DOI: 10.1002/cbic.201402052

Synthesis, Photophysical Properties and Incorporation of a Highly Emissive and Environment-Sensitive Uridine Analogue Based on the Lucifer Chromophore

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

The majority of fluorescent nucleoside analogues used in nucleic acid studies have excitation maxima in the UV region and show very low fluorescence within oligonucleotides (ONs); hence, they cannot be utilised with certain fluorescence methods and for cell-based analysis. Here, we describe the synthesis, photophysical properties and incorporation of a highly emissive and environment-sensitive uridine analogue, derived by attaching a Lucifer chromophore (1,8-naphthalimide core) at the 5-position of uracil. The emissive nucleoside displays excitation and emission maxima in the visible region and exhibits high quantum yield. Importantly, when incorporated into ON duplexes it retains appreciable fluorescence efficiency and is sensitive to the neighbouring base environment. Notably, the nucleoside signals the presence of purine repeats in ON duplexes with an enhancement in fluorescence intensity, a property rarely displayed by other nucleoside analogues.

Introduction

Base-modified fluorescent nucleoside probes that photophysically report subtle changes in their neighbouring base environment have been very useful in designing bioanalytical assays to study the structure and function of nucleic acids.[1,2] As natural nucleobases are essentially nonemissive, useful fluorescence properties have been bestowed by attaching known fluorophores, heterocycles or aromatic rings onto the bases.[2,3] Fluorescent nucleosides have also been developed by using fluorescent heterocycles (e.g., pteridines) and polycyclic aromatic hydrocarbons (PAHs) as base surrogates.[4,5] The fluorescence properties of many of these nucleoside analogues, when incorporated into oligonucleotide (ON) sequences, are influenced by collisional, H-bonding, and stacking interactions with adjacent bases,[6] electron transfer between the fluorescent base and neighbouring bases,[7] solvation/desolvation effects[6] and the conformation of the fluorescent base within the ON.[8] Over the years several such responsive fluorescent nucleoside analogue probes have been implemented in assays, 1) to detect base-pair mismatches,[9] abasic sites,[10] mutagenic nucleobase modifications[11] and electron transfer processes,[12] and 2) to study nucleic acid topologies,[13] enzyme activities[14] and nucleic acid–small molecule and nucleic acid–protein complexes.[15] However, barring a very few examples, emissive nucleoside analogues, when incorporated into ON sequences, experience drastic fluorescence quenching because of interactions with neighbouring bases, thus considerably limiting their application.[16] Notably, purines (especially guanine, G) quench the fluorescence of most fluorophores by a photoinduced electron-transfer process; hence, turn-on fluorescence detection of purine repeats and mismatches has been quite a challenge.[17–19] Therefore, much of the recent effort in the development of new fluorescent nucleoside analogues is directed towards designing environment-sensitive analogues that have excitation and emission maximum in the visible region and high fluorescence efficiency within ONs, with the view of implementing them in both in vitro and in vivo assays.[5,20–23]

Kool and co-workers have assembled a library of DNA-like chains containing different PAH fluorophores ("oligodeoxyfluorosides") that display large Stokes shifts and a wide array of quantum yields and emission wavelengths.[21] These oligodeoxyfluorosides with tunable photophysical properties have been implemented in the detection of molecular species in solution and in the multiplexed imaging of cells. In a somewhat similar approach, siRNAs labelled with multiple phenylpyrrolocytidine residues have been used to monitor (by fluorescence microscopy) the trafficking and silencing activity of siRNA inside living cells.[22] More recently, a quadracyclic adenine analogue and 4-aminophthalimide C-nucleoside have been introduced as fluorescent nucleoside surrogates; when incorporated into ONs these form stable duplexes and retain reasonable fluorescence efficiency, compared to most other fluorescent nucleoside analogues.[23] As a part of the continued efforts to develop new fluorescent probes with useful properties, we report the synthesis and photophysical properties of a highly emissive ribonucleoside analogue, obtained by conjugating the Lucifer chromophore (naphthalimide core) at the 5-position of uridine by an ethynyl linker.[24] The naphthalimide-modified uridine analogue has excitation and emission maxima in the visible region and exhibits excellent fluorescence solvatochromism. Notably, the fluorescence properties of the emissive nucleoside incorporated into ONs is sensitive to the flanking

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

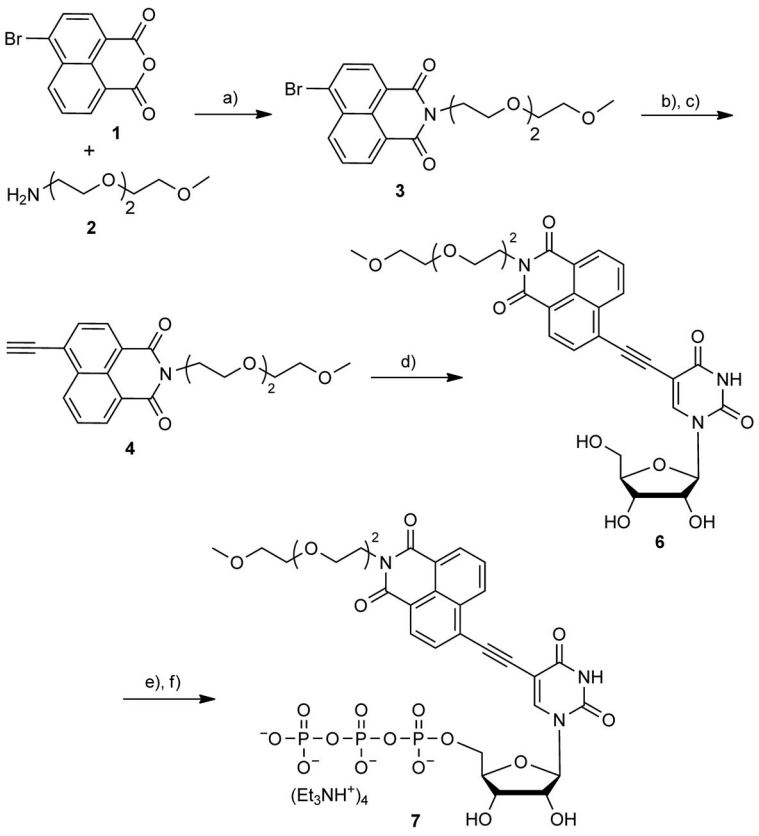
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402052.

bases and base-pair mismatches. In particular, the emissive nucleoside signals the presence of purine repeats (dG and dA) with a significant increase in fluorescence intensity, a property seldom exhibited by most of the fluorescent nucleoside analogues.[17]

Results and Discussion

Synthesis and photophysical properties of naphthalimide-modified uridine 6

The fluorescent probe **6** is based on the 4-amino-1,8-naphthalimide core of Lucifer dyes; this absorbs light in the visible region and exhibits a large Stoke shift and high quantum yield.[25,26] The photophysical properties of the parent naphthalimide, containing the electron-withdrawing imide moiety, largely depend on the substituent at the 4-position of the dye.[27] As the fluorescence properties of the naphthalimide core can be tuned by rational modification, its derivatives have been extensively used as biological markers, sensors and electroluminescent materials, to name a few applications.[26] Encouraged by these reports, it was postulated that coupling 4-ethynyl-1,8-naphthalimide at the 5-position of uridine would impart superior probe-like properties (similar to Lucifer dyes) to the nucleobase. Naphthalimide-modified ribonucleoside **6** was synthesised according to the steps shown in Scheme 1. First, 4-ethynyl-1,8-naphthalimide derivative **4** was prepared by reacting commercially available 4-bromo-1,8-naphthalic anhydride (**1**) with amine-modified tri(ethylene glycol) linker **2**, followed by a Sonogashira cross-coupling reaction with TMS-acetylene. The silyl protecting group was then deprotected in the presence of TBAF to afford **4** in moderate yield. A tri(ethylene glycol) linker was attached at the imide position to enhance the solubility of the naphthalimide derivative in polar solvents for subsequent manipulation. The fluorescent uridine analogue **6** was then obtained by palladium-catalysed cross coupling between the 4-alkyne-modified naphthalimide (**4**) and 5-iodouridine (**5**).



Scheme 1. Synthesis of fluorescent uridine analogue **6** and its corresponding triphosphate **7**.[28] a) ethanol, reflux, 90%; b) TMS-acetylene, $Pd(PPh_3)_4$, CuI, iPr_2NEt, THF, RT, 87%; c) 1 M TBAF, MeOH, 60 °C, 66%; d) 5-iodouridine (**5**), $Pd(PPh_3)_4$, CuI, Et_3N, DMF, RT, 85%; e) $POCl_3$, $(MeO)_3PO$, ~4 °C; f) bis-tributylammonium pyrophosphate, Bu_3N, ~4 °C, 31%.

The ground-state and excited-state electronic properties of nucleoside **6** were evaluated in solvents of different polarities to test the solvatochromic behaviour of the nucleoside. The ground-state electronic spectrum was marginally affected by changes in solvent polarity, however, the excited-state properties were significantly altered (Figure 1, Table 1). In dioxane (the least-polar solvent used in this study) the nucleoside exhibited very strong emission (λ_{em} = 480 nm, quantum yield 87%; Figure 1, Table 1). As the solvent polarity was increased (from dioxane to water) the nucleoside showed a remarkable 48 nm red-shift in the emission band (λ_{em} = 528) and nearly 11-fold quenching of fluorescence intensity. Excited-state lifetime measurements revealed longer lifetimes in nonpolar solvents than in polar solvents (see Figure S1 in the Supporting Information and Table 1). A good positive correlation between Stokes shift in different solvents and $E_T(30)$ value (a microscopic solvent polarity parameter) further indicated the sensitivity of the nucleoside to microenvironment changes (Figure S2). The high fluorescence efficiency, absorption and emission profiles in the visible region and sensitivity to polarity prompted us to study the fluorescence properties of the emissive nucleoside within ONs.

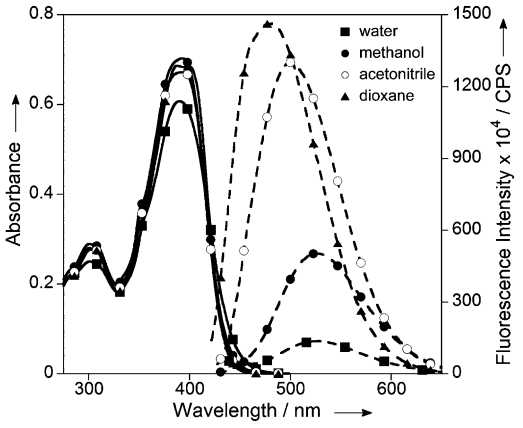


Figure 1. Absorption (25 μM, solid lines) and emission (5.0 μM, dashed lines) spectra of nucleoside **6** in solvents of different polarity. Solutions contained 2.5 and 0.5% DMSO, respectively.[29]

Table 1. Photophysical properties of nucleoside **6**.[28,29]

Solvent	λ_{max}[a] [nm]	λ_{em} [nm]	I_{rel}[b]	Φ[c]	τ_{ave}[c] [ns]
water	391	528	1	0.11	1.03
methanol	393	527	3.7	0.24	1.66
acetonitrile	393	500	9.6	0.67	3.68
dioxane	393	480	10.8	0.87	2.78

[a] Lowest energy maximum. [b] Fluorescence intensity is given relative to that in water. [c] Errors for Φ and τ_{ave} are $\leq$0.003 and $\leq$0.03 ns, respectively.

Enzymatic incorporation of nucleoside 6 into RNA ONs

Primer extension and in vitro transcription reactions in the presence of DNA and RNA polymerases, respectively, and ligation reactions have been effectively used in the synthesis of fluorescently modified DNA and RNA ONs.[15,30–32] Notably, Hirao and co-workers have elegantly used unnatural base pairs that are orthogonal to A–T/U and G–C to site-specifically incorporate fluorescent analogues of unnatural bases into RNA oligonucleotides by in vitro transcription.[15a,33] Thus, we chose to incorporate naphthalimide-modified uridine into RNA ONs by in vitro transcription. The modified UTP **7** was synthesised by treating **6** with anhydrous $POCl_3$ and then with *bis*-tributylammonium pyrophosphate at ~4°C (Scheme 1).[34] The efficiency of T7 RNA polymerase to incorporate **7** into RNA ONs was first tested by radiolabelling experiments with a series of promoter/template DNA duplexes. The duplexes were formed by annealing a T7 RNA polymerase consensus promoter DNA ON with template ONs **T1**–**T5** (Figure 2).[35] The templates were designed to direct the incorporation of the monophosphate of **7** at one or two positions near the promoter region, away from the promoter region or at the 3′-end of the transcript. In vitro transcription reactions were performed in the presence of GTP, CTP, [α-^{32}P]ATP and UTP/**7**, and the radiolabelled transcription products were analysed by polyacrylamide gel electrophoresis under denaturing conditions and phosphorimaged.

When transcription was performed with template **T1**, the RNA polymerase incorporated the monophosphate of **7** at the +7-position to afford the full-length transcript **9** in a good yield (Figure 3, lane 2).[36] The incorporation of this heavier ribonucleotide into the transcript was also evident from the retarded mobility exhibited by transcript **9** (Figure 3, compare lanes

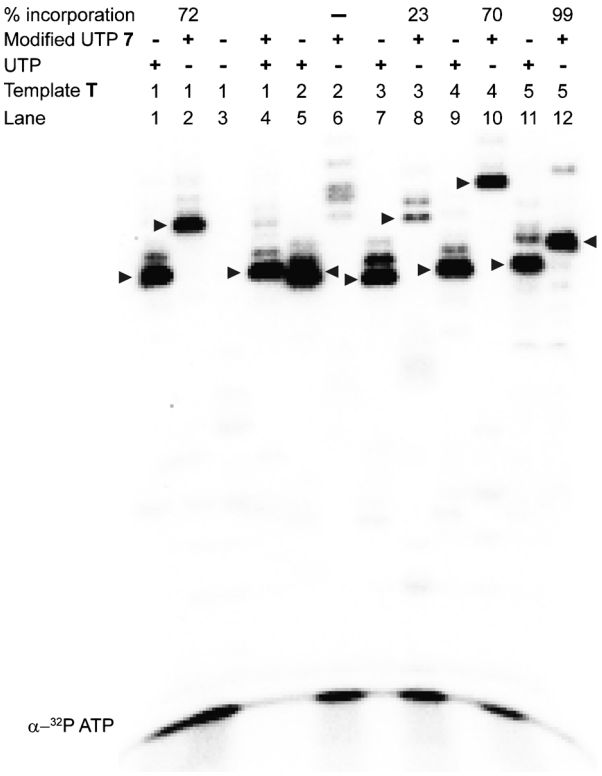


Figure 3. Phosphorimage of transcription products resolved by denaturing PAGE. Transcription was performed with templates **T1**–**T5** and UTP and/or modified UTP (**7**).[29] Incorporation of **7** is given as percent relative to control (natural UTP). Modified full-length transcripts (arrow heads) were determined by mass analysis (Table S1). Incorporation of UTP **7** with **T2** was very low; hence, the band corresponding to the full-length transcript (**10**) could not be assigned (lane 6).

T7 promoter 5'-TAA TAC GAC TCA CTA TAG-3'
DNA template **T1** 3'-ATT ATG CTG AGT GAT ATC GCG GC**A** CGT-5'
+1 +7

T7 RNA polymerase
GTP, CTP, UTP/**7**
α–^{32}P ATP

5'-r($_{ppp}$GCG CCG UGC A)-3' (**8**)
or
5'-r($_{ppp}$GCG CCG **6**GC A)-3' (**9**)

DNA templates		Modified transcripts	
T2	3'-ATT ATG CTG AGT GAT ATC G**A**G GCC CGT-5'	5'-r($_{ppp}$GC**6** CCG GGC A)-3'	(**10**)
T3	3'-ATT ATG CTG AGT GAT ATC GC**A** GCG CGT-5'	5'-r($_{ppp}$GCG **6**CG CGC A)-3'	(**11**)
T4	3'-ATT ATG CTG AGT GAT ATC GCG G**AA** CGT-5'	5'-r($_{ppp}$GCG CC**6** **6**GC A)-3'	(**12**)
T5	3'-ATT ATG CTG AGT GAT ATC GCG GCT CG**A**-5'	5'-r($_{ppp}$GCG CCG AGC **6**)-3'	(**13**)
T6	3'-ATT ATG CTG AGT GAT ATC GCG GT**A** TGT-5'	5'-r($_{ppp}$GCG CCA **6**AC A)-3'	(**14**)
T7	3'-ATT ATG CTG AGT GAT ATC GCG GG**A** GGT-5'	5'-r($_{ppp}$GCG CCC **6**CC A)-3'	(**15**)

Figure 2. Incorporation of UTP **7** into RNA ONs by in vitro transcription with templates **T1**–**T7**.[29]

1 and 2). A control reaction (absence of UTP or **7**) produced no full-length oligoribonucleotide product, thus ruling out misincorporation (Figure 3, lane 3); reaction in the presence of equimolar UTP and **7** indicated preference for UTP over **7** (Figure 3, lane 4). Attempts to introduce the modification near the promoter region (+3 and +4, templates **T2** and **T3**) resulted in very low yields of full-length transcript (Figure 3, lanes 6 and 8). This was not unexpected, as the enzyme is often less tolerant of modifications during the initial phase of polymerisation.[32b] Interestingly, transcription with **T4** results in double incorporation (adjacent positions) with a reasonable efficiency (Figure 3, lane 10); with template **T5** the RNA polymerase incorporated the modification at the 3′-end of the transcript (**13**) with excellent efficiency (Figure 3, lane 12 and Figure S3). Although, internally

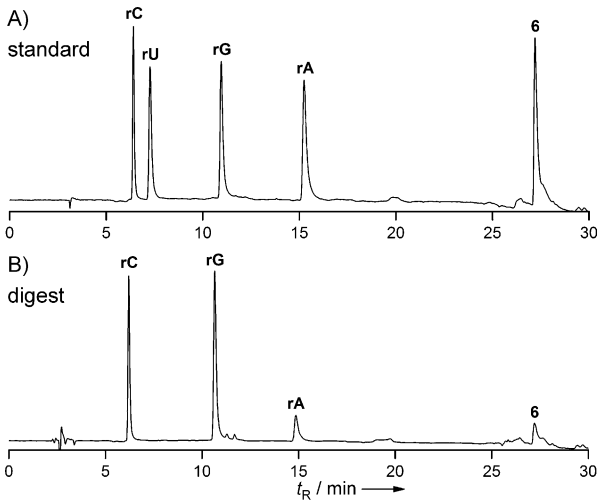


Figure 4. HPLC chromatogram of ribonucleoside products obtained from an enzymatic digestion of transcript **9** at 260 nm.[29] A) Mixture of natural ribonucleosides and modified fluorescent ribonucleoside **6**. B) Digested ON **9**. The fractions corresponding to individual ribonucleoside fractions were further analysed by mass spectroscopy (see Table S2).

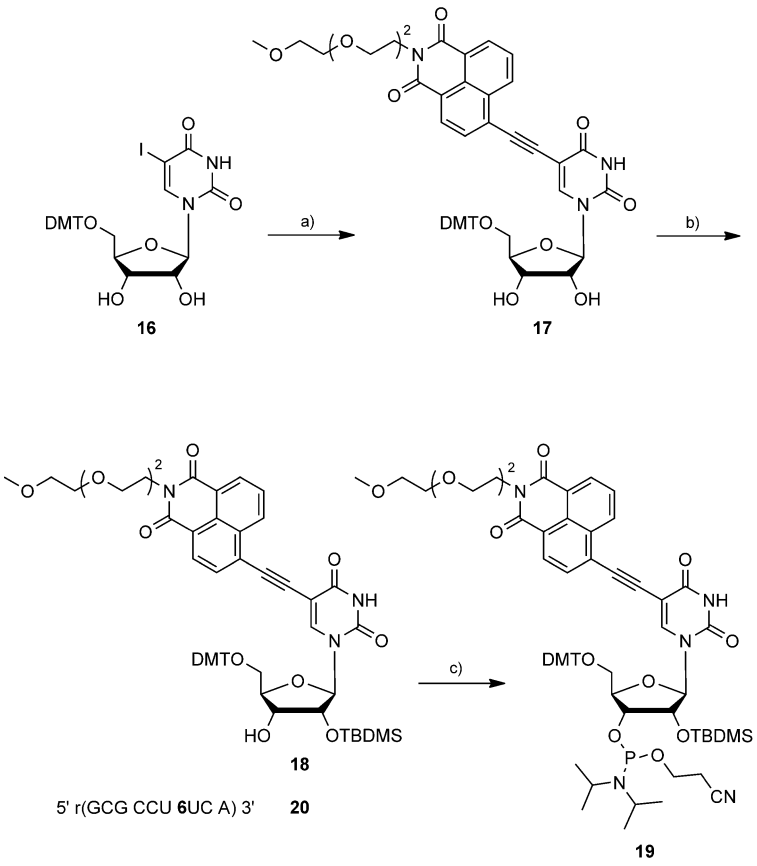
modified transcript **9** and 3′-end modified transcript **13** have same mass, their electrophoretic mobilities were different (Figure 3, compare lanes 2 and 12). Predicting the electrophoretic mobility of modified oligonucleotides based on charge and mass is not straightforward, as the hydrophobicity, hydration, conformation and frictional properties of the appended group (as well as its interaction with medium) can affect mobility.[37] Therefore, the observed difference in mobility could be attributable to differences in the hydrophobicity, conformation and frictional properties between the internal and 3′-end-attached fluorophore.

RNA ONs were isolated from large-scale transcription reactions to further examine the incorporation of the naphthalimide modification into transcripts. Mass analysis confirmed the formation of modified full-length RNA oligonucleotides by in vitro transcription with the fluorescent UTP (Table S1). The presence of **6** in the transcript was also established by enzymatic digestion. RNA ON **9** was subjected to digestion by phosphodiesterase, alkaline phosphatase, RNase A and RNase T1. HPLC analysis of the resulting ribonucleoside products revealed the presence of modified nucleoside in the transcript (Figure 4, Table S2).

Photophysical characterisation of naphthalimide-modified RNA ONs

Interactions with neighbouring bases can affect the photophysical properties of an emissive nucleoside in ONs, by various mechanisms.[6–8,17] Such fluorescent nucleoside analogues are very useful in designing nucleic-acid-based diagnostic tools.[1,2] Therefore, we decided to examine the effect of flanking bases and mismatched base pairs on the fluorescence properties of **6**. RNA ONs **9**, **14** and **15** (containing **6** between rG, rA and rC residues, respectively; "rG" = G residues at each side) were synthesised by large-scale transcription reactions (Figure 2, Figure S4, Table S1).[29] Enzymatic synthesis of an RNA ON with **6** between U residues was not attempted, as it would be difficult to control the site of incorporation in in vitro transcription. In addition, RNA polymerase prefers natural UTP over **7**, thus preventing the introduction of **6** between U residues by transcription reaction (Figure 3, lane 4). Therefore, we synthesised RNA ON **20** (containing **6** in between U residues) by a solid-phase ON synthesis protocol. The 2′-*O*-(*tert*-butyldimethylsilyl)-protected phosphoramidite substrate **19** was synthesised as shown in Scheme 2. The modified phosphoramidite substrate was then site-specifically incorporated into an RNA ON (sequence **20**) by using a standard RNA ON synthesis cycle.[29] The ON was deprotected and purified, and the presence of the fluorescent uridine analogue in the full-length product was confirmed by mass analysis (Figure S5, Table S1).

RNA ONs **9** and **14** (**6** flanked by purine residues, rG and rA, respectively) displayed significantly quenched and slightly blue-shifted emission bands, as compared to the free nucleoside. Whereas ON **20** (**6** between rU residues) exhibited a dis-



Scheme 2. Synthesis of naphthalimide-modified uridine phosphoramidite substrate **19** used in the solid-phase synthesis of RNA ON **20**.[28,29] DMT, 4,4′-dimethoxytrityl; TBDMS, 2′-*O*-*tert*-butyldimethylsilyl. a) **4**, $Pd(PPh_3)_4$, CuI, iPr_2NEt, DMF, RT, 85%; b) $AgNO_3$, TBDMS-Cl, pyridine, THF, RT, 73%; c) $iPr_2NP(Cl)OEtCN$, iPr_2NEt, CH_2Cl_2, RT, 70%.

cernible reduction in fluorescence intensity, ON **15** (**6** in between rC residues) displayed a significant enhancement, compared to the free nucleoside (Figure 5). The effect of neighbouring bases on the fluorescence properties of **6** was further evaluated with duplexes of ONs **9**, **14**, **15** and **20**. The duplexes were assembled by hybridising fluorescent ONs with complementary DNA ONs, such that the emissive nucleoside was opposite to complementary or mismatched bases (Figure 6).

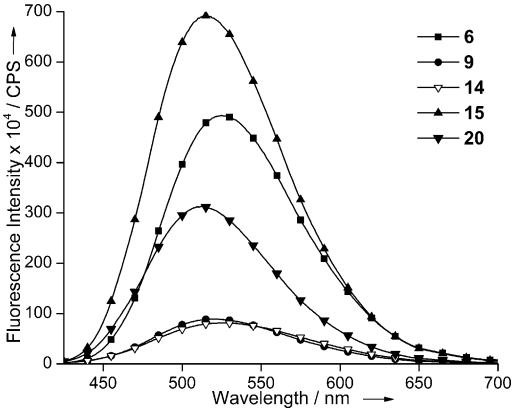


Figure 5. Emission spectra of nucleoside **6** (1 μM) and RNA ONs **9**, **14**, **15** and **20** (1 μM) containing **6** between rG, rA, rC and rU residues, respectively. ONs were excited at 407 nm; excitation and emission slit widths: 6 and 8 nm, respectively.[29]

3'-CGC GGC ACG T-5'	**9A**	3'-CGC GGT ATG T-5'	**14A**
3'-CGC GGC TCG T-5'	**9T**	3'-CGC GGT TTG T-5'	**14T**
3'-CGC GGC GCG T-5'	**9G**	3'-CGC GGT GTG T-5'	**14G**
3'-CGC GGC CCG T-5'	**9C**	3'-CGC GGT CTG T-5'	**14C**
3'-CGC GGG AGG T-5'	**15A**	3'-CGC GGA AAG T-5'	**20A**
3'-CGC GGG TGG T-5'	**15T**	3'-CGC GGA TAG T-5'	**20T**
3'-CGC GGG GGG T-5'	**15G**	3'-CGC GGA GAG T-5'	**20G**
3'-CGC GGG CGG T-5'	**15C**	3'-CGC GGA CAG T-5'	**20C**

Figure 6. Sequences of custom DNA ONs. Hybridisation of **9** with **9A**, **9T**, **9G** and **9C** places the fluorescent nucleoside **6** opposite complementary base dA and mismatched bases dT, dG and dC, respectively.

The naphthalimide modification can potentially affect the hybridisation efficiency of the RNA ONs, and hence, the observed fluorescence profile might reflect a combination of intact duplexes and unhybridised fluorescent ON. Therefore, prior to performing the fluorescence study, the effect of modification on the stability of duplexes was determined by thermal denaturation experiments. Although, the modification had a slight destabilising effect, the naphthalimide-modified ONs formed stable duplexes, with T_m values well above room temperature (Figure S6, Table S3). Hence, destabilisation due to the presence of the modification should not compromise photophysical characterisation of duplexes at room temperature.

When placed opposite complementary or mismatched bases in RNA·DNA heteroduplexes (**9·9A**, **9·9T**, **9·9G** and **9·9C**), **6** flanked by rG residues displayed further quenching in fluorescence intensity, but with no change in emission maximum relative to single stranded RNA ON **9** (Figure 7 A). ON **14** duplexes (**6** flanked by rA and opposite dA, dT or dC bases) showed enhanced fluorescence relative to **14**, but their overall fluorescence efficiencies were weak (Figure 7 B). The fluorescence quenching exhibited for **6** upon incorporation into oligonucleotide duplexes is a common feature shown by most other nucleoside analogues. This quenching might be due to electron transfer between the modified base and neighbouring bases.[7] However, partial stacking interaction and relative conformation of the naphthalimide core with respect to the uracil base in different base environment could also influence the fluorescence properties.[6,8]

RNA ON **15** (**6** between rC residues) showed an intense band with an emission maximum at 515 nm (Figure 8). Surprisingly, the emissive uridine analogue in duplex **15·15G** (consecutive dG residues, **6** opposite dG) displayed more than twofold higher fluorescence intensity, relative to when it was opposite its complementary base (dA, duplex **15·15A**; Figure 8). However, the fluorescence intensity was not significantly higher when the nucleoside analogue was opposite dT or dC (mismatches in **15·15T** and **15·15C**, respectively). We were doubly surprised when the fluorescent uridine analogue was incorporated between rU residues and opposite complementary (**20·20A**) and

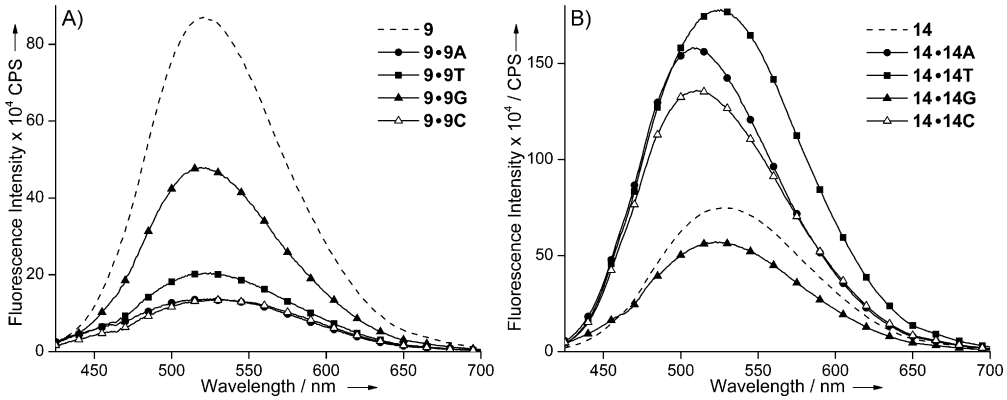


Figure 7. A) Emission spectra of ON **9** (1 μM) and duplexes of **9**. B) Emission spectra of ON **14** (1 μM) and duplexes of **14**. ONs were excited at 407 nm; excitation and emission slit widths: 6 and 8 nm, respectively.[29]

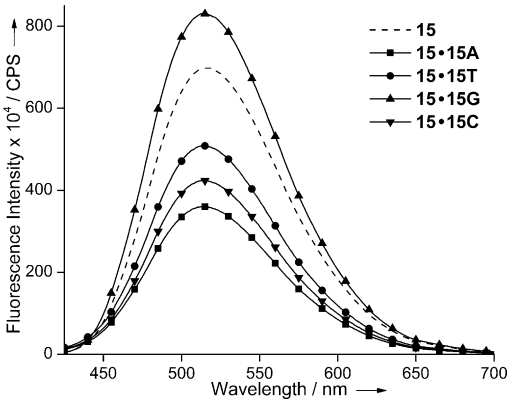


Figure 8. Emission spectra of ON **15** (1 μM) and duplexes of **15**. ONs were excited at 407 nm; excitation and emission slit widths: 6 and 8 nm, respectively.[29]

mismatched bases (**20·20T**, **20·20G**, **20·20C**): significantly higher (more than fourfold) fluorescence intensity, with no apparent change in emission maximum relative to single-stranded ON **20** (Figure 9). This is noteworthy, because, upon incorporation into ONs, the majority of fluorescent nucleoside analogues (including 2-aminopurine) experience drastic quenching in fluorescence intensity due to stacking interactions and or electron transfer.[2,6,17] In particular, this effect is more pronounced when the nucleoside analogue is in the vicinity of purines bases.[17]

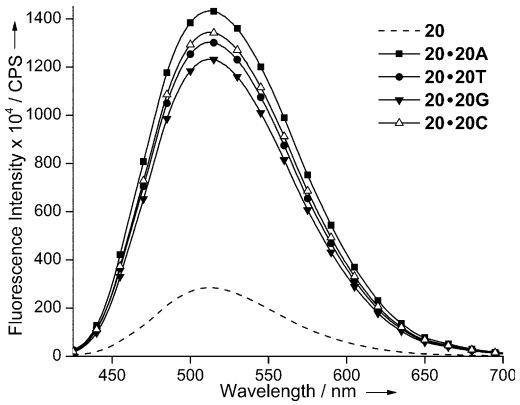


Figure 9. Emission spectra of ON **20** (1 μM) and duplexes of **20**. ONs were excited at 407 nm; excitation and emission slit widths: 6 and 8 nm, respectively.[29]

The fluorescence profile exhibited by **6** in different nucleobase environments is likely attributable to alterations in the relative conformation of the naphthalimide moiety with respect to the uracil base, electron transfer process between the emissive nucleobase and neighbouring bases and solvation/desolvation effects.[6–8,17] Together, these results clearly indicate that the fluorescence properties of the emissive base are sensitive to flanking bases and base-pair substitutions; essentially, these arise from changes in interactions between the modified base and neighbouring bases. The fact that the fluorescence of the majority of fluorophores is quenched by purine residues,[17] the enhancement in fluorescence intensity exhibited by naphthalimide-modified nucleoside in G-rich and A-rich environments is rare and useful.[19] An analogue probe with excitation and emission maxima in the visible region as well as the ability to exhibit enhanced fluorescence efficiency in particular sequence contexts could be highly useful in hybridisation assays to detect nucleobase repeats in nucleic acids (e.g., G-repeats and A-repeats).

Conclusions

Nucleic acids interact with proteins, nucleic acids and small-molecule metabolites; during such recognition events they undergo conformational changes both at the global and nucleoside levels.[38] The changes in nucleoside conformation (e.g., near the protein/ligand binding site) also alter its surrounding physical properties and interactions with adjacent nucleosides. Hence, environment-sensitive fluorescent nucleoside analogues with excitation/emission maxima in the visible region and high quantum yields when within ONs are suitable for both in vitro and cell-based applications, and are highly desirable. In this regard, fluorescent ribonucleoside **6**, based on the Lucifer chromophore, represents a new type of environment-sensitive nucleoside analogue. The triphosphate and phosphoramidite derivative of the naphthalimide-modified nucleoside act as good substrates for the synthesis of fluorescent RNA ONs by in vitro transcription and by solid-phase synthesis, respectively. Furthermore, the emissive nucleoside incorporated into ON duplexes exhibits appreciable fluorescence efficiency, and is responsive to its flanking bases and base-pair substitutions. These favourable photophysical properties and amenability to enzymatic and chemical incorporation underscore the potential of the naphthalimide-modified nucleoside analogue as a probe in the investigation of nucleic acids by fluorescence spectroscopy and microscopy.[39] We are currently evaluating the suitability of the fluorescent nucleoside analogue in probing the cellular uptake and trafficking of RNA ONs (e.g., siRNA) by fluorescence microscopy, and the results will be reported in due course.

Experimental Section

Photophysical characterisation of ribonucleoside analogue 6

Steady-state fluorescence of ribonucleoside analogue in various solvents: Analogue **6** (5 μM) in water, methanol, acetonitrile or dioxane was excited at the respective lowest energy absorption maximum, with excitation and emission slit widths of 1 and 5 nm, respectively. Fluorescence experiments were performed in triplicate in a micro fluorescence cell (path length 1.0 cm; Hellma Analytics, Müllheim, Germany) on a Fluorolog-3 spectrophluorometer (Horiba Jobin Yvon, Kyoto, Japan).

Time-resolved fluorescence measurements: Excited-state lifetimes of **6** in various solvents were determined by using a TCSPC fluorescence spectrophotometer (Horiba Jobin Yvon). Ribonucleoside **6** (5 μM) was excited with a 371 nm NanoLED-371L LED source (IBH/Horiba, Glasgow, UK). Lifetime measurements were performed in

duplicate, and decay profiles were analysed with DAS6 analysis software (IBH). Fluorescence intensity decay kinetics in all solvents were monoexponential, with χ^2 (goodness of fit) values very close to unity.

Enzymatic incorporation of modified triphosphate 7 into RNA ONs

Transcription reactions in presence of [α-^{32}P]ATP: Promoter/template duplexes were constructed by heating equimolar (5 μM) DNA template (**T1**–**T5**) and an 18-mer T7 RNA polymerase consensus promoter DNA sequence in TE buffer (Tris·HCl (10 mM, pH 7.8), EDTA (1 mM), NaCl (100 mM)) at 90 °C for 3 min, and cooling slowly to room temperature. The duplexes were placed in an ice bath for 20 min and stored at −40 °C. Transcription reactions were performed in Tris·HCl (40 mM, pH 7.9) containing annealed template (250 nM), $MgCl_2$ (10 mM), NaCl (10 mM), dithiothreitol (10 mM), spermidine (2 mM), RiboLock RNase inhibitor (1 U μL^{-1}; Thermo Scientific), GTP (1 mM), CTP (1 mM), UTP (1 mM) and/or modified UTP **7** (1 mM), ATP (20 μM), [α-^{32}P]ATP (5 μCi, 2.5 pmol) and T7 RNA polymerase (3 U μL^{-1}) in a total volume of 20 μL for 4 h at 37 °C. Reactions were quenched by adding loading buffer (20 μL; Tris·HCl (10 mM, pH 8), urea (7 M), EDTA (100 mM), Bromophenol Blue (0.05%)), heated (75 °C, 3 min) then cooled in an ice bath. Samples (5 μL) were loaded onto an 18% denaturing polyacrylamide gel. The gel was exposed to X-ray film (10 min), and the exposed film was developed, fixed and dried. The bands were then quantified by using the software GeneTools (Syngene, Cambridge, UK) to determine percentage incorporation of **7** relative to transcription efficiency with natural UTP. All reactions were performed in duplicate, and the standard deviations were found to be ±3%.

Large-scale transcription reactions: Large-scale transcription reactions with templates **T1** and **T3**–**T7** were performed as above but in 250 μL reaction mixtures, to isolate ONs for further characterisation and photophysical studies. GTP, CTP, ATP, UTP or **7** (2 mM each), $MgCl_2$ (20 mM), RiboLock (0.4 U μL^{-1}), annealed template (300 nM) and T7 RNA polymerase (800 U) were mixed and incubated for 12 h at 37 °C. The reaction volume was reduced (to ~1/3) in a SpeedVac, then loading buffer (30 μL) was added. The samples were loaded onto a preparative 20% denaturing polyacrylamide gel and electrophoresed at a constant 24 W (~800 V) for 5 h. The gel was UV shadowed, then the appropriate band was removed, extracted with sodium acetate (0.3 M) and desalted in a Sep-Pak classic C18 cartridge (for purity see Figure S4). Typical transcript yield: 16–19 nmol.

Enzymatic digestion of transcript 9: Fluorescently modified ON **9** (4 nmol) was digested with snake venom phosphodiesterase I (0.015 U), calf intestine alkaline phosphatase (1.5 U μL^{-1}) and RNase A (0.5 μg) in Tris·HCl (100 μL, 50 mM pH 8.5) containing $MgCl_2$ (40 mM) and EDTA(0.1 mM) for 24 h at 37 °C. RNase T1 (0.6 U μL^{-1}) was then added, and the sample was incubated for a further 12 h at 37 °C. The obtained ribonucleoside mixture was analysed by reversed-phase analytical HPLC by using a Luna C18 column (250×4.6 mm, 5 μm; Phenomenex, Torrance, CA) at 260 and 390 nm. Mobile phase A: triethylammonium acetate (50 mM, pH 7.1), mobile phase B: acetonitrile; flow rate: 1 mL min^{-1}; gradient: 0–10% B (20 min), 10–100% B (10 min). Fractions corresponding to individual ribonucleosides were further analysed by mass spectroscopy (Table S2).

Solid-phase synthesis of RNA ON 20: Napthalimide-modified RNA ON **20** was synthesised on a 1 μmol scale CPG solid support (1000 Å) by following a standard solid-phase RNA ON synthesis protocol.[40] Incorporation of regular 2′-*O*-TBDMS-protected phosphoramidites was performed with a coupling time of 10 min; incorporation of fluorescent 2′-*O*-TBDMS-protected phosphoramidite substrate **19** was performed with a coupling time of 30 min (coupling efficiency 20%, based on a trityl monitor assay). The trityl protecting group was deprotected on the synthesiser. The solid support was treated with a solution of methylamine (10 M in ethanol) and water (1:1, 1.5 mL) for 12 h. The mixture was centrifuged, and the supernatant was evaporated to dryness in a SpeedVac. The residue was then dissolved in DMSO (100 μL) and treated with TEA·3HF (150 μL). The sample was heated at 65 °C for 2.5 h and was brought to RT. The completely deprotected ON solution was lyophilised and then purified by 20% denaturing PAGE. The band corresponding to the full-length product was identified by UV shadowing. The ON was extracted with ammonium acetate buffer (0.5 M, 3 mL) and desalted in a Sep-Pak classic C18 cartridge (ε_{260} and MALDI-MS data in Table S1; ON **20** HPLC chromatogram in Figure S5).

Photophysical characterisation of naphthalimide-modified ONs: ONs **9**, **14**, **15** and **20** (10 μM) were annealed to respective complementary custom DNA ONs by heating a mixture (1:1.1) of ONs in cacodylate buffer (20 mM, pH 7.0) with NaCl (500 mM) and EDTA (0.5 mM) at 90 °C for 3 min. Samples were then cooled slowly to RT, then placed in crushed ice for 2 h. Samples were diluted (final 1 μM, with respect to **9**, **14**, **15** and **20**) in cacodylate buffer. Fluorescently modified duplexes were excited at 407 nm (excitation and emission slit widths, 6 and 8 nm, respectively). Fluorescence experiments were performed in triplicate in a micro fluorescence cuvette (path length 1.0 cm, Hellma) on a Horiba Jobin Yvon, Fluorolog-3 at 20 °C.

Acknowledgements

S.G.S. is grateful to CSIR, India (02-0086/12/EMR-II) for a research grant. A.A.T. thanks CSIR, India for a graduate research fellowship.

Keywords: emission spectroscopy · fluorescent nucleoside · G-repeats · nucleoside analogues · RNA

[1] a) U. Asseline, *Curr. Org. Chem.* **2006**, *10*, 491–518; b) K. B. Hall, *Methods Enzymol.* **2009**, *469*, 269–285; c) X. Shi, D. Herschlag, *Methods Enzymol.* **2009**, *469*, 287–302; d) L. M. Wilhelmsson, *Q. Rev. Biophys.* **2010**, *43*, 159–183; e) S. G. Srivatsan, A. A. Sawant, *Pure Appl. Chem.* **2011**, *83*, 213–232.
[2] R. W. Sinkeldam, N. J. Greco, Y. Tor, *Chem. Rev.* **2010**, *110*, 2579–2619.
[3] J. N. Wilson, E. T. Kool, *Org. Biomol. Chem.* **2006**, *4*, 4265–4274.
[4] M. E. Hawkins, *Methods Enzymol.* **2008**, *450*, 201–231.
[5] Y. N. Teo, E. T. Kool, *Chem. Rev.* **2012**, *112*, 4221–4245.
[6] 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.
[7] S. Doose, H. Neuweiler, M. Sauer, *ChemPhysChem* **2009**, *10*, 1389–1398.
[8] R. W. Sinkeldam, A. J. Wheat, H. Boyaci, Y. Tor, *ChemPhysChem* **2011**, *12*, 567–570.
[9] a) A. Okamoto, K. Tanaka, T. Fukuta, I. Saito, *J. Am. Chem. Soc.* **2003**, *125*, 9296–9297; b) A. Okamoto, Y. Saito, I. Saito, *J. Photochem. Photobiol. C* **2005**, *6*, 108–122; c) E. Mayer-Enthart, H.-A. Wagenknecht, *Angew. Chem. Int. Ed.* **2006**, *45*, 3372–3375; *Angew. Chem.* **2006**, *118*, 3451–3453; d) K. Tainaka, K. Tanaka, S. Ikeda, K.-I. Nishiza, T. Unzai, Y. Fujiwara, I. Saito, A. Okamoto, *J. Am. Chem. Soc.* **2007**, *129*, 4776–4784; e) F. Takei, H. Suda, M. Hagihara, J. Zhang, A. Kobori, K. Nakatani, *Chem. Eur. J.* **2007**, *13*, 4452–4457; f) Q. Xiao, R. T. Ranasinghe, A. M. P. Tang, T. Brown, *Tetrahedron* **2007**, *63*, 3483–3490; g) S. G. Srivatsan, H. Weiz-

man, Y. Tor, *Org. Biomol. Chem.* **2008**, *6*, 1334 – 1338; h) H. Gardarsson, A. S. Kale, S. T. Sigurdsson, *ChemBioChem* **2011**, *12*, 567 – 575; i) X. Ming, F. Seela, *Chem. Eur. J.* **2012**, *18*, 9590 – 9600.

[10] a) T. J. Matray, E. T. Kool, *Nature* **1999**, *399*, 704 – 708; b) L. Valis, N. Amann, H.-A. Wagenknecht, *Org. Biomol. Chem.* **2005**, *3*, 36 – 38; c) E. Shipova, K. S. Gates, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2111 – 2113; d) N. J. Greco, Y. Tor, *J. Am. Chem. Soc.* **2005**, *127*, 10784 – 10785; e) A. A. Tanpure, S. G. Srivatsan, *Chem. Eur. J.* **2011**, *17*, 12820 – 12827.

[11] a) N. J. Greco, R. W. Sinkeldam, Y. Tor, *Org. Lett.* **2009**, *11*, 1115 – 1118; b) W. Hirose, K. Sato, A. Matsuda, *Angew. Chem. Int. Ed.* **2010**, *49*, 8392 – 8394; *Angew. Chem.* **2010**, *122*, 8570 – 8572; c) Q. Zhang, Y. Wang, X. Meng, R. Dhar, H. Huang, *Anal. Chem.* **2013**, *85*, 201 – 207.

[12] a) S. O. Kelley, J. K. Barton, *Science* **1999**, *283*, 375 – 381; b) N. A. Grigorenko, C. J. Leumann, *Chem. Commun.* **2008**, 5417 – 5419; c) A. Zahn, C. J. Leumann, *Chem. Eur. J.* **2008**, *14*, 1087 – 1094.

[13] a) A. Nadler, J. Strohmeier, U. Diederichsen, *Angew. Chem. Int. Ed.* **2011**, *50*, 5392 – 5396; *Angew. Chem.* **2011**, *123*, 5504 – 5508; b) S. Müllar, J. Strohmeier, U. Diederichsen, *Org. Lett.* **2012**, *14*, 1382 – 1385; c) I. J. Lee, B. H. Kim, *Chem. Commun.* **2012**, *48*, 2074 – 2076; d) K. T. Kim, B. H. Kim, *Chem. Commun.* **2013**, *49*, 1717 – 1719.

[14] a) S. G. Srivatsan, N. J. Greco, Y. Tor, *Angew. Chem. Int. Ed.* **2008**, *47*, 6661 – 6665; *Angew. Chem.* **2008**, *120*, 6763 – 6767; b) A. S. Wahba, A. Esmaeili, M. J. Damha, R. H. E. Hudson, *Nucleic Acids Res.* **2010**, *38*, 1048 – 1056; c) R. W. Sinkeldam, L. S. McCoy, D. Shin, Y. Tor, *Angew. Chem. Int. Ed.* **2013**, *52*, 14026 – 14030; *Angew. Chem.* **2013**, *125*, 14276 – 14280.

[15] a) R. Kawai, M. Kimoto, S. Ikeda, T. Mitsui, M. Endo, S. Yokoyama, I. Hirao, *J. Am. Chem. Soc.* **2005**, *127*, 17286 – 17295; b) R. Rieder, K. Lang, D. Graber, R. Micura, *ChemBioChem* **2007**, *8*, 896 – 902; c) S. G. Srivatsan, Y. Tor, *Tetrahedron* **2007**, *63*, 3601 – 3607; d) J. Parsons, M. P. Castaldi, S. Dutta, S. M. Dibrov, D. L. Wyles, T. Hermann, *Nat. Chem. Biol.* **2009**, *5*, 823 – 825; e) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009**, *131*, 17605 – 17614; f) J. Riedl, R. Pohl, N. P. Ernsting, P. Orság, M. Fojta, M. Hocek, *Chem. Sci.* **2012**, *3*, 2797 – 2806; g) J. Riedl, P. Ménová, R. Pohl, P. Orság, M. Fojta, M. Hocek, *J. Org. Chem.* **2012**, *77*, 8287 – 8293.

[16] a) P. Cekan, S. T. Sigurdsson, *Chem. Commun.* **2008**, 3393 – 3395; b) K. Börjesson, S. Preus, A. H. El-Sagheer, T. Brown, B. Albinsson, L. M. Wilhelmsson, *J. Am. Chem. Soc.* **2009**, *131*, 4288 – 4293; c) A. A. Tanpure, S. G. Srivatsan, *ChemBioChem* **2012**, *13*, 2392 – 2399.

[17] Purines (especially guanine) are know to quench the fluorescence of several fluorophores in a distance-depended manner by electron transfer. C. A. M. Seidel, A. Schulz, M. H. M. Sauer, *J. Phys. Chem.* **1996**, *100*, 5541 – 5553.

[18] J. Hou, X. Liu, J. Liu, *Biotechnol. Biotechnol. Equip.* **2012**, *26*, 3148 – 3154.

[19] G-repeats (e.g., G-triad) are biologically important structural motifs and are frequently found in nucleic acids (e.g., telomeric and oncogene promoter regions). Hence, development of fluorescence probes that can signal the presence of G-repeats by enhancement in fluorescence intensity is highly desired; a) B. R. Vummidi, J. Alzeer, N. W. Luedtke, *ChemBioChem* **2013**, *14*, 540 – 558; b) Y. Xu, Y. Suzuki, K. Ito, M. Komiyama, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 14579 – 14584.

[20] a) S. M. Park, S.-J. Nam, H. S. Jeong, W. J. Kim, B. H. Kim, *Chem. Asian J.* **2011**, *6*, 487 – 492; b) K. Phelps, A. Morris, P. A. Beal, *ACS Chem. Biol.* **2012**, *7*, 100 – 109; c) A. A. Tanpure, M. G. Pawar, S. G. Srivatsan, *Isr. J. Chem.* **2013**, *53*, 366 – 378.

[21] a) S. S. Tan, S. J. Kim, E. T. Kool, *J. Am. Chem. Soc.* **2011**, *133*, 2664 – 2671; b) J. Guo, S. Wang, N. Dai, Y. N. Teo, E. T. Kool, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3493 – 3498.

[22] A. S. Wahba, F. Azizi, G. F. Deleavey, C. Brown, F. Robert, M. Carrier, A. Kalota, A. M. Gewirtz, J. Pelletier, R. H. E. Hudson, M. J. Damha, *ACS Chem. Biol.* **2011**, *6*, 912 – 919.

[23] a) A. Dierckx, F.-A. Miannay, N. B. Gaied, S. Preus, M. Björck, T. Brown, L. M. Wilhelmsson, *Chem. Eur. J.* **2012**, *18*, 5987 – 5997; b) M. Weinberger, F. Berndt, R. Mahrwald, N. P. Ernsting, H.-A. Wagenknecht, *J. Org. Chem.* **2013**, *78*, 2589 – 2599.

[24] Predicting the photophysical behaviour of nucleoside analogues (free or incorporated into ONs) based on their structure is not simple, and hence, probe design and implementation remain largely empirical tasks.

[25] a) W. W. Stewart, *Nature* **1981**, *292*, 17 – 21; b) A. P. De Silva, H. Q. N. Gunaratne, J.-L. Habib-Jiwan, C. P. McCoy, T. E. Rice, J.-P. Soumillion, *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1728 – 1731; *Angew. Chem.* **1995**, *107*, 1889 – 1891.

[26] S. Banerjee, E. B. Veale, C. M. Phelan, S. A. Murphy, G. M. Tocci, L. J. Gillespie, D. O. Frimannsson, J. M. Kelly, T. Gunnlaugsson, *Chem. Soc. Rev.* **2013**, *42*, 1601 – 1618.

[27] Typically, derivatives containing electron-donating groups at this position exhibit very strong fluorescence arising from the generation of a charge-transfer excited state. R. M. Duke, E. B. Veale, F. M. Pfeffer, P. E. Kruger, T. Gunnlaugsson, *Chem. Soc. Rev.* **2010**, *39*, 3936 – 3953.

[28] See the Supporting Information for details.

[29] See the Experimental Section for details.

[30] a) S. H. Weisbrod, A. Marx, *Chem. Commun.* **2008**, 5675 – 5685; b) F. Wachowius, C. Höbartner, *ChemBioChem* **2010**, *11*, 469 – 480.

[31] a) J. Riedl, R. Pohl, L. Rulíšek, M. Hocek, *J. Org. Chem.* **2012**, *77*, 1026 – 1044; b) B. Holzberger, J. Strohmeier, V. Siegmund, U. Diederichsen, A. Marx, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3136 – 3139.

[32] a) K. Lang, R. Rieder, R. Micura, *Nucleic Acids Res.* **2007**, *35*, 5370 – 5378; b) S. G. Srivatsan, Y. Tor, *Chem. Asian J.* **2009**, *4*, 419 – 427; c) G. Stengel, M. Urban, B. W. Purse, R. D. Kuchta, *Anal. Chem.* **2010**, *82*, 1082 – 1089; d) M. Kimoto, T. Mitsui, S. Yokoyama, I. Hirao, *J. Am. Chem. Soc.* **2010**, *132*, 4988 – 4989; e) Y. Hikida, M. Kimoto, S. Yokoyama, I. Hirao, *Nat. Protoc.* **2010**, *5*, 1312 – 1323; f) M. G. Pawar, S. G. Srivatsan, *Org. Lett.* **2011**, *13*, 1114 – 1117; g) M. G. Pawar, A. Nuthanakanti, S. G. Srivatsan, *Bioconjugate Chem.* **2013**, *24*, 1367 – 1377.

[33] M. Kimoto, T. Mitsui, Y. Harada, A. Sato, S. Yokoyama, I. Hirao, *Nucleic Acids Res.* **2007**, *35*, 5360 – 5369.

[34] J. Ludwig, *Acta Biochim. Biophys. Acad. Sci. Hung.* **1981**, *16*, 131 – 133.

[35] S. G. Srivatsan, Y. Tor, *J. Am. Chem. Soc.* **2007**, *129*, 2044 – 2053.

[36] Trace amounts of N+1, N+2 transcripts are also formed due to random non-encoded incorporation of nucleotides. J. F. Milligan, O. C. Uhlenbeck, *Methods Enzymol.* **1989**, *180*, 51 – 62.

[37] J. P. Peters, S. P. Yelgaonkar, S. G. Srivatsan, Y. Tor, L. J. Maher III, *Nucl. Acid. Res.* **2013**, *41*, 10593 – 10604.

[38] a) M. Mandal, R. R. Breaker, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 451 – 463; b) Z. Shajani, P. Deka, G. Varani, *Trends Biochem. Sci.* **2006**, *31*, 421 – 424; c) K. B. Hall, *Curr. Opin. Chem. Biol.* **2008**, *12*, 612 – 618; d) H. M. Al-Hashimi, N. G. Walter, *Curr. Opin. Struct. Biol.* **2008**, *18*, 321 – 329; e) D. M. J. Lilley, *Philos. Trans. R. Soc. London Ser. B* **2011**, *366*, 2910 – 2917.

[39] Phenylpyrrolocytosine, which exhibits excitation and emission maxima at 360 and 466 nm, respectively, as well as quenching in fluorescence intensity upon hybridisation, has been used in probing siRNA uptake by fluorescence microscopy. See ref. [22].

[40] B. S. Sproat in *Methods in Molecular Biology, Vol. 288: Oligonucleotide Synthesis* (Ed.: P. Herdewijn), Humana, Totowa, **2005**, pp. 17 – 32.

Received: February 21, 2014
Published online on May 23, 2014