Fluorescent Probes

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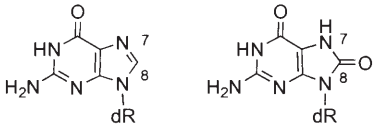
Specific Fluorescent Probe for 8-Oxoguanosine**

*Osamu Nakagawa, Sayaka Ono, Zhichun Li, Akira Tsujimoto, and Shigeki Sasaki**

DNA in living organisms suffers from oxidative damage by reactive oxygen species to form a variety of oxidized nucleosides.[1] 8-Oxoguanosine (8-oxoG) is a representative metabolite derived by the oxidation of guanosine (G; Scheme 1),

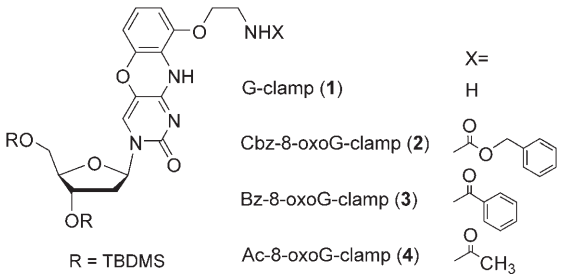


Scheme 1. Structures of guanosine and 8-oxoguanosine. dR: β-D-2′-deoxyribofuranosyl.

and is known to induce G:C to T:A transversion mutations in DNA (C: cytidine; T: thymidine; A: adenosine).[2] The 8-oxoG level is regarded as an index of oxidative damage of cells[3] and is believed to have relevance to some diseases[4] and aging.[5] Thus, versatile methods for the analysis of 8-oxoG are of great significance. Until now HPLC-EC,[6] HPLC/GC-MS,[7] and other techniques[8] have been applied. Recently, antibodies for 8-oxoG have been developed as a tool for its detection in an enzyme-linked immunosorbant assay (ELISA) and the immunohistochemical staining of tissues or cells.[9]

Small molecules with high specificity to 8-oxoG, especially those with fluorescence, will find wider utility for the development of sensors, biological tools, and so on. They may be more beneficial than antibodies in that they are potentially applicable to living cells. In this study, we have attempted to develop fluorescent small molecules for the recognition of 8-oxoG. In a repair enzyme for 8-oxoG (hOGG1), the 7-NH group of 8-oxoG is recognized with one hydrogen bond and no functional group is arranged to bind to the 8-oxygen atom.[10] The difficulty lies in the molecular design of small molecules to discriminate such small differences between G and 8-oxoG. In this paper, the first example of fluorescent molecules with high specificity toward 8-oxoG is reported.

We focused on the tricyclic cytosine derivative with selective affinity toward dG (d: 2′-deoxy) in DNA (G-clamp, **1**).[11] G-clamp (**1**) is composed of phenoxazine with an aminoethoxy unit at the end (Scheme 2). It is reported that

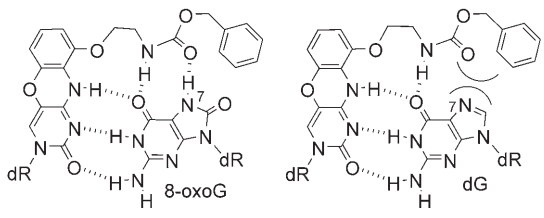


Scheme 2. Structures of G-clamp and 8-oxoG-clamps. Ac: acetyl; Bz: benzoyl; Cbz: benzyloxycarbonyl; TBDMS: *tert*-butyldimethylsilyl.

the specific binding of **1** to dG is due to hydrogen bonding of the amino group of **1** with the O6 atom of dG, in addition to a Watson–Crick-type base pairing between the phenoxazine and the N1, N2, and O6 atoms of dG. In our approach, the derivatives were designed to have an additional functional group, benzyloxycarbonyl (in **2**), benzoyl (in **3**), or acetyl (in **4**), at the aminoethoxy terminal and were named "8-oxoG-clamps" (Scheme 2). It was expected that the carbonyl group would produce attractive forces with 8-oxoG by hydrogen bonding with the 7-NH group on one hand or repulsive forces to the N7 atom of guanosine on the other (Scheme 3).

The functional group of 8-oxoG-clamp derivatives **2**–**4** was efficiently introduced to the amino group of G-clamp. The 3′-O and 5′-O positions of the sugar moiety of **2**–**4** were protected with TBDMS groups to enhance solubility in organic solvents.[12]

All probes (**1**–**4**) possess intrinsic fluorescence at about 450 nm with excitation at 365 nm; therefore, the binding properties were investigated by fluorescence quenching.



Scheme 3. Design concept and proposed complexation of 8-oxoG-clamp (**2** is shown) with 8-oxoG and dG. dR: 3′*O*,5′*O*-di-*tert*-butyldimethylsilyl-β-D-2′-deoxyribofuranosyl.

[*] Dr. O. Nakagawa, S. Ono, Z. Li, A. Tsujimoto, Prof. Dr. S. Sasaki
Graduate School of Pharmaceutical Sciences
Kyushu University
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 (Japan)
Fax: (+81) 92-642-6615
E-mail: sasaki@phar.kyushu-u.ac.jp
Homepage: http://bioorg.phar.kyushu-u.ac.jp/

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Titration experiments were performed by adding 3′O,5′O-disilylated derivatives of dA, dG, dC, dT, or 8-oxo-dG to a chloroform solution of 8-oxoG-clamps **2**–**4** buffered with an organic base and acid. It should be noted that the fluorescence of 8-oxoG-clamp **2** is effectively quenched by the addition of 8-oxo-dG. In a marked contrast, almost no fluorescence quenching was observed with dG. Similarly, dA, dC, and dT did not quench the fluorescence of **2** (Figure 1 a--e), which demonstrates the high selectivity of **2** to 8-oxo-dG. A Job plot of fluorescence quenching clearly indicated a 1:1 ratio for the complex between **2** and 8-oxo-dG (Figure 1 f), which was also

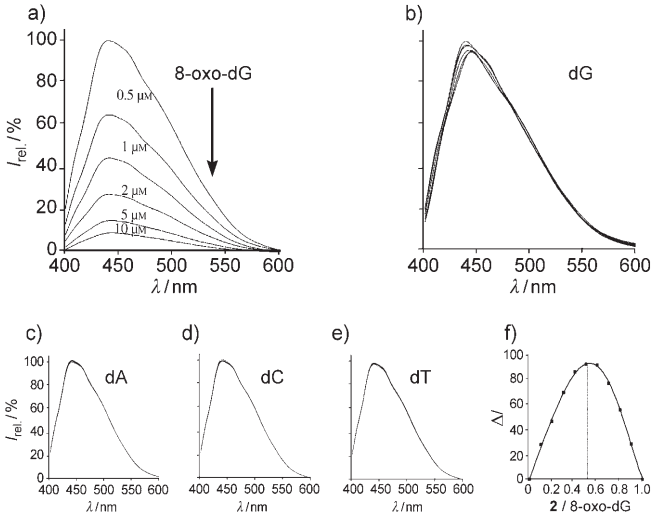

Figure 1. a)–e) Fluorescence titration of Cbz-8-oxoG-clamp (**2**) by 8-oxo-dG, dG, dA, dC, and dT. Titration conditions: 0–10 μM nucleoside was added into a solution of 1 μM **2** in $CHCl_3$ with 0.1% dimethylsulfoxide (DMSO) buffered with 10 mM triethylamine (TEA) and 2.7 mM AcOH at 25 °C. Emission spectra were recorded at $\lambda_{ex} = 365$ nm. f) Job plot with the use of **2** and 8-oxo-dG (total 10 μM). All nucleosides and **2** were used as 3′*O*,5′*O*-di-*tert*-butyldimethylsilyl-2′-deoxynucleosides.

confirmed by ESI-MS measurements.[13] The equilibrium binding constants between the clamp derivatives **1**–**4** and 8-oxo-dG were obtained by these titration experiments and the results are summarized in Table 1. The binding constant of $K_s = 2.3 \times 10^6\,M^{-1}$ for **2** is higher than that of a natural G:C base pair ($K_s = 2.0 \times 10^4$ in $CDCl_3$).[14] The fluorescence of the original G-clamp (**1**) was quenched almost equally by 8-oxo-dG ($K_s = 7.3 \times 10^5\,M^{-1}$) and dG ($K_s = 7.1 \times 10^5\,M^{-1}$), clearly showing that the selectivity of 8-oxoG-clamps **2**–**4** is brought about by the substitution at the amino group of **1**.

Table 1: Binding constants of G-clamp (**1**) and 8-oxoG-clamps **2**–**4** with 8-oxo-dG and dG in $CHCl_3$ buffered at pH 7.[a]

G-clamp (X)	K_s [M^{-1}]				
	8-oxo-dG	dG	dA	dC	dT
1 (H)	7.3×10^5	7.1×10^5	n.q.	n.q.	n.q.
2 (Cbz)	2.3×10^6	n.q.	n.q.	n.q.	n.q.
3 (Bz)	2.9×10^5	n.q.	n.q.	n.q.	n.q.
4 (Ac)	3.0×10^5	n.q.	n.q.	n.q.	n.q.

[a] Conditions: $CHCl_3$ (0.1% DMSO) buffered with 10 mM TEA and 2.7 mM AcOH, 0–10 μM 8-oxo-dG or dG, 1 μM G-clamp (**1**) or 8-oxoG-clamps **2**–**4** at 25 °C, excitation 365 nm. n.q.: no fluorescence quenching.

When the titration experiments were done with 8-oxoG-clamp **2** in chloroform under nonbuffered conditions, fluorescence quenching was observed for both 8-oxo-dG with $K_s = 7.1 \times 10^6\,M^{-1}$ and dG with $K_s = 6.9 \times 10^5\,M^{-1}$.[15] It has been reported that fluorescence quenching by nucleobases takes place through a photoinduced electron transfer mechanism with high dependency on the distance between the chromophores[16] and this may be the case for the fluorescence quenching observed in this study. Hydrogen bonds between **2** and dG might become weaker in the organic buffer system than in chloroform because of the polarity of the buffered solvents, whereas an additional hydrogen bond with the 7-NH group of 8-oxo-dG might contribute to the retention of stability of the complex with **2.** Thus, the selective fluorescence quenching of **2** suggests that, when the two chromophores (**2** and 8-oxo-dG or **2** and dG) are forced into proximity within a tight complex, the fluorescence of **2** is effectively quenched and that **2** and dG dissociate to some extent in the organic buffer system to lose the quenching ability.

In order to characterize the complex structure in detail, 1D and 2D ^1H NMR spectra were measured for **2** and 8-oxo-dG in $CD_3CN/CDCl_3$ (v/v, 6:1) or in CD_2Cl_2 at low temperature. When **2** was added to a solution of 8-oxo-dG, the signals of complexed and free 8-oxo-dG were observed at different chemical shifts (Figure 2 a), which suggests that complexes are formed tightly. The signals of the N^2H (H_d) and N^7H (H_a) protons moved downfield by $\Delta\delta = 3.8$ and 2.2 ppm, respectively. Different regions of the same titration at 0 °C are shown in Figure 2 b and indicate the downfield shift for the H_e proton by complexation. A reverse titration in which 0–2 mM 8-oxo-dG was added to a solution of **2** displayed downfield shifts for the H_b and H_c protons of **2** by $\Delta\delta = 2.3$ and 2.5 ppm, respectively (Figure 2 c). These downfield shifts indicate that these NH protons contribute to complex formation by hydrogen bonds. In the ROESY NMR spectrum of the 1:1 complex of **2** with 8-oxo-dG, clear cross-peaks of H_a–H_b, H_b–H_c, and H_c–H_d were observed (Figure 2 d).[17] In control experiments under the same conditions, **2** did not show significant spectrum changes upon mixing with the derivatives of dA, dT, or dC.[18] The titration with dG indicated lower binding affinity of **2** with dG than with 8-oxo-dG.[18] These NMR results strongly support the fact that **2** and 8-oxo-dG form a complex in the expected manner shown in Figure 2. Formation of highly ordered multiple hydrogen bonds may be responsible for the high selectivity of **2** for 8-oxo-dG.

As fluorescent probes are desired to be applicable in aqueous media, we next examined the fluorescence quenching in water. However, because complexes by hydrogen bonds are hardly formed in water, no quenching was observed. In a preliminary investigation for further development, **2** was solubilized by a detergent (Triton X-100) in water.[19] The fluorescence intensity of **2** increased dramatically at detergent concentrations higher than the critical micellar concentration (cmc), a result indicating that **2** was encapsulated within micelles. Interestingly, the disilylated nucleoside derivative of 8-oxo-dG exhibited highly selective fluorescence quenching

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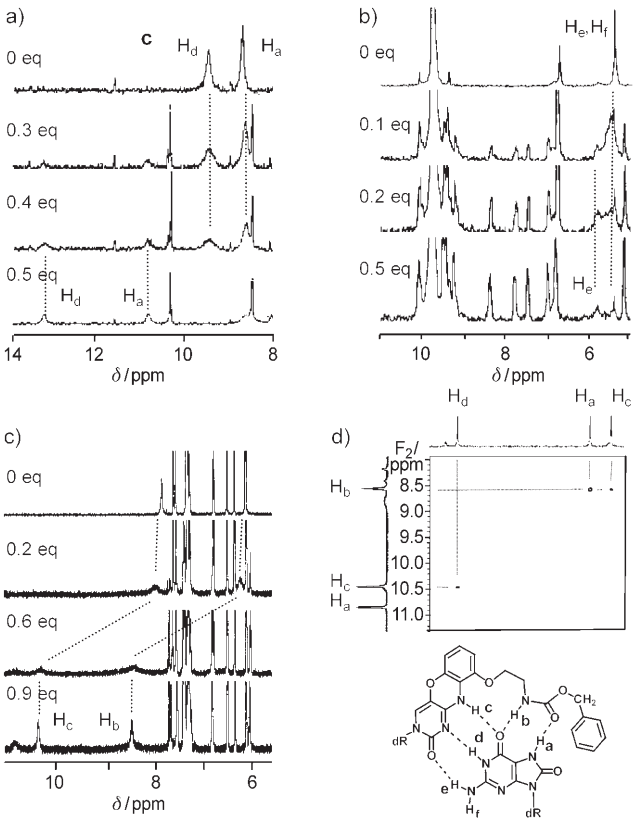

Figure 2. a)–c) ^{1}H NMR spectral change during titration and d) a portion of the ROESY spectrum. a) 0–2 mM **2** was added to 2 mM 8-oxo-dG at −20 °C; b) 0–2 mM **2** was added to 2 mM 8-oxo-dG at 0 °C; c) 0–2 mM 8-oxo-dG was added to 2 mM **2** at 0 °C, d) 2 mM mixture of **2** and 8-oxo-dG (1:1) in CD_2Cl_2 at −10 °C (mixing time = 300 ms). dR = 3′*O*,5′*O*-di-*tert*-butyldimethylsilyl-β-D-2′-deoxyribofuranosyl.

of **2**, similar to that observed in organic solvents (Figure 3a). The same quenching experiments were performed in a system in which dansylamide was cosolubilized by detergent. As dansylamide emits at 520 nm, a longer wavelength than that of **2** (440 nm), with the same excitation at 365 nm, it was expected that quenching of the fluorescence of **2** with the 8-oxo-dG derivative would induce spectral changes in the solution.[20] This expectation was successfully proved by the selective color change with the 8-oxo-dG derivative (Figure 3b). 8-Oxoguanosine derivatives exist in cells as deoxyribosyl and ribosyl derivatives, their phosphates, and components in nucleic acids. Although further efforts are needed to develop a useful system for the selective detection of these metabolites in aqueous media, the results in this study have suggested great potential for 8-oxoG-clamps as new recognition molecules for 8-oxoguanosine.

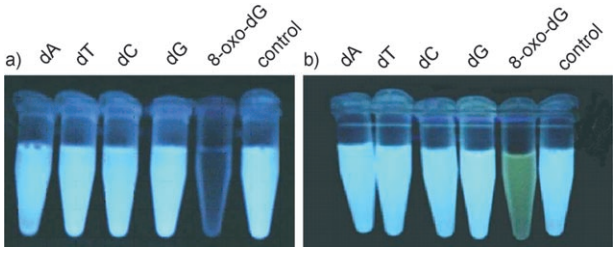

Figure 3. Specific detection of 8-oxo-dG in aqueous media by use of a) only **2** and b) coexisting **2** and dansylamide. Conditions: a) 20 μM each for **2**, 8-oxo-dG, dG, dA, dC, dT; b) as (a) with 40 μM dansylamide; all solubilized in water with 2.5 mM Triton X-100 at 25 °C. Pictures were taken with excitation at 365 nm.

In conclusion, we have developed 8-oxoG-clamps as the first examples of specific fluorescent probes for 8-oxo-dG with the ability for high discrimination from other nucleosides. ^{1}H NMR analysis has indicated that the complexes are formed with highly ordered multiple hydrogen bonds in an expected manner. A preliminary investigation with the use of a detergent showed that 8-oxoG-clamps might be applicable in aqueous media. These results indicate high potential for 8-oxoG-clamps in the development of new analytical systems for 8-oxoguanosine in aqueous media. The development of selective fluorescent probes for other oxidized nucleoside metabolites, such as 8-oxoadenosine, is now in progress.

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