presence of the bromo substituent in the major groove (GAP-Br^7C^7A) did not affect the RNase H activity, while the one in the minor groove (GAP-Br^3C^3A) inhibited the interaction with RNase H.

Formation of the 6-mer RNA fragment can be explained by a previous literature precedent.[23] Inoue et al. reported that efficient cleavage of an RNA strand consisting of a RNA–gapmer duplex by *E. coli* RNase H requires at least one tetradeoxynucleotide cluster in the gapmer sequence which has a similar modification pattern to the GAPs. However, they also found that a gapmer possessing a trideoxynucleotide cluster caused slow cleavage of the RNA strand at a shifted position. Since GAP-Br^3C^3A has a trideoxynucleotide cluster (-TGC-) if the A residue of -TGCA- is substituted with Br^3C^3dA, slow cleavage of the complementary RNA1 at the position between G and U residues was thought to occur to give the 6-mer RNA fragment (see sequences in Table 2). This result supported the theory that the bromo substituent in the GAP-Br^3C^3A inhibited interaction with RNase H in the minor groove.

Conclusions

We have designed a pair of chemical probes to investigate DNA–protein interactions. Each chemical probe consists of ODNs containing 7-Br-7-deazaadenine and 3-Br-3-deazaadenine 2′-deoxynucleosides. When the ODN containing 7-Br-7-deaza-2′-deoxyadenosine formed a duplex, the bromo substituent located in the major groove of the duplex inhibited interaction with NF-κB. In contrast, the duplex containing 3-Br-3-deaza-2′-deoxyadenosine, the bromo substituent of which is located in the minor groove, did not interact with RNase H. As a result, the utility of the chemical probes anticipated in this paper was corroborated by the model proteins described. In addition, the use of ODNs containing 7-deazaadenine and 3-deazaadenine 2′-deoxynucleosides, together with the pair of chemical probes, afforded information on the requirement for nitrogen atoms as H-bonding acceptors located in either the major or minor groove. Accordingly, the combined use of ODNs containing 7-Br-7-deazaadenine, 3-Br-3-deazaadenine, 7-deazaadenine, and 3-deazaadenine 2′-deoxynucleosides should represent a set of versatile chemical probes for the investigation of DNA–protein interactions. Furthermore, since a series of the corresponding ribonucleosides is also readily available,[13,24] this concept could be applied to the investigation of RNA–protein interactions. Proteins related to RNAi machinery would be an attractive target, research on which is currently in progress.

Experimental Section

Synthesis of ODNs: ODNs were synthesized on a DNA synthesizer (Applied Biosystems Model 3400) by use of phosphoramidite units **10**, **13**, **24**, and **29** and commercially available deoxynucleoside and 2′-OMe nucleoside phosphoramidite units at 1 μmol scale by the standard procedure. For the incorporation of the deazaadenosine and 2′-OMe derivatives into the ODNs, solutions in dry acetonitrile (0.1 M) with coupling times of 10 min were used. After completion of synthesis, the CPG support was treated with concentrated NH_4OH at 55 °C for 16 h, and the support was filtered off. The released ODN protected with a DMTr group at the 5′-end was chromatographed on a C-18 silica gel column (1 × 12 cm) with a linear gradient of acetonitrile (from 0 to 40%) in TEAA buffer (pH 7.0, 0.1 N). The fractions were concentrated, and the residue was treated with aqueous AcOH (80%) at room temperature for 15 min. The solution was concentrated, and the residue was co-evaporated with H_2O. The residue was dissolved in H_2O, and the solution was washed with AcOEt. The H_2O layer was then concentrated to give a deprotected ODN. The ODN was further purified by reversed-phase HPLC, on a J′sphere ODN M80 column (4.6 × 150 mm, YMC) with a linear gradient of acetonitrile (from 10 to 25% over 30 min) in TEAA buffer (pH 7.0, 0.1 N) to give highly purified ODNs. The structures of a series of NF1s and NF2s were confirmed by complete hydrolysis, followed by analysis of the resulting nucleoside units by HPLC (Supporting Information). The structures of a series of GAPs were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF/MS) spectrometry on a Voyager-DE pro: GAP-C^7A, calculated mass: $C_{103}H_{134}N_{37}O_{64}P_9$ 3191.6 $[M+H]^+$, observed mass: 3191.5; GAP-C^3A, calculated mass: $C_{103}H_{134}N_{37}O_{64}P_9$ 3191.6 $[M+H]^+$, observed mass: 3191.0; GAP-Br^7C^7A, calculated mass: $C_{103}H_{133}BrN_{37}O_{64}P_9$ 3269.5 $[M+H]^+$, observed mass: 3269.6; GAP-Br^3C^3A, calculated mass: $C_{103}H_{133}BrN_{37}O_{64}P_9$ 3269.5 $[M+H]^+$, observed mass: 3268.9.

T_m measurements: Each sample containing an appropriate duplex (3 μM) in a buffer of sodium cacodylate (pH 7.0, 10 mM) with NaCl (100 mM) was heated at 95 °C for 5 min, cooled gradually to an appropriate temperature, and used for the T_m measurement. Thermal-

ly induced transitions of each mixture of ODNs were monitored at 260 nm on a Beckman DU 650 spectrophotometer. The sample temperature was increased at a rate of 0.5 °C min^{-1}.

Interaction with NF-κB—a competition assay: A mixture of ^{32}P-labeled dsNF-A (10 nM), the competitor duplex (several concentrations, shown in Figure 2), and human recombinant NF-κB (50 nM, Promega) in a buffer (total 20 μL) containing HEPES (pH 7.5, 10 mM), $MgCl_2$ (10 mM), LiCl (50 mM), NaCl (100 mM), spermidine (1 mM), poly dI-dC (10 nM), BSA (0.2 mg mL^{-1}), IGEPAL CA-630 (0.05%), and glycerol (10%) was incubated at 4 °C for 30 min. The samples were loaded onto native polyacrylamide gel (8%, 39:1 acrylamide/bisacrylamide), which was subjected to electrophoresis at 4 °C (1×TBE, 145 V, 40 min). Quantitative measurements of bound and free oligodeoxynucleotides were carried out by image-analyzer (BAS-2500, Fuji Co., Ltd).

Interaction with RNase H—determination of $t_{1/2}$ and kinetic parameters of the RNase H reaction: Unlabeled RNA1 containing ^{32}P-labeled RNA1 (0.5 μM) was annealed with each GAP (0.5 μM). The resulting duplexes were incubated in a buffer (total 20 μL) containing Tris-HCl (pH 7.5, 40 mM), $MgCl_2$ (8 mM), and DDT (5 mM) supplemented with *E. coli* RNase H (TaKaRa) at 30 °C. At each time point (0, 2, 5, 10, 20, and 30 min), aliquots (2 μL) of the reaction mixture were taken, and the reactions were terminated by addition of the stop solution (10 μL). The samples were loaded onto denatured polyacrylamide gel (20%), which was subjected to electrophoresis at room temperature (1×TBE, 1200 V, 1.5 h). Quantitative measurements of RNA1 and the cleaved fragment were carried out with an image-analyzer. As in the case of the determination of kinetic parameters, various concentrations of the duplexes (0.5, 1.5, 4.5, and 13.5 μM) were treated under the same conditions, and the cleavage rates were determined. Lineweaver–Burk plots gave straight lines, and the kinetic parameters for each duplex were obtained from these plots.

Keywords: chemical probes · deoxyadenosine · DNA recognition · DNA–protein interactions · oligonucleotides

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Received: October 1, 2007
Published online on January 24, 2008

