DNA-Damage Detection

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Selective Detection of 5-Formyl-2′-deoxyuridine, an Oxidative Lesion of Thymidine, in DNA by a Fluorogenic Reagent**

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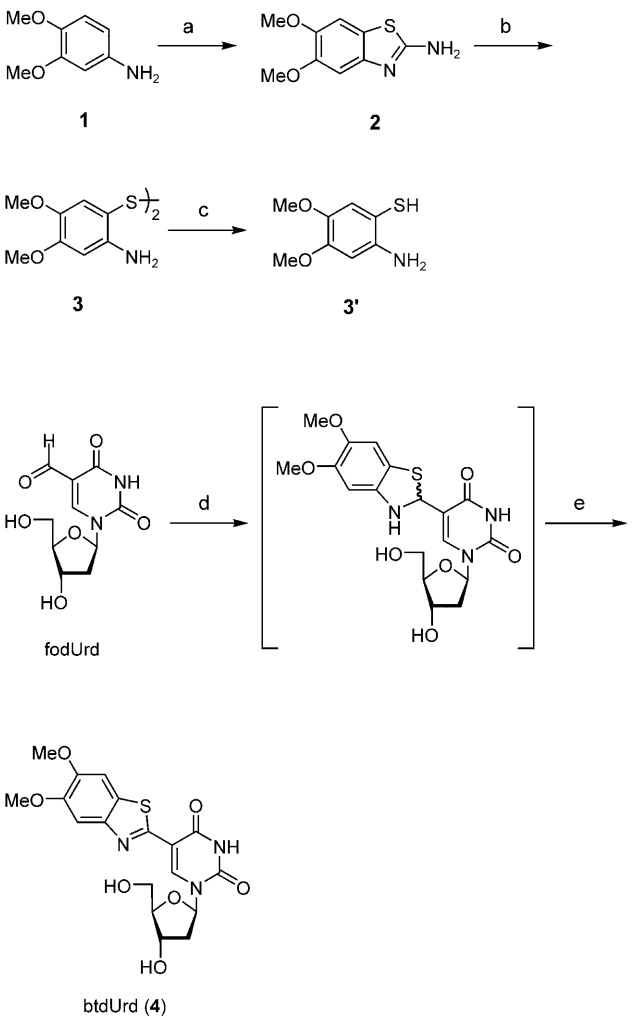
DNA in living cells is damaged by reactive oxygen species derived from UV light, ionizing radiation, and cellular respiration. 5-Formyl-2′-deoxyuridine (fodUrd), an oxidized thymidine lesion generated in yields comparable to that of 2′-deoxy-8-oxoguanosine,[1] induces mutation in DNA (AT-to-GC transition) through the mispairing of an ionized form of fodUrd with 2′-deoxyguanosine during DNA replication.[2] It appears that the formation of fodUrd may cause carcinogenicity and/or aging of cells.[3] A selective and more convenient method for detecting fodUrd would be highly desirable, since the existing methods are complicated, involve time-consuming analysis by HPLC and/or mass spectrometry following complete enzymatic hydrolysis of the target DNA, and require isotope-labeled fodUrd (^{13}C and/or ^{15}N) as an internal standard.[4]

Some other approaches have been developed for the detection of DNA damage.[5] Molecular recognition through the formation of a base pair between an oligonucleotide probe containing a synthetic complimentary nucleobase and O^6-benzyl-dG was developed by Gong and Sturla.[6] A common method is to detect DNA damage with enhanced reactivity by selective tagging with a molecule that contains a reporter group. Ide et al. first demonstrated the detection of abasic sites by the use of an aldehyde reactive probe.[7] Other selective detection methods with chemical reagents have recently been reported.[8] However, the signal-to-noise (S/N) ratio is a problem in these methods, as the fluorescent and/or colorimetric molecules used have higher background signals.

Herein, we report a new concept for the simple detection of fodUrd in damaged DNA with a fluorogenic reagent. The reagent 2-amino-4,5-dimethoxythiophenol (**3′**) shows no fluorescence before reaction with the target fodUrd in DNA. However, upon the reaction of **3′** with fodUrd, the formyl group at the 5-position of fodUrd is converted into a benzothiazol-2-yl group, which is directly conjugated with the uracil group. This ring system is similar to luciferin, which undergoes the luciferase reaction to produce luminescence. Several 5-heteroaryl 2′-deoxyuridines with good fluorescence properties have been reported.[9] Thus, fodUrd in DNA could be detected directly by fluorescence measurement without enzymatic hydrolysis of the target DNA to its corresponding nucleosides, HPLC separation, and analysis.

First, we synthesized the reagent bis(4,5-dimethoxyanilin-2-yl)disulfide (**3**) from 3,4-dimethoxyaniline (**1**) in two steps (Scheme 1).[10] Since oxidation by air leads to the spontaneous dimerization of reagent **3′**, the disulfide **3** was reduced to **3′** by the addition of dithiothreitol (DTT) just before treatment with fodUrd. Oxidation with H_2O_2 in the presence of



Scheme 1. Synthesis of btdUrd (**4**): a) NH_4SCN, Br_2, AcOH, 10°C, 78%; b) KOH, ethylene glycol, H_2O, reflux, 82%; c) DTT, DMF; d) **3′**, DMF; e) H_2O_2, $Sc(OTf)_3$, H_2O, 75% (2 steps). DMF = *N*,*N*-dimethylformamide, Tf = trifluoromethanesulfonyl.

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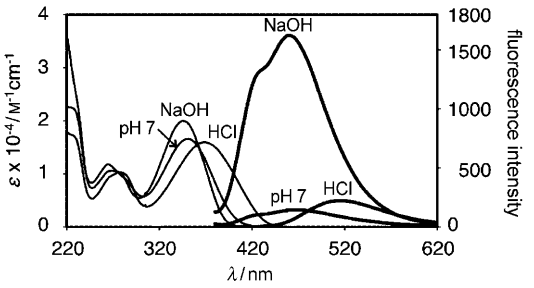

4	λ_{max}^{abs} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{max}^{flu} [nm]	Stokes shift [nm]	Φ	Brightness $\Phi \times \varepsilon_{max}$
aqueous HCl (100 mM)	368	15900	514	146	0.160	2500
phosphate buffer (10 mM, pH 7.0)	351	16600	467	116	0.074	1200
aqueous NaOH (100 mM)	345	20000	458	113	0.701	14000

Figure 1. UV absorption, fluorescence spectra, and photophysical data of **4**. Thin lines show UV absorbance; thick lines show fluorescence. Fluorescence spectra (5×10^{-7} M) were measured by excitation at the wavelength of each absorbance maximum at 25 °C in aqueous HCl (100 mM), phosphate buffer (10 mM, pH 7.0), and aqueous NaOH (100 mM). The quantum yield (Φ) was obtained from the half width of the spectrum by comparison with a known compound (quinine in 0.1 M H_2SO_4).

$Sc(OTf)_3$[11] then gave 5-(5,6-dimethoxybenzothiazol-2-yl)-2′-deoxyuridine (btdUrd, **4**) in 75% yield.

The UV absorbance and fluorescence spectra of **4** were dependent on the pH value of the solution containing **4** (Figure 1). BtdUrd showed much weaker fluorescence under acidic (aqueous HCl, 100 mM) and neutral conditions (phosphate buffer, 10 mM, pH 7.0), with a quantum yield of 0.160 and 0.074, respectively, than under basic conditions. In aqueous NaOH (100 mM), btdUrd showed the strongest fluorescence at 458 nm (excitation at 345 nm), with a quantum yield of 0.701 and a Stokes shift of 113 nm (Figure 1). These are excellent values for a fluorescent compound in aqueous solution,[12] and the results suggest that deprotonation at N3 of the uracil moiety contributes to the strong fluorescence and high quantum yield.

Next, we synthesized a 15-mer oligodeoxyribonucleotide (ODN) containing fodUrd (ODN-^{fo}U)[13] and treated it with **3′** under our optimized conditions (Figure 2). Thus, ODN-^{fo}U (1.0 μM) and **3** (50 μM), pretreated with DTT (250 μM) at room temperature for 30 minutes, underwent the desired reaction in acetate buffer (10 mM, pH 5.0) at room temperature for 2 hours to give the ODN containing **4**, ODN-^{bt}U, in greater than 95% yield. No further oxidation reaction was necessary to generate ODN-^{bt}U when a lower concentration (1.0 μM) of ODN-^{fo}U was used in an aqueous buffer. Dissolved oxygen could be responsible for oxidation of the intermediate.

The fluorescence intensity (λ_{ex}: 345 nm, λ_{em}: 458 nm) of the crude ODN-^{bt}U was measured in aqueous NaOH (100 mM) after removal of the excess reagents by extraction from the reaction mixture with AcOEt. We observed a linear

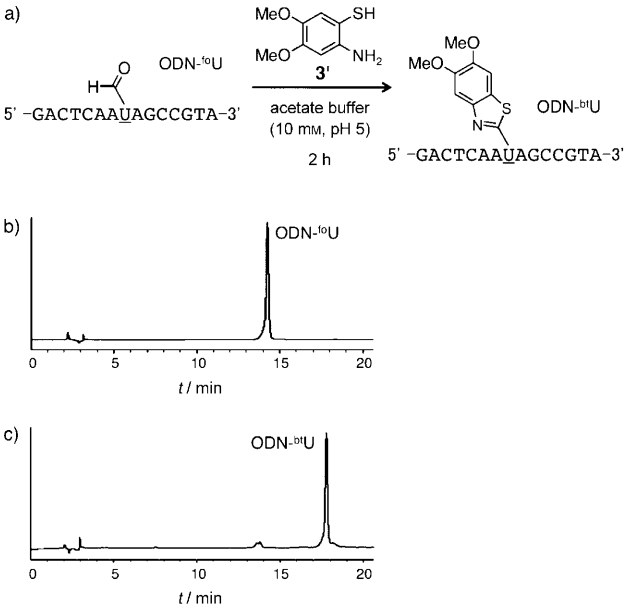

Figure 2. Conversion of ODN-^{fo}U into ODN-^{bt}U with **3′**. a) Reaction scheme with the sequences of ODN-^{fo}U and ODN-^{bt}U; b) reversed-phase HPLC chromatogram of ODN-^{fo}U (before reaction); c) reversed-phase HPLC chromatogram of ODN-^{bt}U (after reaction).

correlation between the concentration of ODN-^{bt}U and the fluorescence intensity over the range 1–100 pmol (Figure 3). In control experiments, we also measured the fluorescence intensity (λ_{ex}: 345 nm, λ_{em}: 458 nm) of the 15-mers ODN-T, which does not contain fodUrd, and ODN-AP, which contains one abasic site, after their fluorogenic reaction with **3** under the same conditions. Since abasic sites are generated as a major aldehyde source in DNA at a rate of approximately 10^4 abasic sites per cell per day,[14] if an aldehyde reacts with **3′** and the resulting benzothiazole derivative generates fluorescence, the selective fluorogenic detection of fodUrd may be disturbed. However, there was no fluorescence of ODN-T and ODN-AP at 458 nm (although the abasic site reacted with **3′** in 58% yield, as detected by HPLC: see Figures S2 and S3 in the Supporting Information). According to the data in Figure 3, the detection limit of our method is around

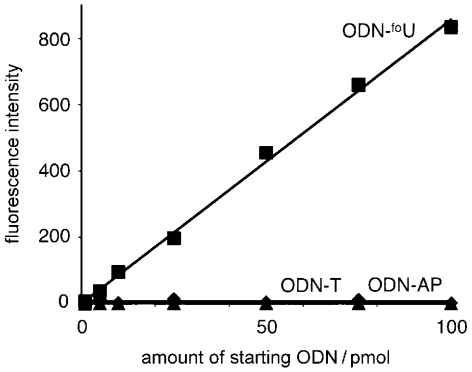

Figure 3. Fluorescence intensity (at 458 nm) of the reaction mixture containing various amounts of the ODN derived from ODN-^{fo}U (square), ODN-AP (diamond), and ODN-T (triangle) in aqueous NaOH (100 mM). Fluorescence measurements were preformed at 25 °C with excitation at 345 nm.

150 fmol, as the minimum S/N ratio is 3:1 (ODN-^{fo}U signal to ODN-T noise). Although this sensitivity is not yet sufficient, detection by this method is more accurate than by previous methods.[4] Hydrolysis by enzymatic digestion often failed around nonnatural nucleotide moieties, and this failure made detection by HPLC and/or mass spectrometry difficult. However, our method can be used to detect even the presence of nonhydrolyzed fodUrd in an oligonucleotide by direct fluorescence measurement.

We also attempted the reaction of ODN-^{fo}C, which contains a 5-formyl-2′-deoxycytidine (fodCyd) moiety,[15] with **3′**, because fodCyd may be a product of an oxidized lesion of 5-methyl-2′-deoxycytidine at 5-methyl CpG sequences in DNA under oxidation conditions.[1b,14] Since no reaction was observed by HPLC (see Figure S4 in the Supporting Information), the existence of fodCyd in DNA may not disturb the selective detection of fodUrd. We also attempted the detection of ODN-^{fo}U in the presence of ODN-AP (0–50 equiv) and found that ODN-AP did not interfere with the formation of ODN-^{bt}U nor with the fluorescence detection of ODN-^{bt}U (see Figure S5 in the Supporting Information).

Finally, γ-irradiated calf-thymus DNA was prepared for the detection of fodUrd formation. First, we confirmed the chemical reactivity of **3′** with fodUrd in γ-irradiated DNA after enzymatic digestion followed by HPLC analysis (see Figure S6 in the Supporting Information). FodUrd was found at a retention time of about 10 minutes. After fluorogenic derivatization by **3**, the fodUrd peak disappeared, and a new peak appeared around 26 minutes. DNA (50 μg) subjected to γ irradiation in various doses (0–300 Gy) was treated with **3′** (500 μM) in acetate buffer (10 mM, pH 5.0) at room temperature for 2 hours, and the reaction mixture was then extracted three times with EtOAc. After redissolution of the DNA in NaOH (100 mM), the fluorescence emission at 458 nm (λ_{ex}: 345 nm) was measured to estimate the yield of btdUrd and calibrated by using an identical ODN containing btdUrd (ODN-^{bt}U; Figure 4). The results showed that 8.9 fodUrd/10^6 bases/Gy could be detected by our method (equivalent to 0.94 Gy/min ^{60}Co γ irradiation) from the calibration curve. This result is in reasonable agreement with the formation rate of 15.3 fodUrd/10^6 bases/Gy (equivalent to 20 Gy/min ^{60}Co γ irradiation) reported by Frelon et al.,[4a] although the γ-irradiation conditions differed.

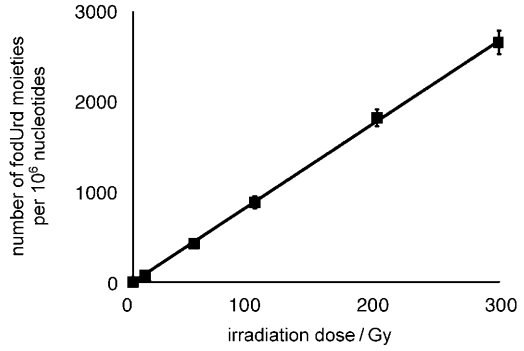

Figure 4. Quantification of fodUrd in γ-irradiated calf-thymus DNA (50 μg) at various irradiation doses by calculation from the calibration curve of ODN-^{fo}U (Figure 3). Each value was calculated from three independent experiments; the error bars indicate standard deviation. Fluorescence measurements (at 458 nm) were performed at 25 °C with excitation at 345 nm.

In conclusion, we have developed a new method for the selective and convenient detection of the oxidized lesion of thymidine, 5-formyl-2′-deoxyuridine (fodUrd), in DNA by treatment with the fluorogenic reagent **3′**. Our method does not need long reaction times, any enzymatic digestion, HPLC separation, or mass spectrometric analysis. Abasic sites and fodCyd in DNA do not disturb fluorescence detection by our fluorogenic method. Therefore, the aminothiophenol reagent **3′** would be useful for the selective detection and measurement of fodUrd formed in DNA exposed to various forms of oxidative stress.

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