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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Adenosine-1,3-diazaphenoxazine Derivative for Selective Base Pair Formation with 8-Oxo-2′-deoxyguanosine in DNA

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Supporting Information

ABSTRACT: The selective detection of 8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA without chemical or enzymatic treatment is an attractive tool for genomic research. We designed and synthesized the non-natural nucleoside analogue, the adenosine-1,3-diazaphenoxazine (Adap) derivative, for selective recognition of 8-oxo-dG in DNA. This study clearly showed that Adap has a highly selective stabilizing effect on the duplex containing the Adap−8-oxo-dG base pair. Furthermore, the fluorescent property of Adap was shown to be useful for the selective detection of 8-oxo-dG in the duplex DNA. To the best of our knowledge, this is the first successful demonstration of a non-natural nucleoside with a high selectivity for 8-oxo-dG in DNA.

Cellular DNA is continuously exposed to a variety of chemically reactive species such as alkylating agents, reactive oxygen species (ROS), etc., resulting in DNA damage that may increase the risk of developing diseases. 8-Hydroxy-2′-deoxyguanosine [or 8-oxo-2′-deoxyguanosine (8-oxo-dG)], the representative damaged nucleoside, is generated by the oxidation of 2′-deoxyguanosine triphosphate (dGTP) in the triphosphate nucleotide pool or 2′-deoxyguanosine (dG) in DNA.[1] As 8-oxo-dG forms a base pair not only with dC but also with dA, 8-oxo-dGTP and 8-oxo-dG induce transversion mutation from the GC to the TA base pair during DNA replication.[2] It has been shown that the intracellular 8-oxo-dG level is relevant to some diseases and aging.[3−5] Therefore, it is of significant importance to develop methods for selective detection of 8-oxo-dG. The 8-oxo-dG nucleoside or its base is routinely analyzed by HPLC−EC, HPLC/GC−MS, and ELISA.[6−8] Also, recent papers have reported that 8-oxo-dG in DNA can be detected by the reaction of a biotin-conjugated spermine derivative.[9] Nevertheless, there is a still strong demand for an efficient detection method for 8-oxo-dG in DNA in its intact form without degradation. We previously reported the 8-oxo-G clamp as a selective fluorescence probe for 8-oxo-G.[10,11] The TBDMS-protected 8-oxo-G clamp forms multiple H bonds with 8-oxo-dG, causing selective fluorescence quenching in organic solvents. When the 8-oxo-G clamp was incorporated into an oligodeoxynucleotide (ODN), selective fluorescence quenching as a result of hybridization with the complementary DNA incorporating 8-oxo-dG was observed.[12] However, there was a drawback to the use of the 8-oxo-G clamp in ODNs: the detection selectivity for 8-oxo-dG was highly dependent on the DNA sequence. Together with the fact that the 8-oxo-G clamp did not increase the thermal stability toward 8-oxo-dG in DNA, it was suggested that the 8-oxo-G clamp hardly formed a selective base pair with 8-oxo-dG in the DNA duplex.[12] These results led us to design a new nucleoside analogue that recognizes and forms a selective base pair with 8-oxo-dG in DNA. Thus, a 2′-deoxyadenosine derivative having a 1,3-diazaphenoxazine unit (termed "Adap") that can form multiple H bonds with 8-oxo-dG on both the Watson−Crick face and the Hoogsteen face was designed (Figure 1). Adap has the useful property of selectively increasing the thermal stability of duplexes containing an Adap−8-oxo-dG pair and also results in selective fluorescence quenching with 8-oxo-dG in DNA.

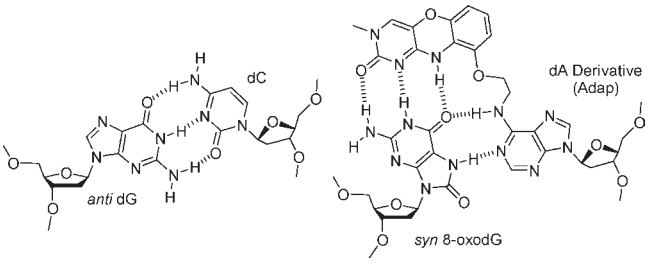


Figure 1. Concept of *A*denosine-1,3-*diaza*phenoxazine (Adap) for the recognition of 8-oxo-dG in DNA.

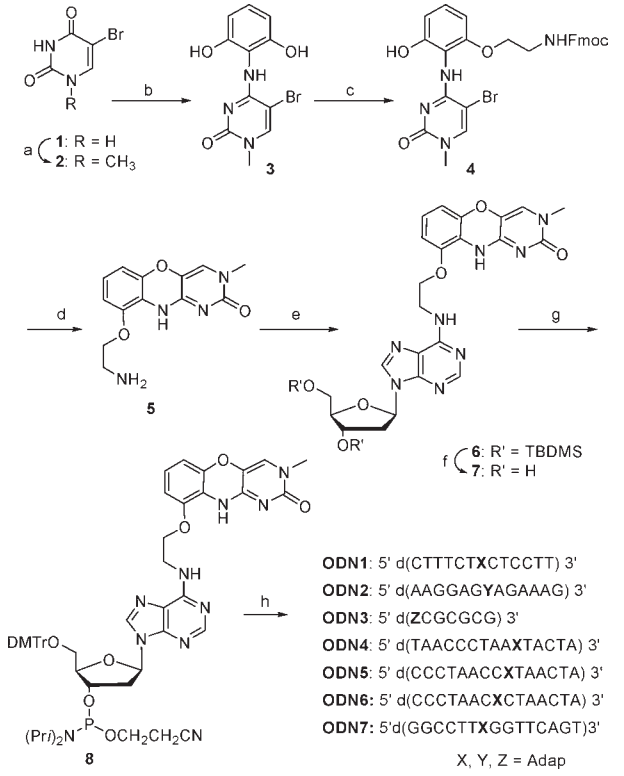
When designing a recognition molecule, we focused on the conformational isomers around the glycosidic bond of 8-oxo-dG in DNA (Figure 1). The base of dG adopts the anti conformation, whereas that of 8-oxo-dG prefers the syn conformation. The 8-oxo-dG base in the syn conformation forms a base pair with dA between the Hoogsteen face of 8-oxo-dG and the Watson−Crick face of dA. It was expected that a recognition molecule having the adenosine skeleton together with the cytosine skeleton might effectively discriminate 8-oxo-dG from dG. Thus, the new dA derivative was designed to have the 1,3-diazaphenoxazine unit as a cytosine unit connected to the 6-amino group of dA through the ethoxy spacer. The 1,3-diazaphenoxazine skeleton might form H bonds with the Watson−Crick face of 8-oxo-dG in the major groove of DNA (Figure 1). The new *a*denosine-1,3-*diaza*phenoxazine analogue has been called Adap.

The synthesis of Adap is summarized in Scheme 1. *N1*-Methyl-5-bromouracil (**2**) was prepared from 5-bromouracil (**1**), HMDS, and methyl iodide and then subjected to coupling with 2,6-dihydroxyaniline to afford **3**. *N*-Fmoc-2-aminoethanol was connected with **3** by the Mitsunobu reaction to produce **4**, which was treated with 7 M ammonia in methanol to effect the

Received: January 13, 2011
Published: April 27, 2011

dx.doi.org/10.1021/ja200327u | *J. Am. Chem. Soc.* 2011, 133, 7272–7275

Scheme 1. Synthesis of Adap and ODNs[a]



[a] Conditions: (a) HMDS, reflux, and then CH_3I, MeCN, r.t. to reflux, 68%. (b) PPh_3, CCl_4, CH_2Cl_2, reflux, then 2,6-dihydroxyaniline, DBU, 45%. (c) *N*-Fmoc-2-aminoethanol, PPh_3, DIAD, CH_2Cl_2, 96%. (d) Ammonia (7 M in MeOH), 100%. (e) 2′*O*,5′*O*-Di-TBS-6-chloroadenosine, DIPEA, 1-propanol, reflux, 44%. (f) TBAF, THF, 100%. (g) (1) DMTrCl, pyridine; (2) 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphorodiamidite, DIPEA, CH_2Cl_2; 73% for two steps. (h) DNA automated synthesizer.

removal of the Fmoc protecting group from the amino group together with the cyclization reaction, yielding the 1,3-diazaphenoxazine derivative **5**. 2′*O*,5′*O*-Di-TBS-6-chloroadenosine[13] was coupled with **5** in the presence of diisopropylethylamine (DIPEA) under reflux conditions to give **6**. After removal of the TBDMS groups of **6** with TBAF, the resulting diol compound **7** was transformed into the amidite precursor **8** according to the conventional method. Amidite **8** was applied to the automated DNA synthesizer to incorporate Adap into the ODNs. Adap was incorporated in the middle of the pyrimidine strand of **ODN1**, in the middle of the purine strand of **ODN2**, and at the 5′ end of **ODN3**. ODNs **ODN4**−**7** were designed for biologically relevant DNA sequences. After cleavage from the resin and purification by reversed-phase HPLC, the structures of the ODNs were confirmed by MALDI−TOF MS analysis.

The thermal stabilization effects of Adap were investigated using the 13-mer duplex formed with **ODN1** and **ODN2** by measuring the T_m values at different duplex concentrations in a buffer containing 100 mM NaCl and 10 mM sodium phosphate at pH 7.0 (representative melting curves are shown in Figure S1 in the Supporting Information). The thermodynamic parameters were obtained from van't Hoff plots (Figure S2) using different ODN concentrations and are summarized in Table 1. The data obtained for the natural ODNs are also shown for comparison.

Table 1. Thermodynamic Parameters for the Duplex Formed between ODN1 and ODN2[a]

X in **ODN1**	Y in **ODN2**	T_m (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal K^{-1} mol^{-1})	$\Delta G°_{310K}$ (kcal/mol)
G	C	44.1	−100.7 ± 6	−288 ± 20	−11.3 ± 0.2
C	G	48.2	−114.7 ± 18	−328 ± 55	−13.1 ± 0.8
A	T	40.9	−90.3 ± 7	−259 ± 22	−10.1 ± 0.2
T	A	41.9	−92.9 ± 10	−266 ± 38	−10.4 ± 0.3
A	G	33.1	−94.6 ± 4	−280 ± 13	−7.8 ± 0.04
G	A	35.4	−71.1 ± 8	−201 ± 25	−8.7 ± 0.2
C	oxo-G	44.3	−98.3 ± 8	−281 ± 23	−11.3 ± 0.2
A	oxo-G	39.8	−91.4 ± 3	−263 ± 9	−9.7 ± 0.05
oxo-G	C	42.3	−96.8 ± 8	−278 ± 25	−10.6 ± 0.2
oxo-G	A	39.2	−96.1 ± 7	−279 ± 21	−9.6 ± 0.1
Adap	A	31.6	−100 ± 16	−299 ± 51	−7.1 ± 0.4
Adap	C	36.5	−124. ± 3	−372 ± 10	−8.8 ± 0.01
Adap	T	39.6	−79.7 ± 9	−226 ± 30	−9.7 ± 0.3
Adap	G	33.9	−70.8 ± 7	−201 ± 22	−8.3 ± 0.1
Adap	oxo-G	**47.2**	−86.5 ± 3	−241 ± 10	−11.7 ± 0.1
A	Adap	30.4	−90.0 ± 10	−267 ± 31	−7.0 ± 0.2
C	Adap	32.2	−63.9 ± 8	−180 ± 24	−8.0 ± 0.2
T	Adap	36.8	−85.5 ± 6	−247 ± 21	−8.8 ± 0.09
G	Adap	33.2	−70.4 ± 5	−201 ± 17	−8.0 ± 0.1
oxo-G	Adap	**45.9**	−125 ± 12	−363 ± 39	−12.4 ± 0.4

[a] UV melting profiles were measured using a solution containing each ODN strand at a concentration of 2 μM in 100 mM NaCl and 10 mM sodium phosphate buffer at pH 7.0. The data were analyzed by the melt curve processing program. These values were determined by van't Hoff plots with five data points (1−12 μM) and the curve-fitting method. 8-Oxoguanosine is abbreviated as oxo-G.

The combination of dC and 8-oxo-dG showed a T_m of 44.3 °C, which is 3.9 °C lower than the value for the corresponding natural CG pair (48.2 °C). This is probably because Watson−Crick base-pair formation involving dC requires the unfavorable anti conformation of 8-oxo-dG. On the other hand, the dA−8-oxo-dG combination provided a T_m value similar to that for the dA−dT base pair (39.8 vs 40.9 °C) and higher than that for the mismatched dA−dG pair (39.8 vs 33.1 °C) because dA forms the base pair with the favorable syn conformation of 8-oxo-dG. It should be noted that selective stabilization was observed for the combination of Adap in **ODN1** and 8-oxo-dG in **ODN2** (T_m = 47.2 °C for 8-oxo-dG vs 31.6, 36.5, 39.6, and 33.9 °C for dA, dC, T, and dG, respectively). The high selectivity is represented by the significant difference of 13.3 °C between the T_m values for dG and 8-oxo-dG. Interestingly, Adap formed a base pair with T at a slightly lower stability than the natural dA−T pair, while a greater stabilizing effect was obtained with the Adap−8-oxo-dG combination. This result indicates the possible and expected formation of additional H bonds of Adap with the Watson−Crick face of 8-oxo-dG. In the different combination in which Adap was incorporated in the purine strand of **ODN2**, a highly selective stabilizing effect was observed for the 8-oxo-dG−Adap combination (T_m = 45.9 °C for 8-oxo-dG vs 30.4, 32.2, 36.8, and 33.2 °C for dA, dC, T, and dG, respectively). From its large, planar and hydrophobic structure, one may expect stacking interactions for Adap. To check whether such effects were included, thermodynamic parameters were obtained using **OND3** containing Adap or dA at the 5′ dangling end. It was shown that Adap produced no significant stabilizing effect on **ODN3**, indicating that

dx.doi.org/10.1021/ja200327u | *J. Am. Chem. Soc.* 2011, 133, 7272–7275

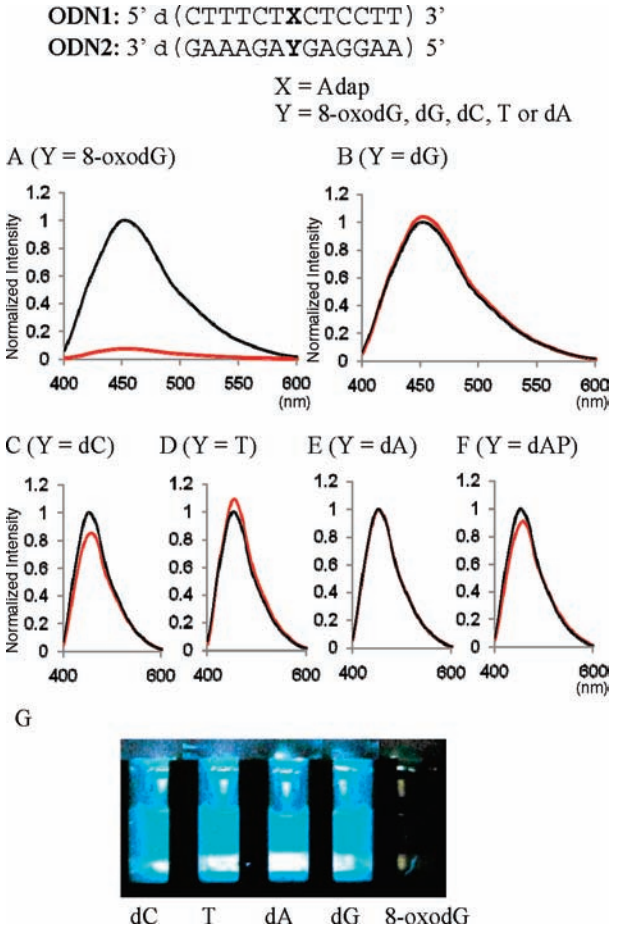


Figure 2. Fluorescence quenching of **ODN1** containing Adap. (A−F) Fluorescence spectra of 50 nM **ODN1** (X = Adap) and 50 nM **ODN2** with Y = (A) 8-oxo-dG, (B) dG, (C) dC, (D) T, (E) dA, and (F) dAP [a stable abasic site (tetrahydrofuran ring)]. Excitation at 365 nm was used, and the solvent was a buffer containing 100 mM NaCl and 10 mM sodium phosphate buffer at pH 7.0 and 30 °C. The black curves represent the spectra of **ODN1**, and the red curves show those after the addition of **ODN2**. (G) Photos of solutions of the ODNs (5 μM each) under the above conditions.

nonselective hydrophobic interactions or stacking interactions are not provided by Adap (Table S1). The circular dichroism spectrum showed that typical B-type DNA was not disturbed in the presence of the Adap−8-oxo-dG pair (Figure S3). Thus, it has been clearly demonstrated that Adap is the first nucleoside analogue having a highly selective stabilizing effect with 8-oxo-dG in DNA.

We next applied Adap to the fluorescent detection of 8-oxo-dG in DNA. In our previous study, the fluorescence of the 1,3-diazaphenoxazine unit of the 8-oxo-G clamp was selectively quenched by complexation with 8-oxo-dG. Therefore, quenching of the fluorescence spectra of **ODN1** containing Adap was measured in the absence and the presence of **ODN2** having 8-oxo-dG, dG, dC, dT, dA, or dAP. Effective fluorescence quenching was observed only for the duplex containing **ODN2** with Y = 8-oxo-dG (Figure 2A). No quenching was observed in the presence of **ODN2** incorporating dG, dC, T, dA, or dAP (Figure 2B−F). The quenching efficiency was so high that the presence of 8-oxo-dG in the duplex DNA could be detected visually (Figure 2G). Higher quenching efficiencies were observed at lower temperatures and higher ODN concentrations (Figure S4). Also, no or much less quenching was induced by the

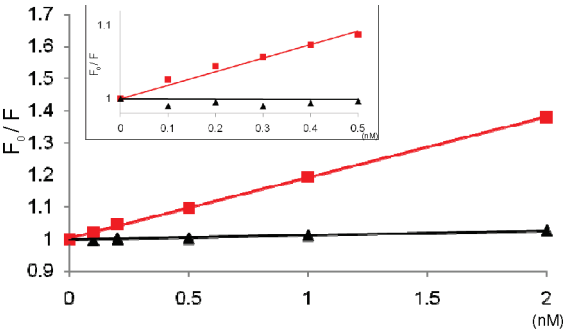


Figure 3. Stern−Volmer plots for **ODN1** (X = Adap) duplexed with **ODN2** containing Y = 8-oxo-dG (■, red line) and dG (▲, black line). The fluorescence spectra were measured using 10 nM **ODN1** with excitation at 365 nm under the same buffer conditions as in Figure 2. F_0 and F are the fluorescence intensities at 452 nm without and **ODN2** (0, 0.1, 0.2, 0.5, 1, 2 nM), respectively. The inset shows plots at lower **ODN2** concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 nM).

control ODN substrates having 8-oxo-dG at the positions other than the complementary site of **ODN2** (Figure S5). **ODN2** (Y = Adap) containing Adap in the purine strand also exhibited selective fluorescence quenching when duplexed with **ODN1** (X = 8-oxo-dG) containing 8-oxo-dG in the pyrimidine strand (Figure S6). These results indicate that the formation of a tight complex between Adap and 8-oxo-dG plays a key role in the efficient and selective fluorescence quenching. We also tested the fluorescence quenching properties of TBDMS-protected Adap (**6**) in organic solvents. In contrast to the quenching in the duplex DNA, the fluorescence of **6** was nonselectively quenched by the addition of the TBDMS-protected 8-oxo-dG and dG (Figure S7). These quenching results indicate that the selective quenching of Adap by 8-oxo-dG in the duplex DNA may require a restricted environment of the duplex DNA that enables Adap to form a base pair with the syn conformation of 8-oxo-dG. The plots of quenching efficiency of **ODN1** were linear at low concentrations of **ODN2** (Figure 3). This graph indicates that the probe having Adap can accurately detect 8-oxo-dG at ~100 pM concentrations of DNA under these conditions.

To test whether Adap can detect 8-oxo-dG in biologically relevant DNA sequences, a preliminary investigation was performed using the telomere sequence. It has been reported that the oxidation of dG in the telomere sequence induces 8-oxo-dG more efficiently than that in non-telomere sequences.[14] Chemical oxidation by $H_2O_2/CuCl_2$ induced 8-oxo-dG formation at the underlined positions in the telomere sequence of 5′-d(TAG TAG TTA GGG TTA GGG TTA GGG TTA GGG)-3′. Accordingly, **ODN4**, **-5**, and **-6** were designed for the detection of 8-oxo-dG at the positions of dG^6, dG^{10}, and dG^{11}, respectively, where the superscript number represents the position starting from the 5′ end. Each ODN showed selective fluorescence quenching for 8-oxo-dG at the respective target site (Figure S8A−C). Another selective detection was demonstrated using **ODN7** for 8-oxo-dG in the sequence 5′-d(ATA ATG ACT GAA CC 8oxo-G AAG GCC GGT TC)-3′, which was reported to induce efficient G-to-T transversion mutation in a yeast strain lacking the *rad30* gene (Figure S8D).[15] These preliminary data demonstrate the potential utility of Adap for the analysis of 8-oxo-dG in biologically relevant sequences.

It should be noted that 8-oxo-dG in the long 80-mer DNA was detected by **ODN1** containing Adap with a similar efficiency. No quenching was observed with the 80-mer DNA that did not contain 8-oxo-dG (Figure S9). The 80-mer **ODN8**, which

dx.doi.org/10.1021/ja200327u | *J. Am. Chem. Soc.* 2011, 133, 7272–7275

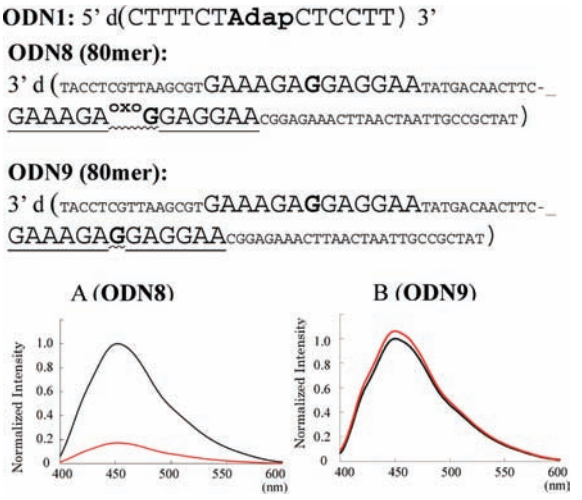


Figure 4. Fluorescence quenching of **ODN1** by 80-mer ODNs. The conditions were described in Figure 2, except for the length of the ODN.

contained 8-oxo-dG in one region complementary with **ODN1** and an additional dG in the other complementary region, was used to test whether 8-oxo-dG can be efficiently detected in the presence of a competitive similar sequence in the same DNA strand. As Figure 4A illustrates, the fluorescence of Adap in **ODN1** was effectively quenched by the 80-mer **ODN8**. **ODN9** with two mismatched sites did not cause fluorescence quenching (Figure 4B). These results indicate that the ODN containing Adap may potentially be used for the specific detection of 8-oxo-dG in long DNA substrates, regardless of the presence of competitive DNA substrates or sequences in the same strand.

Adap exhibited a selective stabilizing effect on 8-oxo-dG in the duplex DNA without disruption of the typical B-DNA conformation. Our previous studies showed that efficient fluorescence quenching of the 1,3-diazaphenoxazine part results from close contact with 8-oxo-dG in the complex.[10,11] Therefore, selective and efficient fluorescence quenching is indicative of the selective complex formation between Adap and 8-oxo-dG in the duplex DNA. The thermal stabilization and selective fluorescence response of Adap to 8-oxo-dG represent its superiority over the previous 8-oxo-G clamp. In spite of its large, planar, hydrophobic nature, Adap did not show significant stacking or hydrophobic interactions. To obtain insight into the complex structure, duplex DNA having the Adap−8-oxo-dG complex was simulated by molecular modeling. The complex was first simulated using monomeric Adap and 8-oxo-dG in the syn conformation, which showed that the complex is stabilized by multiple H bonds (Figure S10A). Upon optimization, duplex DNA containing this complex showed no disruption of either the complex structure or the B-DNA conformation, in which the 1,3-diazaphenoxazine part of the Adap−8-oxo-dG base pair is located in the major groove without causing any steric repulsions (Figure S10B,C). In contrast to Adap, the previous 8-oxo-G clamp did not show thermal stabilization effects toward 8-oxo-dG in the duplex DNA, probably because it can form complex only with 8-oxo-dG in the less stable anti conformation. Further analysis (e.g., by ^{1}H NMR spectroscopy) is needed to clarify the origin of the selective stabilizing effect of Adap.

In conclusion, we have shown that Adap has a highly selective stabilizing effect on the duplex containing the Adap−8-oxo-dG base pair. Furthermore, the fluorescent property of Adap has been shown to be useful for the selective detection of 8-oxo-dG in DNA. To the best of our knowledge, this is the first successful demonstration of a non-natural nucleoside with a high selectivity for 8-oxo-dG in DNA. Further application of the new Adap−oxo-dG base pair, such as the use of the Adap−triphosphate derivative, is now in progress.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, thermodynamic results, and fluorescence data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We are grateful for the support by a Grant-in-Aid for Scientific Research (S) from the JSPS and by CREST from JST.

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