Synthesis and Photophysical Characterisation of a Fluorescent Nucleoside Analogue that Signals the Presence of an Abasic Site in RNA

ARTICLE *in* CHEMBIOCHEM · NOVEMBER 2012

Impact Factor: 3.09 · DOI: 10.1002/cbic.201200408 · Source: PubMed

2 AUTHORS:

Arun Tanpure
Indian Institute of Science Education and R…
10 PUBLICATIONS **124** CITATIONS
SEE PROFILE

Seergazhi G Srivatsan
Indian Institute of Science Education and R…
30 PUBLICATIONS **768** CITATIONS
SEE PROFILE

CHEMBIOCHEM

ChemPubSoc Europe

DOI: 10.1002/cbic.201200408

Synthesis and Photophysical Characterisation of a Fluorescent Nucleoside Analogue that Signals the Presence of an Abasic Site in RNA

Arun A. Tanpure and Seergazhi G. Srivatsan*[a]

The synthesis and site-specific incorporation of an environment-sensitive fluorescent nucleoside analogue (**2**), based on a 5-(benzofuran-2-yl)pyrimidine core, into DNA oligonucleotides (ONs), and its photophysical properties within these ONs are described. Interestingly and unlike 2-aminopurine (a widely used nucleoside analogue probe), when incorporated into an ON and hybridised with a complementary ON, the emissive nucleoside **2** displays significantly higher emission intensity than the free nucleoside. Furthermore, photophysical characterisation shows that the fluorescence properties of the nucleoside analogue within ONs are significantly influenced by flanking bases, especially by guanosine. By utilising the responsiveness of the nucleoside to changes in base environment, a DNA ON reporter labelled with the emissive nucleoside **2** was constructed; this signalled the presence of an abasic site in a model depurinated sarcin/ricin RNA motif of a eukaryotic 28S rRNA.

Introduction

Fluorescent nucleoside analogue probes are very useful biophysical tools for investigating the structure and binding properties of nucleic acids.[1] Unlike many proteins that show intrinsic fluorescence due to the presence of environment-sensitive fluorescent aromatic amino acids (e.g., tryptophan), nucleic acids lack intrinsic fluorescence, as natural nucleobases are essentially nonemissive.[2] Consequently, several nucleoside analogue probes with useful fluorescence properties have been developed by 1) using naturally occurring fluorescent heterocycles and polycyclic aromatic hydrocarbons (PAHs) as nucleobase surrogates, 2) attaching known fluorophores to the nucleobases or sugars (e.g., pyrene, anthracene, phenanthroline, bipyridine and terpyridine), and 3) extending the π conjugation by attaching heterocycles to nucleobases.[1e,3] For example, pterdines, a class of naturally occurring planar heterobicyclic compounds that are structurally similar to purines, have been used as nucleobase surrogates to develop highly emissive nucleoside analogues.[4] In a similar approach, Kool and co-workers developed PAH base analogues in which the nucleobases were replaced with fluorescent PAHs (e.g. pyrene, perylene and phenanthrene).[1c] Such highly nonpolar analogues lacking the Watson–Crick hydrogen-bonding face have been effectively implemented in investigating the shape-complementarity requirement in base-pair formation, the electron transfer process in nucleic acids, and to promote intercalation.[5–7] Fusing additional aromatic rings onto pyrimidines and purines also enhances π conjugation, thus generating "size-expanded" fluorescent nucleobase analogues.[1f] The Saito group has developed several such fluorescent ring-expanded nucleobases for homogeneous single nucleotide polymorphism (SNP) analysis.[1a]

While the majority of the above analogues structurally deviate from the natural nucleosides, isomorphic analogues have a similar size, shape and Watson–Crick hydrogen bonding face as that of the native bases, and hence, when incorporated, they minimally perturb the structure and function of target oligonucleotides (ONs).[1e] In particular, 2-aminopurine (2-AP), an isomorphic adenosine analogue that is highly responsive to changes in its local environment, is one of the most extensively used nucleoside analogues in nucleic acid-based diagnostics and discovery assays.[8,9] Pyrrolo-C (pC) and more recently, its derivative 6-phenylpyrrolo-C (PhpC) have been introduced as isosteric cytosine (C) analogues.[10,11] As free nucleosides they display moderate quantum yields, but these drop drastically when the nucleosides are placed in a double-stranded oligonucleotide. This property of pC and PhpC has been elegantly used in monitoring RNA secondary structure, and activity of the RNase H enzyme, respectively.[10,11] In another design strategy, structurally non-invasive pyrimidine analogues have been developed by attaching or fusing five- or six-membered heterocyclic rings onto pyrimidines. Some of these analogues display favourable photophysical properties and have been effectively implemented in assays to probe nucleic acid conformation and function.[12]

Although a significant number of environment-sensitive base-modified nucleoside analogues with improved fluorescence properties have been developed,[13,14] only a very few nucleosides that retain reasonable quantum yield when incorporated into ONs have been implemented in biophysical assays to study the dynamics and functions of nucleic acids.[15] Therefore, we sought to develop environment-sensitive nu-

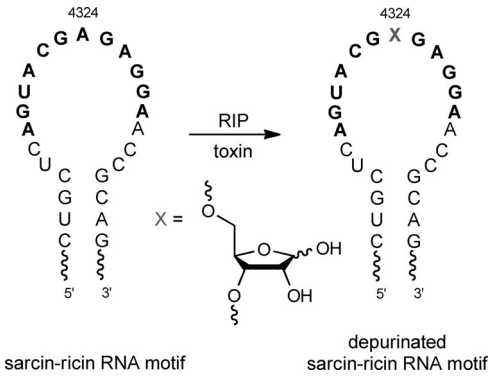
[a] *A. A. Tanpure, Dr. S. G. Srivatsan*
Department of Chemistry
Indian Institute of Science Education and Research
900, NCL Innovation Park, Dr. Homi Bhabha Road
Pune 411008 (India)
E-mail: srivatsan@iiserpune.ac.in

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201200408.

cleoside analogue probes that exhibit minimal structural perturbation and display emission in the visible region with an appreciable quantum yield when incorporated into ONs.

By drawing inspiration from a naturally occurring fluorescent amino acid (tryptophan), we have developed a focused series of base-modified emissive pyrimidine ribonucleoside analogues by tagging indole, *N*-methylindole, benzothiophene or benzofuran at the 5-position of uracil (Figure S1).[16] In particular, benzothiophene- and benzofuran-conjugated uridine derivatives are reasonably emissive, with emission maxima in the visible region, and display excellent fluorescence solvatochromism. We hypothesised that the responsiveness of these ribonucleoside analogues to changes in polarity and the neighbouring base environment (when incorporated into RNA ONs) could also be employed to explore the structure and recognition properties of DNA ONs. In order to investigate the photophysical consequences of placing the emissive nucleoside within a DNA ON, we chose to incorporate the 2′-deoxy version of the benzofuran-conjugated pyrimidine analogue.[17] Here, we describe the synthesis, photophysical characterisation and incorporation of benzofuran-conjugated 2′-deoxyuridine analogue **2** into DNA ONs. Remarkably, upon incorporation into single-stranded and double-stranded oligonucleotides the emissive nucleoside shows significantly enhanced emission intensity compared with the free nucleoside, a property that is rarely displayed by most of the fluorescent nucleoside analogues. Furthermore, by using fluorescence spectroscopy we investigated the photophysical behaviour of the emissive nucleoside incorporated into DNA ONs in different base environments. Finally, as proof of the responsiveness of the nucleoside to environmental changes, we describe the ability of a DNA ON reporter labelled with modified nucleoside **2** to signal the presence of an abasic site in a model depurinated sarcin/ricin RNA motif of a eukaryotic 28S rRNA.

Unlike DNA abasic sites, which are common DNA lesions, RNA abasic sites are rare; they are almost uniquely associated with the depurination activity of ribosome inactivating protein (RIP) toxins.[18] These toxins (e.g., ricin and saporin) arrest the function by binding to a highly conserved RNA motif in eukaryotic 28S rRNA (the "sarcin/ricin loop") that interacts with elongation factors essential for protein synthesis, and depurinating a specific adenosine residue (A4324) in the loop (Scheme 1).[19] Consequently, the elongation factors necessary for translation no longer bind to the depurinated ribosome, thereby leading to cell death.[20] In particular, ricin has been considered as a potential bioterrorism agent because of its high toxicity, easy procedures for isolation from natural sources and the lack of effective treatment against its exposure.[21] Traditionally, RIP toxins have been detected by using enzyme-linked immunosorbent assays (ELISAs) and antibody-based immunoassays.[22] Methods have also been developed to monitor the specific depurination activity of RIP toxins by radiolabelling, immunoaffinity chromatography, electro-chemiluminescence and mass spectrometry.[23] Usually these methods are laborious and involve elaborate assay setups and radiolabelling procedures. Alternatively, a few abasic-site-sensitive fluorescence probes have provided effective tools to directly detect



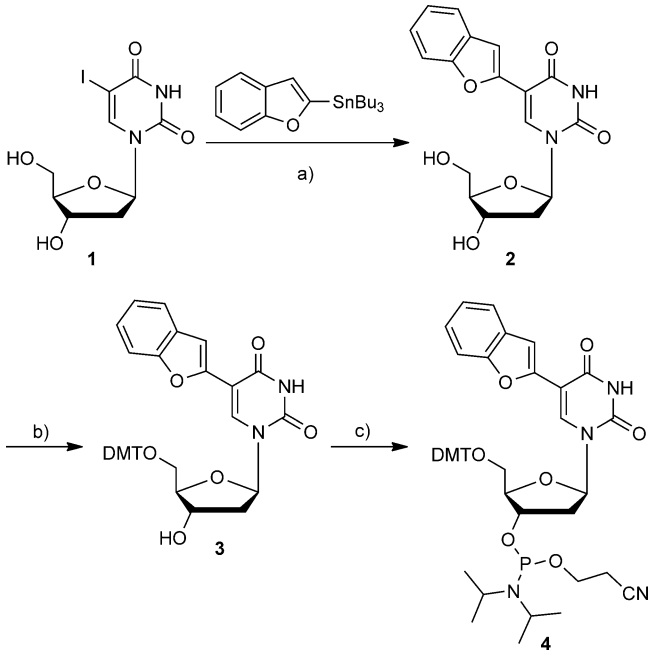
Scheme 1. Sequence of the sarcin/ricin hairpin motif of ribosomal RNA. The conserved residues are shown in bold. RIP toxins catalytically depurinate residue A_{4324} to produce the depurinated RNA motif. The structure of the RNA abasic site (**X**) is also shown.

the formation of abasic sites in RNA.[24] Nevertheless, abasic site detection assays that are compatible with screening formats are highly desirable for the discovery of RIP inhibitors.

Results and Discussion

Synthesis and photophysical characterisetion of nucleoside 2

The fluorescent deoxyuridine analogue, 5-(benzofuran-2-yl)-2′-deoxyuridine **2** was synthesised under typical Stille cross-coupling reaction conditions by reacting 5-iodo-2′-deoxyuridine **1** with 2-(tri-*n*-butylstannyl)benzofuran in the presence of a palladium catalyst, $Pd(PPh_3)_2Cl_2$ (Scheme 2).[25] Many fluorescent nucleoside analogue probes that have been utilised for studying the structure of nucleic acids photophysically respond to solvent polarity and viscosity changes.[1e] Therefore, before incorporation into oligonucleotides, the photophysical properties of nucleoside **2** were evaluated by performing UV absorption, and steady-state and time-resolved fluorescence spectroscopic measurements in solvents of differing polarity and viscosity. The ground-state electronic spectrum of **2** in water revealed distinct absorption bands at 265, 272 and 322 nm (Figure 1). When measured in different solvents, the absorption maxima of **2** were marginally affected by solvent polarity. However, the excited-state electronic properties were substantially influenced by changes in solvent polarity. Upon excitation of the nucleoside at its lowest energy maximum (322 nm) in aqueous solution, it displayed a strong emission band in the visible region ($\lambda_{em} = 446$ nm, Figure 1, Table 1). As the solvent polarity was gradually decreased (water to dioxane), the nucleoside exhibited up to nearly 2.5-fold quenching in fluorescence intensity and a significant hypsochromic shift (from 446 to 406 nm). Furthermore, the quantum yields determined in various solvents followed a similar decreasing trend as that for fluorescence intensity (Table 1). A positive correlation between the Stokes shift determined in various solvents and Reichardt's microscopic solvent polarity parameter, $E_T(30)$, also revealed the sensitivity of the nucleoside to changes in solvent-polarity environment (Figure 2).[27]



Scheme 2. Synthesis of fluorescent nucleoside, 1-(2-deoxy-β-D-ribofuranosyl)-5-(benzofuran-2-yl)uracil **2** and the corresponding phosphoramidite substrate **4**.[25] a) $Pd(PPh_3)_2Cl_2$, dioxane, 90 °C, 97%; b) 4,4′-dimethoxytritylchloride (DMT-Cl), 4-dimethylaminopyridine (DMAP), pyridine, RT, 67%; c) $iPr_2NP(Cl)OEtCN$, iPr_2NEt, CH_2Cl_2, RT, 51%.

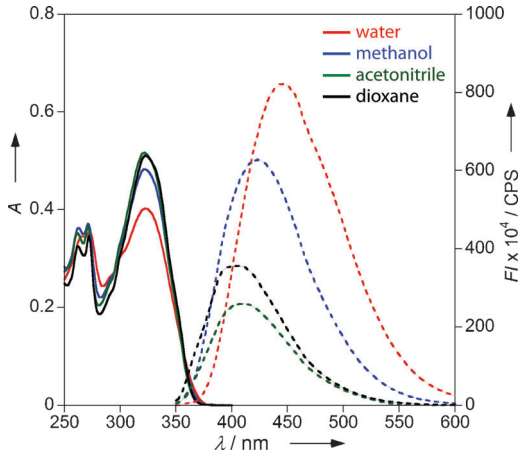


Figure 1. Absorption (——) and emission (-----) spectra of nucleoside **2** (25 and 5.0 μM, respectively) in different solvents. FI = fluorescence intensity. For fluorescence studies, samples were excited at 322 nm, and excitation and emission slit widths were 3 and 5 nm, respectively.[26]

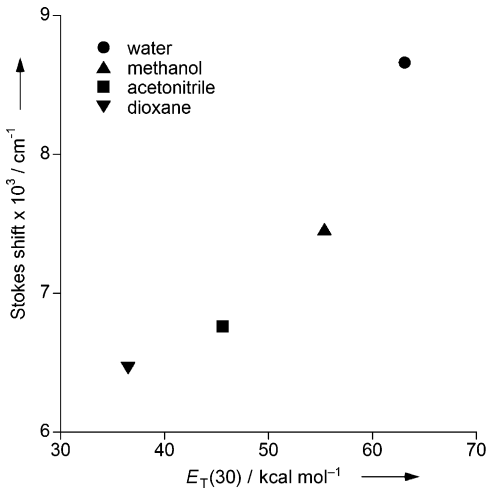


Figure 2. Plot of Stokes shift versus $E_T(30)$, a microscopic solvent polarity parameter.

The effect of solvent polarity on the excited-state decay kinetics of nucleoside **2** was also investigated by time-resolved fluorescence spectroscopy. The nucleoside had the highest excited-state lifetime in water (2.38 ns), and this decreased significantly in dioxane (0.33 ns, Table 1, Figure S2). The ratio of radiative (k_r) to nonradiative (k_{nr}) decay rate constants, determined by lifetime and quantum yield in different solvents, revealed that the radiative pathway is considerably favoured in polar solvents compared with nonpolar solvents (Table 1).

The relative conformation of the pyrimidine ring and conjugated aromatic ring separated by a rotatable aryl–aryl bond in modified nucleoside analogues, which has a direct impact on the conjugation and, hence, fluorescence properties, has been shown to be sensitive to molecular crowding and viscosity.[28] In comparison with low-viscosity media, viscous media restrict free rotation, thereby resulting in structural rigidification and usually enhanced fluorescence emission.[29] When incorporated into ONs, a conjugated nucleoside analogue can undergo structural rigidification–derigidification by interactions with neighbouring bases during a folding or recognition process.[28] In order to assess the effects of changes in conformation of the benzofuran moiety relative to the nucleobase, further fluorescence characterisation of nucleoside **2** were performed in solvents of similar polarity but differing viscosity. As the solvent viscosity increased, from ethylene glycol ($\eta_{25\,°C} = 16.1$ cP) to glycerol ($\eta_{25\,°C} = 934$ cP), the nucleoside shows enhancing emission intensity (nearly 1.5-fold higher in glycerol, relative to ethylene glycol) with no apparent change in emission maximum (Figure 3 and Table S1). Lifetime and anisotropy measurements also revealed a longer decay time and a higher anisotropy value in glycerol than in ethylene glycol, which is consistent with the viscosity difference

Table 1. Photophysical properties of **2** in different solvents.[25,26]

Solvent	λ_{max}[a] [nm]	λ_{em} [nm]	I_{rel}[b]	Φ[c]	τ_{ave}[c] [ns]	k_r/k_{nr}
water	322	446	1.0	0.19	2.38	0.24
methanol	322	423	0.8	0.12	0.78	0.13
acetonitrile	322	411	0.3	0.04	0.27	0.04
dioxane	322	406	0.4	0.07	0.33	0.08

[a] λ_{max} corresponding to the lowest energy maximum is given. [b] Relative emission intensity is given relative to the intensity in water. [c] Standard deviations for Φ and τ_{ave} were $\leq$0.002 and 0.02 ns, respectively.

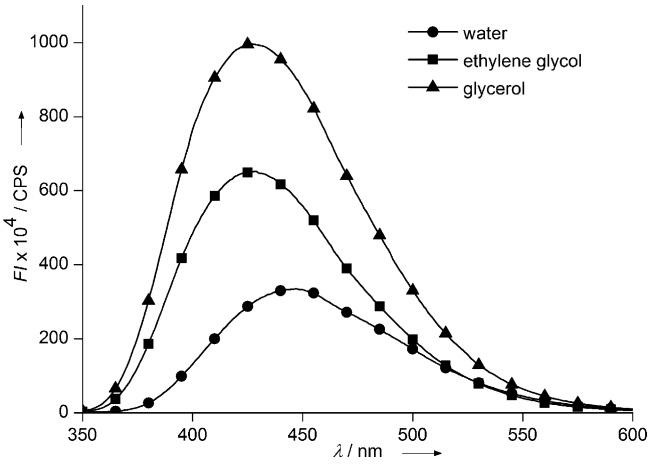


Figure 3. Emission spectra of nucleoside **2** (5.0 μM) in solvents of varying viscosity. FI = fluorescence intensity. Samples were excited at 322 nm, and excitation and emission slit widths were 1 and 10 nm, respectively.[26]

between the solvents (Table S1). Taken together, emission in the visible region with a reasonable quantum yield and the dual-sensitivity of the nucleoside fluorescence to solvent polarity and viscosity prompted us to study the responsiveness of the emissive nucleoside within DNA ONs.

Incorporation of nucleoside 2 into DNA ONs

The phosphoramidite substrate **4**, which is necessary for the solid-phase ON synthesis, was prepared by first protecting the 5′-hydroxyl with a dimethoxytrityl group, followed by phosphitylation of the 3′-hydroxyl in the presence of 2-cyanoethyl diisopropylchlorophosphoramidite (Scheme 2).[25] To study the effect of flanking bases on the fluorescence of the modified nucleoside, a series of DNA ONs (**5**–**8**) was synthesised in which **2** was placed between different bases (Figure 4). The modified phosphoramidite was incorporated into 17-mer DNA ONs under standard solid-phase ON synthesis conditions. The deprotected ONs were then purified by PAGE under denaturing conditions. The integrity of full-length modified ONs was confirmed by mass analysis (Figure S3, Table S2). The presence of a benzofuran moiety can potentially perturb the structure of an ON, and hence the formation of a stable duplex with its complementary ON. The stability of a duplex assembled by hybridising one of the modified ONs (**8**) with its complementary ON (**8c**) was studied by a UV-thermal denaturation experiment. This showed only a small difference in T_m between the modified duplex **8·8c** and the unmodified duplex **9·8c**, thus indicating that the benzofuran modification has an only marginal effect on duplex stability (Figure S4, Table S3).

5′-GCGATCAA**2**AACTAGCG-3′	**5**
5′-GCGATCAT**2**TACTAGCG-3′	**6**
5′-GCGATCAC**2**CACTAGCG-3′	**7**
5′-GCGATCAG**2**GACTAGCG-3′	**8**
5′-GCGATCAGTGACTAGCG-3′	**9**
5′-CGCTAGTTATTGATCGC-3′	**5c**
5′-CGCTAGTAAATGATCGC-3′	**6c**
5′-CGCTAGTGAGTGATCGC-3′	**7c**
5′-CGCTAGTCACTGATCGC-3′	**8c**
5′-CGCTAGTCTCTGATCGC-3′	**10**
5′-CGCTAGTCGCTGATCGC-3′	**11**
5′-CGCTAGTCCCTGATCGC-3′	**12**

Figure 4. Sequences of fluorescently modified (**5**–**8**) and custom synthesised (**9**–**12** and **5c**–**8c**) DNA oligonucleotides (**9** is a control: unmodified DNA). While hybridisation of **5**–**8** with **5c**–**8c**, respectively, places nucleoside **2** opposite its complementary base, hybridisation of **8** with **10**–**12** places **2** opposite mismatched bases.[26]

Photophysical characterisation of nucleoside 2 in different base environment

The photophysical properties of fluorescent nucleosides incorporated into ONs can be altered by a variety of mechanisms, such as stacking of the chromophore to flanking bases, collisional and hydrogen-bond interactions with neighbouring bases, the solvation–desolvation effect, rigidification–derigidification of the chromophore and excited-state processes with neighbouring bases.[8,28,30,31] The influence of neighbouring bases on the fluorescence properties of **2** was studied by performing steady-state fluorescence measurements with DNA ONs **5**–**8** and duplexes constructed by hybridising **5**–**8** with their respective complementary ONs (**5c**–**8c**, Figure 4). Single-stranded ONs **5**–**7** (emissive nucleoside between dA, dT or dC residues, respectively) exhibited significantly enhanced emission compared with the free nucleoside (Figure 5). Interestingly, when incorporated into duplexes **5·5c** and **6·6c**, nucleoside **2** showed enhanced emission compared with the respective single-stranded ONs (Figure 5, Figure S5). This observation is particularly noteworthy because the majority of emissive nucleoside analogues (e.g., 2-AP, pyrroloC)[10,32] show progressive fluorescence quenching upon incorporation into single-stranded and double-stranded ONs, a major impediment to the use of many such analogues in in vivo assays.

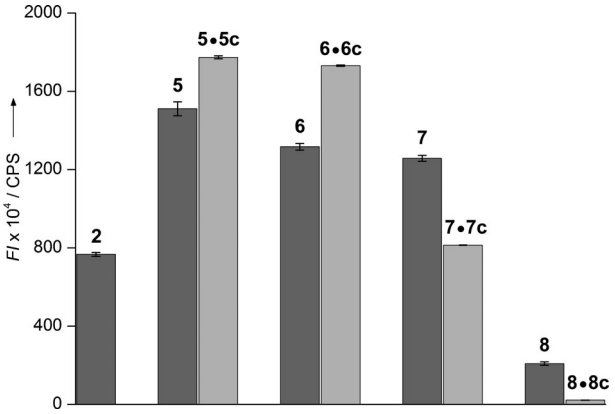


Figure 5. Fluorescence intensity (FI) of modified ONs (**5**–**8**, 1 μM) and corresponding duplexes (1 μM) in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA) at their respective emission maxima (422–444 nm, see Table S4). Samples were excited at 330 nm; excitation and emission slit widths were 3 and 10 nm, respectively.[25,26]

However, ON **8**, in which **2** is flanked by guanosine residues, displayed a slightly blue-shifted and markedly less-intense (ca. threefold) emission band compared with the free nucleoside **2** (Figures 5 and S5). The quenching effect was more pronounced (~tenfold) when the emissive nucleoside was placed opposite the complementary base in the "perfect" duplex **8·8c**. 2-AP and several other fluorescent nucleoside analogues exhibit similar fluorescence intensity quenching when incorporated into ONs.[1e,8,32] Furthermore, duplexes **8·10**, **8·11** and **8·12**, in which the emissive nucleoside is opposite mismatched bases, showed similar fluorescence quenching to that of the "perfect" duplex **8·8c** (Figure 6). This fluorescence quenching,

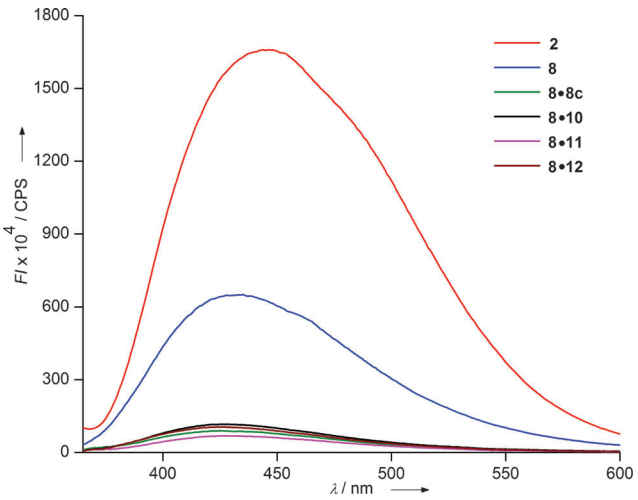


Figure 6. Emission spectra of **2**, **8** and duplexes assembled by hybridising **8** with complementary (**8c**) and mismatched ONs (**10**–**12**). FI = fluorescence intensity. Samples were excited at 330 nm; excitation and emission slit widths were 7 and 10 nm, respectively.[26]

along with a small spectral blue shift, might be attributable to a combination of the following: an electron transfer process between the fluorescent nucleoside and adjacent guanosine residues,[30,31,33] a desolvation effect[8] and alterations in the conformation of the benzofuran moiety relative to the nucleobase.[28] These results clearly reveal the influence of neighbouring bases on the fluorescence of nucleoside **2**. Such a property has been employed in devising nucleic acid-based molecular beacons and in the detection of a specific mismatch in DNA duplexes.[1a,34]

Fluorescence detection of an RNA abasic site

Early methods to detect abasic sites were largely focused on the detection of abasic sites in DNA as these are formed spontaneously or as intermediates in the base-excision repair process of damaged nucleobases.[35,36] These methods relied on the specific and irreversible reaction between aldehyde-reactive probes and abasic sites of isolated DNA.[37] Later, Greenberg developed a more sensitive method by utilising biotinylated cysteine to specifically detect the oxidised form of an abasic lesion, 2-deoxyribonolactone.[38] Matray and Kool used a pyrene-modified deoxyribonucleotide triphosphate, which was preferentially incorporated opposite an abasic site by DNA polymerase, to identify the presence of abasic sites.[5,39] Alternatively, fluorescence-based methods with probes that show changes in their emission properties when adjacent or opposite abasic sites were found to be more useful as they offered direct detection of abasic sites in DNA and RNA.[24,40,41] Shipova and Gates developed one of the first fluorimetric assays to monitor the time-depended generation of abasic sites by using 2-AP-labelled DNA hairpin constructs.[41] In this study 2-AP was placed adjacent to a guanine residue that was alkylated by leinamycin[42] and subsequently excised to produce an abasic site adjacent to the fluorescent probe. Upon treatment with leinamycin, 2-AP positively reported the formation of abasic sites with a significant enhancement in fluorescence intensity. By adopting a similar approach, pyrene- and thiophene-modified fluorescent nucleoside analogues and a fluorescent ligand that specifically binds to an abasic site were used in monitoring the formation of abasic sites in RNA oligonucleotides.[24]

Our previous study revealed that a short RNA ON reporter containing the benzofuran-conjugated ribonucleoside analogue could specifically signal the presence of a DNA abasic site in an RNA–DNA heteroduplex.[16a] This observation encouraged us to study the impact of placing the deoxyribonucleoside **2** opposite an RNA abasic site. As before, a series of DNA–RNA heteroduplexes was assembled by annealing modified DNA ONs **5**–**8** to the respective complementary RNA ONs **13c**–**16c** or to ONs **13a**–**16a** containing a chemically stable abasic site surrogate, tetrahydrofuran (Figures 4 and 7A). Nucleoside **2** (flanked by dA, dT or dC), when placed opposite an abasic site in duplexes **5·13a**, **6·14a** or **7·15a**, showed discernible quenching in fluorescence intensity compared with the corresponding "perfect" duplexes **5·13c**, **6·14c** and **7·15c** (Figure 7B). However, duplex **8·16c** and abasic-site-containing duplex **8·16a**, in which the nucleoside is flanked by dG residues, exhibited very weak fluorescence.[33]

To further assess the ability of emissive nucleoside to detect the presence of an abasic site in a biologically relevant RNA motif, we decided to use model RNA ONs **17** and **18** (Figure 8A). ON **17**, which contains the conserved sarcin/ricin loop region of eukaryotic 28S rRNA, is a commonly used substrate to study the depurination activity of RIPs.[23b,24] ON **18** contains a chemically stable abasic site substitute, tetrahydrofuran, and acts as a model of the depurinated product that would be obtained upon depurination of substrate **17** by RIP toxins.[24b,d] A complementary DNA ON **19** labelled with **2** was synthesised; upon hybridisation to **17** or **18**, this would place **2** opposite a complementary base and an abasic site, respectively (Figure 8A).[43] A duplex of fluorescent ON and RIP RNA substrate (**19·17**) showed a strong emission band, the intensity of which was nearly 1.5-fold higher than that for single-stranded **19** (Figure 8B). Interestingly, the duplex of ON probe and depurinated product mimic (**19·18**) exhibited drastically quenched emission (ca. eightfold) relative to that of duplex **19·17** (Figure 8B). Although the exact morphology of the nucleoside **2** in **19·18** is unknown, we believe that the observed quenching in

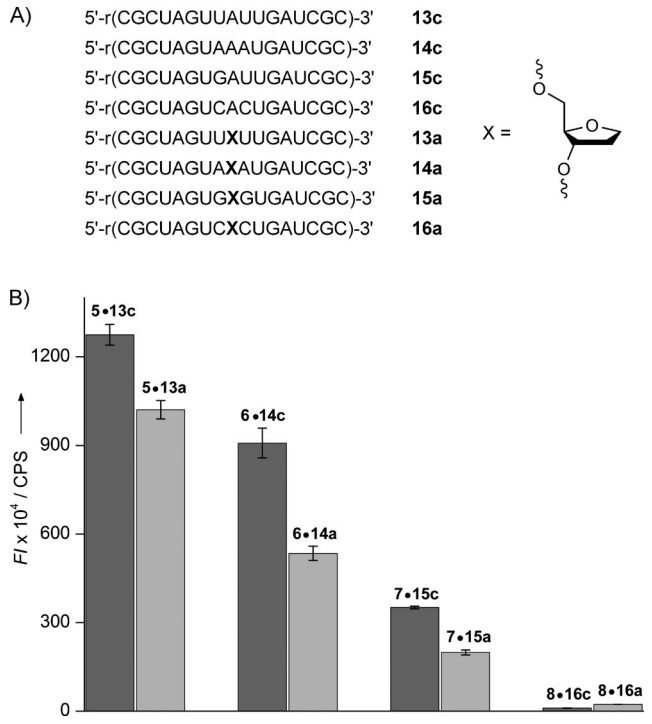


Figure 7. A) Sequences of synthetic RNA oligonucleotides. While hybridisation of **5**–**8** with **13c**–**16c**, respectively, places nucleoside **2** opposite its complementary base, hybridisation of **5**–**8** with **13a**–**16a** places it opposite a chemically stable abasic site surrogate **X**. B) Fluorescence intensity (FI) of heteroduplexes (1 μM) in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA) at 430 nm. Samples were excited at 330 nm; excitation and emission slit widths were 3 and 10 nm, respectively.[25,26]

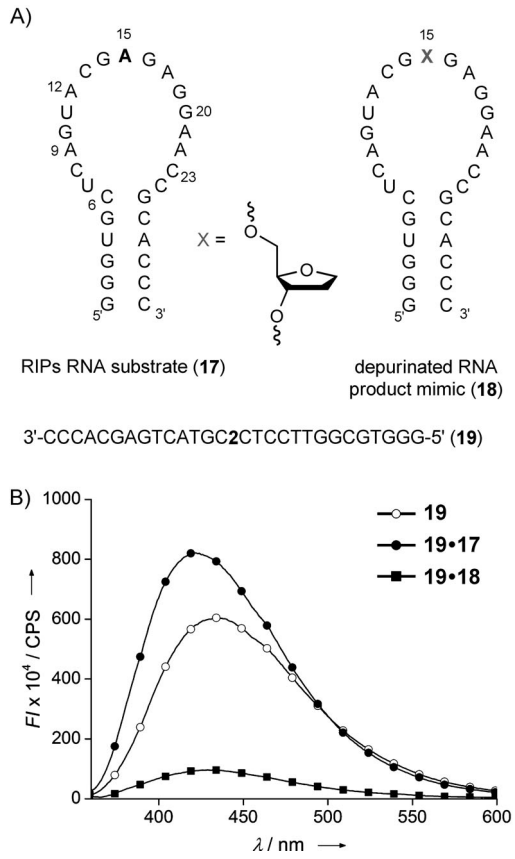


Figure 8. A) Sequence of synthetic RNA (**17**, RIP substrate) and depurinated product mimic (**18**) containing a chemically stable abasic site substitute (X). Adenosine residue A15 (A4324 in rat 28S rRNA) is specifically depurinated by RIP toxins to produce an abasic site. cDNA (**19**) containing fluorescent nucleoside **2** is also shown. B) Emission spectra (1 μM) of substrate (**19·17**) and depurinated product duplexes (**19·18**). FI = fluorescence intensity. Samples were excited at 330 nm; excitation and emission slit widths were 7 and 10 nm, respectively.[25,26]

fluorescence intensity might be attributable to the following reasons. In duplex **19·17** the benzofuran moiety (tagged at the 5-position of the base) is extrahelical and projects towards the major groove, and hence it is less stacked and located away from the guanosine residues of ON **17** (Figure S6). However, in duplex **19·18** the benzofuran ring is presumably intrahelical, because **2** opposite an abasic site can potentially undergo *anti*-to-*syn* conformational change as it is not restricted by complementary base pairing.[28] In this situation the fluorophore is better stacked and closer to the guanosine residues of **18,** thereby resulting in quenching of fluorescence.[44] Together, it can be inferred that **2** in duplex **19·18** signals the presence of an abasic site in RNA, albeit with quenched emission. This attribute of the emissive nucleoside can be potentially implemented in a fluorescence hybridisation assay to detect the depurination activity of highly toxic RIPs.[24]

Conclusions

A microenvironment-sensitive fluorescent 2′-deoxyuridine analogue that is based on a 5-(benzofuran-2-yl)pyrimidine core and that displays emission in the visible region has been incorporated into DNA ONs. Notably, upon incorporation into duplexes, nucleoside **2** remarkably maintains its fluorescence efficiency, a property that is seldom exhibited by the majority of fluorescent nucleoside analogues. Furthermore, the ability of the fluorescent analogue incorporated into a DNA ON reporter to detect the presence of an abasic site in RNA highlights the potential of **2** as a fluorescent probe. As the conformation of the emissive nucleoside, its surrounding environment and/or its interaction with neighbouring bases are likely to be altered during a folding or recognition event, it is expected that this environment-sensitive nucleoside analogue will be a useful probe in studying the structure and function of nucleic acids.

Experimental Section

Photophysical characterisation of benzofuran-conjugated 2′-deoxyuridine 2

Steady-state fluorescence in various solvents: Modified nucleoside **2** (5 μM) in water, methanol, acetonitrile or dioxane was excited at its lowest energy absorption maximum (322 nm), with excitation and emission slit widths of 3 nm and 5 nm, respectively. In the case of ethylene glycol and glycerol the excitation and emission slit widths were 1 nm and 10 nm, respectively. All solutions contained DMSO (0.5%). Fluorescence experiments were performed in triplicate in a micro fluorescence cell (path length 1.0 cm; Hellma Optics, Jena,

Germany) on a FluoroLog-3 fluorescence spectrophotometer (Horiba, Kyoto, Japan).

Time-resolved fluorescence measurements: Excited-state lifetimes of **2** in various solvents were determined by using a time correlated single photon counting (TCSPC) fluorescence spectrophotometer (Horiba). In water, methanol, ethylene glycol and glycerol the concentration of **2** was 5 μM; in acetonitrile and dioxane it was 250 μM. In water, methanol, ethylene glycol and glycerol, **2** was excited by using a NanoLED 339 nm source (IBH/Horiba, Glasgow, UK) with a band pass of 4 nm, and fluorescence emission was collected. Similarly, **2** in acetonitrile and dioxane was excited by using a NanoLED 375 nm diode laser source (IBH) with a band pass of 12 nm. Lifetime measurements were performed in duplicate, and decay profiles were analysed with DAS6 analysis software (IBH). Fluorescence intensity decay kinetics in water and ethylene glycol were found to be monoexponential, whereas in other solvents they were found to be biexponential, with χ^2 (goodness of fit) values very close to unity.

Synthesis and purification of modified ONs: Benzofuran-modified DNA ONs **5**–**8** and **19** were synthesised on a 1.0 μmol scale (1000 Å CPG solid support). Phosphoramidite **4**[25] was site-specifically incorporated into the ONs by a standard DNA ON synthesis protocol with a final trityl-off step (coupling efficiencies were 60–75%). The solid support was treated with aqueous ammonium hydroxide (3 mL, 30%) for 15 h at ~50 °C. The aqueous ammonium hydroxide solution was evaporated to dryness on a Speed Vac, and deprotected ON products were purified by 20% polyacrylamide gel electrophoresis under denaturing conditions. Respective modified ON products were visualised by UV shadowing; product bands were excised from the gel and transferred to a Poly-Prep column (Bio-Rad). The gel pieces were crushed with a sterile glass rod, and ONs were extracted in sodium acetate buffer (0.3 M, 3 mL) for 12 h. The resulting solutions were filtered and desalted in Sep-Pak classic C18 cartridges (Waters). See Table S2 for ε_{260} and MALDI-MS data of the modified ONs.

Steady-state fluorescence of modified DNA ONs: ONs **5**–**8** (10 μM) were annealed to their respective complementary ONs (**5c**–**8c**, 11 μM) by heating in cacodylate buffer (20 mM pH 7.0) with NaCl (100 mM) and EDTA (0.5 mM) at 90 °C for 3 min. Samples were then cooled slowly to RT and placed on crushed ice for 2 h. Samples were diluted (final modified ON concentration, 1 μM) in cacodylate buffer. By following the above procedure, DNA–RNA heteroduplexes (1 μM) were assembled between modified DNA ONs **5**–**8** and the respective complementary RNA ONs **13c**–**16c** or abasic-site containing ONs **13a**–**16a**. Fluorescently modified duplexes were excited at 330 nm (excitation and emission widths: 3 and 10 nm, respectively). Fluorescence experiments were performed in triplicate in a micro fluorescence cuvette at RT. Duplexes **19·17** and **19·18** were also prepared as above, and fluorescence spectra were recorded by exciting the samples at 330 nm (excitation and emission widths: 7 nm and 10 nm, respectively).

Acknowledgements

S.G.S and A.A.T thank the Department of Science and Technology, India (SR/S1/OC-51/2009) and the Council of Scientific and Industrial Research, India for financial support and graduate research fellowship, respectively.

Keywords: abasic site · DNA · emission spectroscopy · fluorescent nucleoside · RNA

[1] a) A. Okamoto, Y. Saito, I. Saito, *J. Photochem. Photobiol. C* **2005**, *6*, 108–122; b) U. Asseline, *Curr. Org. Chem.* **2006**, *10*, 491–518; c) J. N. Wilson, E. T. Kool, *Org. Biomol. Chem.* **2006**, *4*, 4265–4274; d) X. Shi, D. Herschlag, *Methods Enzymol.* **2009**, *469*, 287–302; e) R. W. Sinkeldam, N. J. Greco, Y. Tor, *Chem. Rev.* **2010**, *110*, 2579–2619; f) L. M. Wilhelmsson, *Q. Rev. Biophys.* **2010**, *43*, 159–183; g) S. G. Srivatsan, A. A. Sawant, *Pure Appl. Chem.* **2011**, *83*, 213–232; h) K. Phelps, A. Morris, P. A. Beal, *ACS Chem. Biol.* **2012**, *7*, 100–109.

[2] a) J.-M. L. Pecourt, J. Peon, B. Kohler, *J. Am. Chem. Soc.* **2000**, *122*, 9348–9349; b) E. Nir, K. Kleinermanns, L. Grace, M. S. de Vries, *J. Phys. Chem. A.* **2001**, *105*, 5106–5110.

[3] R. T. Ranasinghe, T. Brown, *Chem. Commun.* **2005**, 5487–5502.

[4] M. E. Hawkins in *Topics in Fluorescence Spectroscopy, DNA Technology, Vol. 7* (Ed: J. R. Lakowicz), Kluwer Academic/Plenum Publishers, New York, **2003**, pp. 151–175.

[5] T. J. Matray, E. T. Kool, *Nature* **1999**, *399*, 704–708.

[6] N. A. Grigorenko, C. J. Leumann, *Chem. Commun.* **2008**, 5417–5419.

[7] C. Wanger, H.-A. Wagenknecht, *Org. Lett.* **2006**, *8*, 4191–4194.

[8] a) D. C. Ward, E. Reich, L. Stryer, *J. Biol. Chem.* **1969**, *244*, 1228–1237; b) E. L. Rachofsky, R. Osman, J. B. A. Ross, *Biochemistry* **2001**, *40*, 946–956.

[9] a) M. J. Rist, J. P. Marino, *Curr. Org. Chem.* **2002**, *6*, 775–793; b) T. Xia, *Curr. Opin. Chem. Biol.* **2008**, *12*, 604–611; c) K. B. Hall, *Methods Enzymol.* **2009**, *469*, 269–285.

[10] R. A. Tinsley, N. G. Walter, *RNA* **2006**, *12*, 522–529.

[11] a) D. A. Berry, K.-Y. Jung, D. S. Wise, A. D. Sercel, W. H. Pearson, H. Mackie, J. B. Randolph, R. L. Somers, *Tetrahedron Lett.* **2004**, *45*, 2457–2461; b) A. S. Wahba, A. Esmaeili, M. J. Damha, R. H. E. Hudson, *Nucleic Acids Res.* **2010**, *38*, 1048–1056.

[12] a) S. G. Srivatsan, Y. Tor, *Tetrahedron* **2007**, *63*, 3601–3607; b) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009**, *131*, 17605–17614; c) Y. Xie, T. Maxson, Y. Tor, *J. Am. Chem. Soc.* **2010**, *132*, 11896–11897.

[13] Fluorescent deoxyribonucleosides: a) G. Stengel, J. P. Gill, P. Sandin, L. M. Wilhelmsson, B. Albinsson, B. Nordén, D. P. Millar, *Biochemistry* **2007**, *46*, 12289–12297; b) M. Mizuta, K. Seio, K. Miyata, M. Sekine, *J. Org. Chem.* **2007**, *72*, 5046–5055; c) F. Seela, V. R. Sirivolu, *Org. Biomol. Chem.* **2008**, *6*, 1674–1687; d) A. Dumas, N. W. Luedtke, *J. Am. Chem. Soc.* **2010**, *132*, 18004–18007; e) W. Hirose, K. Sato, A. Matsuda, *Angew. Chem.* **2010**, *122*, 8570–8572; *Angew. Chem. Int. Ed.* **2010**, *49*, 8392–8394; f) A. Omumi, D. G. Beach, M. Baker, W. Gabryelski, R. A. Manderville, *J. Am. Chem. Soc.* **2011**, *133*, 42–50; g) T. Ehrenschwender, H. A. Wagenknecht, *J. Org. Chem.* **2011**, *76*, 2301–2304; h) A. Nadler, J. Strohmeier, U. Diederichsen, *Angew. Chem.* **2011**, *123*, 5504–5508; *Angew. Chem. Int. Ed.* **2011**, *50*, 5392–5396; i) H. Gardarsson, A. S. Kale, S. T. Sigurdsson, *ChemBioChem* **2011**, *12*, 567–575; j) J. Riedl, R. Pohl, L. Rulíšek, M. Hocek, *J. Org. Chem.* **2012**, *77*, 1026–1044; k) K. K. Karlsen, A. Pasternak, T. B. Jensen, J. Wengel, *ChemBioChem* **2012**, *13*, 590–601.

[14] Fluorescent ribonucleosides: a) I. Hirao, Y. Harada, M. Kimoto, T. Mitsui, T. Fujiwara, S. Yokoyama, *J. Am. Chem. Soc.* **2004**, *126*, 13298–13305; b) M. Kaul, C. M. Barbieri, D. S. Plich, *J. Am. Chem. Soc.* **2006**, *128*, 1261–1271; c) J. Parsons, T. Hermann, *Tetrahedron* **2007**, *63*, 3548–3552; d) K. Lang, R. Rieder, R. Micura, *Nucleic Acids Res.* **2007**, *35*, 5370–5378; e) R. Rieder, K. Lang, D. Graber, R. Micura, *ChemBioChem* **2007**, *8*, 896–902; f) H. S. Jeong, S. Kang, J. Y. Lee, B. H. Kim, *Org. Biomol. Chem.* **2009**, *7*, 921–925; g) H. Peacock, O. Maydanovych, P. A. Beal, *Org. Lett.* **2010**, *12*, 1044–1047; h) U. Förster, K. Lommel, D. Sauter, C. Grünewald, J. W. Engels, J. Wachtveitl, *ChemBioChem* **2010**, *11*, 664–672; i) D. Shin, R. W. Sinkeldam, Y. Tor, *J. Am. Chem. Soc.* **2011**, *133*, 14912–14915; j) A. R. Hernández, E. T. Kool, *Org. Lett.* **2011**, *13*, 676–679.

[15] a) P. Sandin, K. Börjesson, H. Li, J. Mårtensson, T. Brown, L. M. Wilhelmsson, B. Albinsson, *Nucleic Acids Res.* **2008**, *36*, 157–167; b) G. Stengel, M. Urban, B. W. Purse, R. D. Kuchta, *Anal. Chem.* **2010**, *82*, 1082–1089.

[16] a) A. A. Tanpure, S. G. Srivatsan, *Chem. Eur. J.* **2011**, *17*, 12820–12827; b) M. G. Pawar, S. G. Srivatsan, *Org. Lett.* **2011**, *13*, 1114–1117.

[17] Benzofuran-conjugated uridine has better quantum yield than the benzothiophene analogue (see ref. [16]).

[18] a) L. Barbieri, M. G. Battelli, F. Stirpe, *Biochim. Biophys. Acta Rev. Biomembr.* **1993**, *1154*, 237 – 282; b) L. Roncuzzi, A. Gasperi-Campani, *FEBS Lett.* **1996**, *392*, 16 – 20.
[19] a) Y. Endo, A. Glück, I. G. Wool, *J. Mol. Biol.* **1991**, *221*, 193 – 207; b) P. Ghosh, J. K. Batra, *Biochem. J.* **2006**, *400*, 99 – 104.
[20] a) D. Moazed, J. M. Robertson, H. F. Noller, *Nature* **1988**, *334*, 362 – 364; b) Y. Endo, Y.-L. Chan, A. Lin, K. Tsurugi, I. G. Wool, *J. Biol. Chem.* **1988**, *263*, 7917 – 7920.
[21] a) J. Audi, M. Belson, M. Patel, J. Schier, J. Osterloh, *J. Am. Med. Assoc.* **2005**, *294*, 2342 – 2351; b) Centers for Disease Control and Prevention, *Morb Mortal Wkly Rep.* **2003**, *52*, 1129 – 1131; c) M.-C. Ho, M. B. Sturm, S. C. Almo, V. L. Schramm, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 20276 – 20281.
[22] a) M. A. Poli, V. R. Rivera, J. F. Hewetson, G. A. Merrill, *Toxicon* **1994**, *32*, 1371 – 1377; b) C. Lubelli, A. Chatgilialoglu, A. Bolognesi, P. Strocchi, M. Colombatti, F. Stirpe, *Anal. Biochem.* **2006**, *355*, 102 – 109; c) R. E. Fulton, H. G. Thompson, *J. Immunoassay Immunochem.* **2007**, *28*, 227 – 241.
[23] a) M. Zamboni, M. Brigotti, F. Rambelli, L. Montanaro, S. Sperti, *Biochem. J.* **1989**, *259*, 639 – 643; b) S. Tang, R.-G. Hu, W.-Y. Liu, K.-C. Ruan, *Biol. Chem.* **2000**, *381*, 769 – 772; c) S.-A. Fredriksson, A. G. Hulst, E. Artursson, A. L. de Jong, C. Nilsson, B. L. M. van Baar, *Anal. Chem.* **2005**, *77*, 1545 – 1555; d) W. K. Keener, V. R. Rivera, C. C. Young, M. A. Poli, *Anal. Biochem.* **2006**, *357*, 200 – 207; e) A. J. Haes, B. C. Giordano, G. E. Collins, *Anal. Chem.* **2006**, *78*, 3758 – 3764; f) F. Becher, E. Duriez, H. Volland, J. C. Tabet, E. Ezan, *Anal. Chem.* **2007**, *79*, 659 – 665.
[24] A very few fluorescence-based methods are available to detect the RNA abasic sites: a) S. Roday, M. B. Sturm, D. Blakaj, V. L. Schramm, *J. Biochem. Biophys. Methods* **2008**, *70*, 945 – 953; b) S. G. Srivatsan, N. J. Greco, Y. Tor, *Angew. Chem.* **2008**, *120*, 6763 – 6767; *Angew. Chem. Int. Ed.* **2008**, *47*, 6661 – 6665; c) Q.-Q. Tan, D.-X. Dong, X.-W. Yin, J. Sun, H.-J. Ren, R.-X. Li, *J. Biotechnol.* **2009**, *139*, 156 – 162; d) A. A. Tanpure, P. Patheja, S. G. Srivatsan, *Chem. Commun.* **2012**, *48*, 501 – 503.
[25] See the Supporting Information for details.
[26] See the Experimental Section for details.
[27] C. Reichardt, *Chem. Rev.* **1994**, *94*, 2319 – 2358.
[28] R. W. Sinkeldam, A. J. Wheat, H. Boyaci, Y. Tor, *ChemPhysChem* **2011**, *12*, 567 – 570.
[29] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, New York, **2006**.
[30] a) M. Kawai, M. J. Lee, K. O. Evans, T. M. Nordlund, *J. Fluoresc.* **2001**, *11*, 23 – 32; b) J. M. Jean, K. B. Hall, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 37 – 41.
[31] a) C. A. M. Seidel, A. Schulz, M. H. M. Sauer, *J. Phys. Chem.* **1996**, *100*, 5541 – 5553; b) S. O. Kelley, J. K. Barton, *Science* **1999**, *283*, 375 – 381; c) M. Torimura, S. Kurata, K. Yamada, T. Yokomaku, Y. Kamagata, T. Kanagawa, R. Kurane, *Anal. Sci.* **2001**, *17*, 155 – 160.
[32] Depending on the neighbouring base environment, 2-AP within oligonucleotides exhibits up to 100-fold reduction in quantum yield (see ref. [8]).
[33] Among the four nucleosides, guanosine has a prominent photoinduced electron-transfer fluorescence quenching effect on several fluorophores including 2-AP. See refs. [30] and [31].
[34] T. Heinlein, J.-P. Knemeyer, O. Piestert, M. Sauer, *J. Phys. Chem. B* **2003**, *107*, 7957 – 7964.
[35] a) T. Lindahl, *Nature* **1993**, *362*, 709 – 715; b) K. S. Gates, T. Nooner, S. Dutta, *Chem. Res. Toxicol.* **2004**, *17*, 839 – 856; c) H. A. Dahlmann, V. G. Vaidyanathan, S. J. Sturla, *Biochemistry* **2009**, *48*, 9347 – 9359.
[36] If unrepaired, DNA abasic sites potentially lead to mutation or toxicity; a) L. A. Loeb, B. D. Preston, *Annu. Rev. Genet.* **1986**, *20*, 201 – 230; b) W. Zhou, P. W. Doetsch, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6601 – 6605.
[37] a) M. Talpaert-Borl, M. Liuzzi, *Biochim. Biophys. Acta Gene Struct. Expression* **1983**, *740*, 410 – 416; b) H. Ide, K. Akamatsu, Y. Kimura, K. Michiue, K. Makino, A. Asaeda, Y. Takamori, K. Kubo, *Biochemistry* **1993**, *32*, 8276 – 8283; c) J. Lhomme, J. F. Constant, M. Demeunynck, *Biopolymers* **1999**, *52*, 65 – 83; d) D. Boturyn, J.-F. Constant, E. Defrancq, J. Lhomme, A. Barbin, C. P. Wild, *Chem. Res. Toxicol.* **1999**, *12*, 476 – 482; e) H. Atamna, I. Cheung, B. N. Ames, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 686 – 691; f) D. Chakravarti, A. F. Badawi, D. Venugopal, J. L. Meza, L. Z. Crandall, E. G. Rogan, E. L. Cavalieri, *Mutat. Res.* **2005**, *588*, 158 – 165; g) E. Fundador, J. Rusling, *Anal. Bioanal. Chem.* **2007**, *387*, 1883 – 1890.
[38] a) K. Sato, M. M. Greenberg, *J. Am. Chem. Soc.* **2005**, *127*, 2806 – 2807; b) S. Dhar, T. Kodama, M. M. Greenberg, *J. Am. Chem. Soc.* **2007**, *129*, 8702 – 8703.
[39] Deoxy-C-nucleoside containing a non-hydrogen-bonding and non-shape-complementary base surrogate, 2,2′-bipyridyl unit, has been used in the detection of DNA abasic sites: C. Brotschi, A. Häberli, C. J. Leumann, *Angew. Chem.* **2001**, *113*, 3101 – 3103; *Angew. Chem. Int. Ed.* **2001**, *40*, 3012 – 3014.
[40] Fluorescence detection of abasic sites in DNA: a) E. L. Rachofsky, E. Seibert, J. T. Stivers, R. Osman, J. B. A. Ross, *Biochemistry* **2001**, *40*, 957 – 967; b) T. Hirose, T. Ohtani, H. Muramatsu, A. Tanaka, *Photochem. Photobiol.* **2002**, *76*, 123 – 126; c) K. Yoshimoto, S. Nishizawa, M. Minagawa, N. Teramae, *J. Am. Chem. Soc.* **2003**, *125*, 8982 – 8983; d) L. Valis, N. Amann, H. A. Wagenknecht, *Org. Biomol. Chem.* **2005**, *3*, 36 – 38; e) N. J. Greco, Y. Tor, *J. Am. Chem. Soc.* **2005**, *127*, 10784 – 10785; f) Z. Xu, Y. Sato, S. Nishizawa, N. Teramae, *Chem. Eur. J.* **2009**, *15*, 10375 – 10378.
[41] E. Shipova, K. S. Gates, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2111 – 2113.
[42] T. Nooner, S. Dutta, K. S. Gates, *Chem. Res. Toxicol.* **2004**, *17*, 942 – 949.
[43] The UV-thermal melting profiles indicate that both RIP substrate **17** and product mimic **18** form stable duplexes with fluorescently modified DNA oligonucleotide **19** (see Figure S4).
[44] Quenching by electron transfer process depends on the distance between the fluorophore and quencher (e.g., guanosine). See ref. [33].

Received: June 14, 2012
Published online on October 15, 2012

