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Sequence selective formation of 1,N^6-ethenoadenine in DNA by furan-conjugated probe

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ABSTRACT

1,N^6-Ethenoadenosine derivatives have been applied as fluorescence probes in various fields of biochemistry and molecular biology. We developed a 1,N^6-ethenoadenosine-forming reaction at a target adenine in DNA duplex and applied it to a mutation diagnosis. Furan-derivatized oligodeoxyribonucleotides were synthesized and fluorescence properties were studied in the presence of complementary strand under oxidative conditions. Strong emissions at 430 nm were observed in the presence of the complementary strand with an adenine in front of furan moiety.

Since Kochetkov et al.[1] first reported that adenine and cytosine derivatives are capable of reacting with chloroacetaldehyde to give imidazo[2,1-*i*]purine '1,N^6-ethenoadenine' and imidazo[1,2-*c*]pyrimidine '3,N^4-ethenocytosine' derivatives, respectively, various kinds of ethylene bridged nucleo-bases have been synthesized.[2–4] The presence of a fused imidazole ring alters the fluorescence properties of the nucleo-bases. In contrast 3,N^4-ethenocytosine has much weaker fluorescence (quantum yield 0.003)[5] under acidic conditions, 1,N^6-ethenoadenine has strong fluorescence (quantum yield 0.6) in the long-wave UV region under neutral pH conditions.[6] 1,N^6-Ethenoadenine, whose fluorescent emission highly depend on the surrounding environment, is suitable as a fluorescent probe, and a large number of studies using 1,N^6-ethenoadenine have been carried out to analyze the structure and function of nucleic acids,[7] as well as enzymatic studies[8,9] and binding and conformational studies.[10,11] Several groups have recently reported that etheno-nucleoside derivatives are generated by the reaction of nucleo-bases and lipid peroxidation products derived from endogeneous sources under physiological conditions.[12] In particular, 4-oxo-2-nonenal, which is derived from linoleic acids, showed very high reactivity toward nucleo-bases with an exocyclic amino group. The reaction between deoxyadenosine and 4-oxo-2-nonenal has been well analyzed by Blair and co-workers[13–15] (Fig. 1). Diastereomeric substituted 1,N^6-ethanodeoxyadenosine adducts were first formed by incubation for 24 h at 37 °C. After incubation of diastereomeric isomers at 60 °C for 24 h, a dehydrated adduct which was identified as a substituted 1,N^6-etheno-deoxyadenosine, 1″-[3-(2′-deoxy-β-D-erythropentafuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]heptan-2″-one, by ^{1}H NMR and LC/MS^n was observed as the main product with a concomitant decrease in 1,N^6-ethanodeoxyadenosine adducts. With development of ultrasensitive methods using an immunoaffinity-HPLC-fluorescence method[16] and ^{32}P-postlabeling technique,[17] 1,N^6-ethenoadenine is used as the biomarker of oxidation stress in vivo[18] and genetic damage associated with certain cancer risk factor.[19]

We report herein the development of a 1,N^6-ethenoadenine-forming reaction at a target adenine in DNA duplex and application to the diagnosis of a one-point variation in DNA using the fluorescence properties of 1,N^6-ethenoadenine derivatives. Most of the probes that have previously been reported and used in fluorescence measurements contain extrinsic fluorophores such as rhodamine, fluorescein, and cyanine derivatives. As long as these extrinsic dyes are being used, fluorescent quenchers or washing steps are indispensable for the diagnosis of DNA variation. Selective 1,N^6-ethenoadenine formation using a γ-oxo-α,β-unsaturated aldehyde group with adenine moiety in the DNA sequences enabled homogeneous diagnosis of an adenine-related one-point variation in DNA.

The synthetic procedure for **Fr-ODN** is shown in Scheme 1. Since the furan ring is a chemically stable synthetic equivalent of 4-oxo-2-butenal, 2-substituted furans are convenient for the preparation of γ-oxo-α,β-unsaturated aldehyde group.[20] Halia et al. have reported the synthesis of oligodeoxynucleotides containing a furan moiety linked to the 2′ position of 2′-amino-2′-deoxyuridine.[21] The furan-derivatized oligodeoxynucleotides gave interstrand crosslinked products in the presence of a complementary strand using *N*-bromosuccinimide which is effective for oxidation

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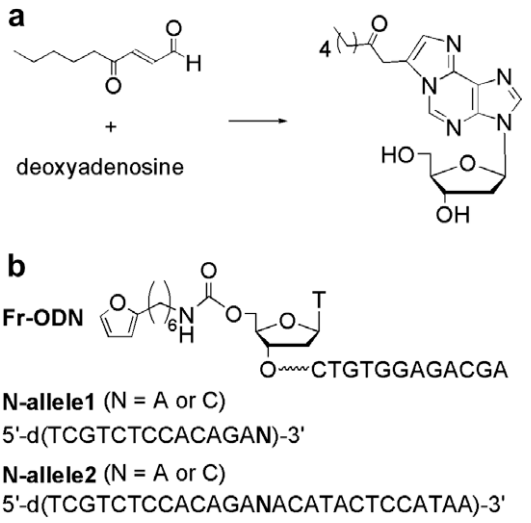


Figure 1. (a) A substituted 1,N^6-ethenodeoxyadenosine generated by the reaction of doxyadenosine and 4-oxo-2-nonenal. (b) Furan-conjugated oligodeoxynucleotide (**Fr-ODN**), N allele1, and N allele2 (N = A or C) used in this study.

of furan moiety into γ-oxo-α,β-unsaturated aldehyde group. By reference to Halia's procedure, we prepared oligodeoxyribonucleotides containing a furan moiety at 5′ position using unmodified oligodeoxynucleotides as the framework of the probe that is easily purchased and cost-effective. 2-(6′-Iodohexyl)furan was synthesized by alkylation of furyllithium with 1,6-diiodohexane. In the following substitution from iodide to the phthalimide group, methanolic hydrazine was used for the conversion of the phthalimide group to the primary amino group **3** with an overall yield of 14% from commercially available furan. After the furan derivative with primary amino group (**3**) was incorporated via a carbamate linkage to the 5′ position of the d(TCTGTGGAGACGA) on CPG support, the oligonucleotide was released from the support and deprotected under basic conditions. **Fr-ODN** was purified by reversed-phase HPLC and analyzed by ESI-TOF (ESI-TOF $[M-5H]^{-5}$ calcd 840.2, found 840.2).

To estimate the reaction conditions for 1,N^6-ethenoadenosine formation, the duplex stabilities of **Fr-ODN** hybridized with target DNA strands were examined (Table 1). The sequence of target DNA strands with the point mutation site is the antisense sequence of JAK2 gene, which is associated with chronic myeloproliferative disease(CMPDs).[22] The T_m values of the duplex with **Fr-ODN** and the complementary strands are decreased by 0.3–1.5 °C compared with that of the non-modified DNA duplex. These results suggested that the attachment of a furan derivative at the 5′ end of the probe ODN slightly destabilized the duplex.

Table 1
T_m values of duplexes between **Fr-ODN** and N allele 1

Fr-ODN/A allele 1	52.8 °C (−1.5)
Fr-ODN/C allele 1	52.0 °C (−0.3)
5′-d(TCTGTGGAGACGA)-3′/ 3′-d(NAGACACCTCTGCT)	54.3 °C (N = A) 52.3 °C (N = C)

Conditions: [**Fr-ODN**] = 2.5 μM, [N allele 1] = 2.5 μM, 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl.

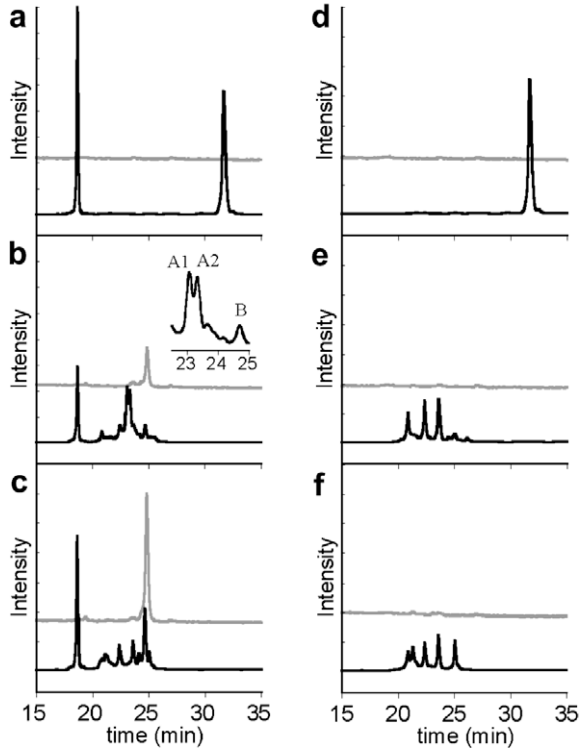
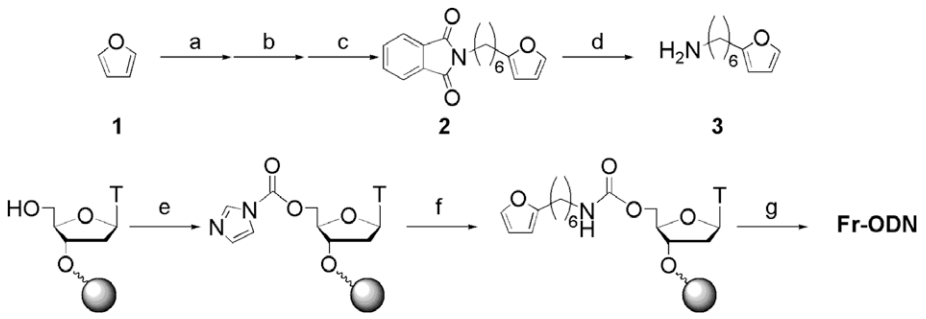


Figure 2. Reversed-phase HPLC profiles of reaction mixtures of **Fr-ODN** (a)–(c) in the presence and (d)–(f) absence of A allele1. The reaction mixtures were incubated at 37 °C for 2 h, then at 60 °C for 2 h in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and analyzed at 0 h (a, d), 2 h (b, e), and 4 h (c, f) after the addition of *N*-bromosuccinimide.The black line indicated absorption intensity monitored at 260 nm. The gray line indicated emission at 430 nm with excitation at 310 nm. The magnified HPLC spectrum from 22.5 min to 25 min of Figure 2b was shown in inset (b), which contain peaks Al, A2 and B.

Formation of the self-adducts in the presence of 4 equiv of *N*-bromosuccinimide was first monitored by reversed-phase HPLC (Fig. 2d–f). **Fr-ODN** completely disappeared within 5 min of the addition of *N*-bromosuccinimide, and the overlapped small peaks appeared from 20 min to 25 min in HPLC profiles. It was consid-



Scheme 1. Reagents and conditions: (a) BuLi, THF, −78 °C, 20 min; (b) 1,6-diiodohexane, 2 h; (c) potassium phthalimide, DMF, rt, 3 h, 16% (from **1**); (d) H_2NNH_2, MeOH, reflux, 87%; (e) d-(TCT GTG GAG ACG A)-CPG, carbonyldiimidazole, dioxane, rt, 2 h; (f) **3**, DMSO, 60 °C, 2 h; (g) NH_3aq, 55 °C, 8 h, 36%.

ered that the furan that was tethered with ODN was immediately oxidized and converted to highly reactive species, which was presumed to be a 4-oxo-alkenal derivative. Reaction mixtures of **Fr-ODN** and A allele 1 were analyzed after incubation at 37 °C for 2 h, then at 60 °C for 2 h. In the presence of A allele 1, which forms an adenine overhang duplex with **Fr-ODN**, additional three peaks, other than self-adducts, were newly observed in reversed-phase HPLC profiles after incubation for 2 h at 37 °C (Fig. 2b). Twin larger peaks (A1 and A2) were observed around 23 min and a smaller peak (B) was observed at 25 min. Following 2 h incubation at 60 °C, peak A1 and A2 had practically disappeared and peak B was increased. Peak B was clearly detected by fluorescence detection at ex 310 nm, em 430 nm as depicted in Figure 2c (gray line).

Mass spectrum analysis of peak B revealed that peak B is likely a dehydrated crosslink product between A allele 1 and oxidized **Fr-ODN** (MALDI-TOF calcd 8412.6, found 8414.2). The isolated yield of peak B was 17%. Blair reported that three major adducts (α1, α2, and β) were observed in the reaction between 4-oxo-2-nonenal and deoxyadenosine.[14] α1 and α2, which are two isomeric substituted ethano adducts, were observed after 2 h incubation at 37 °C. Adduct β was observed following 2 h incubation at 60 °C. According to the reported mechanism, peak A1 and A2 are thought to be derived from the duplexes containing the diastereomeric adducts, and peak B is thought to be derived from the duplex containing an ethno adduct.

The fluorescence properties of a substituted 1,N^6-ethenodeoxyadenosine, 1″[3-(2′-deoxy-β-D-erythropentafuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]heptane-2″-one, which was synthesized according to previous procedures,[14] were studied as a reference compound. A fluorescence emission peak of the substituted 1,N^6-ethenodeoxyadenosine was observed at 430 nm on excitation at 310 nm ($\Phi_F = 0.14$) (Fig. 3a). Both fluorescence excitation maximum and emission maximum shifted to longer wavelength compared to those of 1,N^6-ethenodeoxyadenosine ($\Phi_F = 0.56$). We next examined fluorescence properties of **Fr-ODN** under oxidative conditions after addition of *N*-bromosuccinimide in the presence of complementary DNAs. Fluorescence spectrum of **Fr-ODN** in the presence of A allele 1 was measured with excitation at 310 nm after incubation for 2 h at 37 °C, then at 60 °C for 2 h. Fluorescence emissions with a maximum wavelength at 430 nm was observed, and the shape of obtained spectrum was very similar to that of the substituted 1,$\boldsymbol{N}^6$-ethenodeoxyadenosine (Fig. 3a). These results strongly suggested that etheno adducts were formed in the reaction mixture between **Fr-ODN** and A allele 1. The time dependence of the fluorescence intensity of the reaction mixtures at 430 nm is shown in Figure 3b. The fluorescence intensity of **Fr-ODN** at 37 °C was gently increased in the presence of A allele 1 with the course of time, and sharply increased at 60 °C, whereas fluorescence intensities of **Fr-ODN** in the absence of A allele 1 were very little increased. The fluorescence intensity of **Fr-ODN** in the presence of C allele 1 was almost the same as that in the absence of A allele 1. The rate of dehydration of the ethanoadenosine derivative depends on the reaction temperature and pH conditions; fluorescence intensity at 430 nm in the absence and presence of N allele 1 (N = A or C) was therefore measured under various pH conditions. Whereas fluorescence intensity of 1,N^6-ethenoadenosine hardly change under these conditions, that of the reaction mixtures in the presence of A allele 1 was increased under acidic conditions. As a result of increased fluorescence intensity, the fluorescence intensity in the presence of A allele 1 was seven times larger than that of C allele 1 at pH 6.0. The concentration dependence of the fluorescence intensity of 2.5 μM **Fr-ODN** was measured in the presence of various concentrations of the N allele 1 (N = A or C) (Fig. SI 1). A allele 1 and C allele 1 were clearly distinguishable at a concentration of 0.5 μM. Finally, the influence of the 3′-neighboring base at mutation site was estimated using N allele 2. Although the 3′-neighboring base of SNP site is adenine, the fluorescence intensity of the reaction mixture (N = A) is much lager than that of N = C (Fig. 3d). These results suggest that **Fr-ODN** could be applied to the diagnosis of adenine-related DNA mutation.

This Letter describes mutation detection by fluorescent measurements using an ethenoadenosine-forming reaction. We have synthesized the oligonucleotide containing a furan derivative at the 5′ end. It was revealed from reversed-phase HPLC profiles and fluorescent measurements that furan-conjugated oligonucleotides reacted with complementary DNAs under oxidative conditions and an etheno adduct was formed in the presence of A allele DNA. The study reported here is thoroughly based on the unique chemical properties of γ-oxo-α,β-unsaturated aldehyde, which would bring an advance in the current biotechnologies.

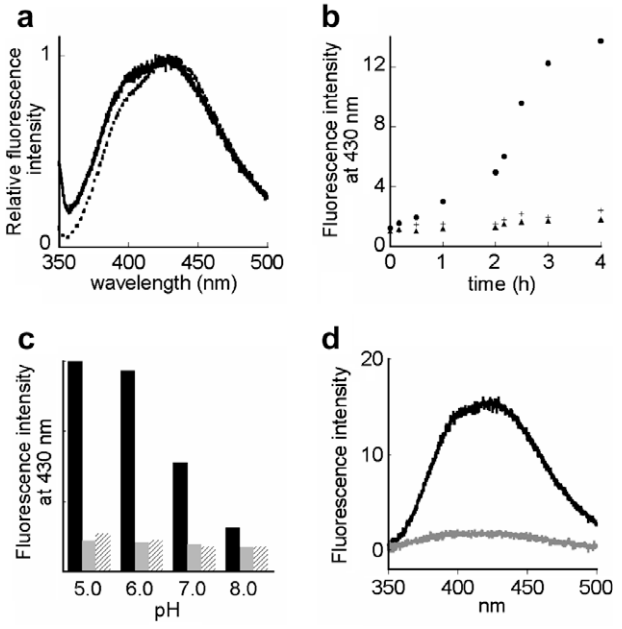


Figure 3. (a) Relative fluorescence spectra of 2.5 μM **Fr-ODN** in the presence of 2.5 μM A allele 1 (solid line) and a substituted 1,N^6-ethenodeoxyadenosine (dotted line). λ_{ex} = 310 nm (b) Time course of the fluorescence intensity of 2.5 μM **Fr-ODN** in the presence of 2.5 μM N allele l (N = A: black line, N=C: gray line) at 430 nm. Reaction mixtures were incubated at 37 °C for 2 h, then at 60 °C for 2 h in 10 mM sodium phosphate buffer containing 100 mM NaCl. (c) pH dependence of the fluorescence intensity at 430 nm of 2.5 μM **Fr-ODN** in the absence (shaded bar) or presence of 2.5 μM N allele 1 (N = A: black bar, N = C: gray bar), (d) Fluorescence spectra of 2.5 μM **Fr-ODN** in the presence of 2.5 μM N allele 2 (N = A: black line, N = C: gray line).

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.092.

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