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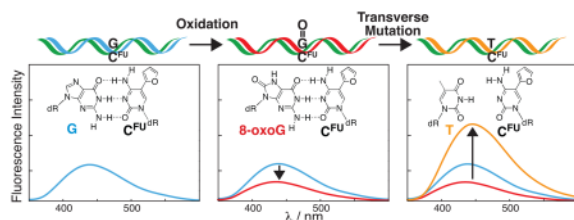
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An Emissive C Analog Distinguishes between G, 8-oxoG and T

Nicholas J. Greco, Renatus W. Sinkeldam, and Yitzhak Tor

Department of Chemistry and Biochemistry, University of California, San Diego 9500 Gilman Drive, La Jolla, CA 92093-0358 ytor@ucsd.edu

Abstract



A minimally disruptive fluorescent dC analog provides a rapid and non-destructive method for in vitro detection of G, 8-oxoG and T, the downstream transverse mutation product.

One of the most thoroughly examined DNA base modifications is 7,8-dihydro-8-oxoguanine (8-oxoG), a mutagenic product of oxidative damage by reactive oxygen species.¹ The presence of 8-oxoG is frequently viewed as a marker for cellular oxidative stress, a condition that has been linked to carcinogenesis.² The significance of this seemingly minor base damage results from 8-oxoG's ability to deceive DNA polymerases and form a stable (*syn*)8-oxoG•A base pair by presenting its Hoogsteen face thereby mimicking T (Figure 1).³ Unless repaired, this might cause G to T transversion mutations during DNA synthesis (Figure 1).⁴ Not surprisingly, base-excision repair mechanisms have evolved to correct such deleterious base modification products, and the bacterial and human enzymes have been thoroughly studied.⁵

A non-destructive and real-time fluorescence-based detection of 8-oxoG, its repair and induced mutation processes in oligonucleotides, could significantly advance the in vitro biochemical evaluation of this important DNA lesion.⁶ It would complement existing methods that rely on chromatographic, electrophoretic and immunological methods.^{7,8} Toward this end, we hypothesized that one could take advantage of the distinct redox properties of 8-oxoG.⁹ Electrochemical measurements show that 8-oxoG is more easily oxidized compared to G ($E_{1/2} \approx 0.75$ and 1.3 vs. NHE, respectively).^{9a,10} Consequently, chemical approaches relying on 8-oxoG's susceptibility to oxidation and covalent trapping of the oxidized products in duplex DNA have been reported.¹¹ Since fluorescence quenching frequently occurs via photoinduced electron transfer (PET) mechanisms,¹² we suspected that 8-oxoG is likely to be a more effective quencher of certain fluorophores compared to G, its precursor.¹³ Here we describe the design, synthesis, photophysical evaluation, incorporation and implementation of a simple isomorphous fluorescent dC analog **5** that, upon incorporation into an oligonucleotide, photophysically distinguishes between 8-oxoG and G on the complementary strand. Not only is the damaged 8-oxoG-containing duplex highly quenched and the "repaired" G-containing duplex more emissive, the transverse mutated duplex containing T instead of G displays the

most intense emission. This furan-containing emissive nucleobase therefore provides signature emission profiles for all key nucleobases involved in this DNA damage pathway (Figure 1).¹⁴

We have been developing simple and minimally perturbing emissive nucleobases for the detection of nucleic acids lesions.^{15,16} The primary design principle dictates maintaining the highest possible structural similarity to the natural nucleobases, while significantly improving their photophysical properties. Specifically, an isolated absorption band for selective excitation, enhanced quantum yield over the native nucleobases and sensitivity to changes in the microenvironment are desired. Useful uridine-based nucleosides, fulfilling these criteria, were obtained by conjugating five-membered aromatic heterocycles such as furan at the 5 position (e.g., **2**).^{15,16,17,18} Among the various heterocycles conjugated to dU (i.e., furan, thiophene, oxazole, thiazole) the furan moiety was found to yield the most favorable photophysical characteristics.^{15,17a} We therefore anticipated the analogous furan modified cytosine nucleobase to be emissive and responsive.

The 5-modified nucleosides are easily obtained using a coupling reaction between the 5-iodo substituted dU (**1**) and the corresponding stannylated heterocycles (Scheme 1). Conversion of the acetate protected furan-modified dU analog **3** to the desired dC analog **5** is accomplished by activation of the 4 position as an aryl sulfonate ester followed by a displacement reaction with ammonia,¹⁹ providing the fully deprotected furan-modified dC analog **5** (Scheme 1).^{17a,20} Silyl protection of the furan-modified dU **4** facilitated the conversion to the dC analog **6** with retention of hydroxyl protection thus allowing for standard benzamide protection of the exocyclic amine to give **7**. Desilylation and protection of the 5'-hydroxyl as the 4,4'-dimethoxytrityl derivative (**9**) followed by phosphorylation of the unprotected 3'-hydroxyl afforded **10**, the building block necessary for automated DNA synthesis (Scheme 1).²⁰

The absorption spectrum of an aqueous solution of **5** shows, in addition to the typical high energy band seen in the parent nucleoside, a clear shoulder at ~310 nm (Figure 2). Excitation at this wavelength yields an emission profile with a maximum at 443 nm, which tails deeply into the visible range, with a relative quantum yield of 0.02 (Table 1).^{20,21} Lowering solvent polarity results in a better defined long wavelength absorption band ($\lambda_{\text{max}} = 309$ nm) and a hypsochromic shift in emission maxima ($\lambda_{\text{em}} = 421$ nm), which is associated with a hypochromic effect ($I_{\text{Water/Dioxane}} = 3$) (Figure 2, Table 1).

Emission spectra of **5** in dioxane-water mixtures provide a more detailed view of the hypsochromic shift that arises upon decreasing the polarity of the chromophore's microenvironment (Figure S2.1).²⁰ Plotting the corrected emission energy maximum of **5** vs. the microenvironment polarity described by $E_{\text{T}}(30)$ values,²² results in a linear correlation (Figure 3).²³ Stern-Volmer titrations were conducted to determine the differences between G and 8-oxoG's quenching abilities (Figure 4). Rewardingly, while G minimally impacted the emission of **5** ($K_{\text{sv}} = 0.004 \text{ mM}^{-1}$), 8-oxoG was found to be a very effective quencher, even at low concentrations ($K_{\text{sv}} = 16.5 \text{ mM}^{-1}$).²⁰

To investigate the potential of the emissive nucleoside to photophysically discriminate between G, 8-oxoG and T, an oligonucleotide that contains **5** at a central position was synthesized using **10** and standard solid-phase synthesis protocols (Figure 5).²⁰ All modified oligonucleotides were characterized using MALDI TOF MS.²⁰ Since modified C residues can, in principle, deaminate to yield the corresponding U derivatives, special care was taken to unequivocally verify the presence of **5** and absence of **2** in the modified oligonucleotide **11**. Enzymatic digestion reactions, followed by HPLC analysis against all authentic nucleosides verified the presence of intact **5** and absence of **2** in the modified oligonucleotide **11** (Figure S6.1, Table S6.1).²⁰

Oligonucleotide **11**, containing the furan functionalized dC **5** was hybridized to three complementary oligonucleotides that contain either G (oligo **12**), 8-oxoG (oligo **13**) or T (oligo **14**) opposite the emissive nucleotide (Figure 5). Thermal denaturation (Table 2) and CD studies show no appreciable difference between the modified (**11•12**, **11•13** and **11•14**) and unmodified duplexes (**15•12**, **15•13** and **15•14**, respectively) suggesting the small furan modification does not disrupt duplex formation, stability or structure (Figure S8.1 and S9.1).²⁰

The emission spectra of the furan dC containing duplexes, in both low (100 mM) and elevated (500 mM) ionic strength are shown in Figure 6.²³ While the perfect duplex **11•12**, where G is placed opposite **5**, is significantly emissive, placing **5** opposite 8-oxoG (**11•13**) leads to considerable emission quenching (ca. two-fold), as hypothesized. Nucleoside **5** therefore clearly distinguishes between G and 8-oxoG, its oxidized product. In contrast, duplex **11•14**, where **5** is placed opposite T, a substantial emission enhancement (ca. four-fold compared to **11•13**) is observed (Figure 6, Table 3). Nucleoside **5** thus reports the presence of T, the ultimate transversion mutation product resulting from G oxidation to 8-oxoG, via enhanced emission. These observations, illustrating signature emission profiles for duplexes containing G, 8-oxoG and T, suggest that the furan-modified dC nucleoside **5** could be utilized to follow, in vitro, DNA damage and its repair via this pathway using such emissive oligonucleotides.

The observed fluorescence quenching of **5** by 8-oxoG can be understood by the lower redox potential of 8-oxoG and concomitant higher potency as an excited state quencher compared to G (Figure 4). Several observations collectively suggest that the substantial fluorescence enhancement observed for the **5•T** mismatch in **11•14** is due to exposure of the emissive nucleoside to a more polar environment, likely extrahelical. Thermal denaturation data illustrates that the dC/T containing duplex **15•14** (as well as the analogous modified duplex **11•14**) are the least stable (Table 2). This is consistent with previous observations illustrating this particular dual pyrimidine mismatch to be rather unfavorable,²⁴ therefore suggesting a local structural perturbation. Fluorescence experiments carried out at elevated ionic strengths (500 mM) for all duplexes resulted in similar observations (Figure 6), rendering the potential higher abundance of the more emissive single strand due to poor hybridization unlikely.²⁰

Further support for the proposed extrahelical residency of the fluorescent nucleobase is obtained by interpolating the emission energy of modified duplexes containing **5** and environmental polarity using the linear correlation shown above (Figure 3, Table 3). The emission spectra of the furanyl modified duplexes **11•12** and **11•13**, where furanyl dC **5** can pair in a Watson–Crick fashion, display emission maximum that correlate to relatively apolar environments (Figures 3 and 6, Table 3), while duplex **11•14**, where furanyl dC **5** is unable to pair in a W-C fashion with T, shows a red shifted emission maximum that correlates to a polar environment, in agreement with an extrahelical disposition.

In summary, we have shown that an isomorphous fluorescent nucleoside **5**, which upon incorporation results in no observable perturbation of the duplex shape and stability, is a valuable probe for the detection of G, 8-oxoG and its transverse mutation product T by eliciting markedly different emission intensities in conjunction with changes in emission maxima. The effective synthesis and incorporation of **5** could facilitate rapid and non-destructive real-time fluorescence-based methods for the in vitro monitoring of this DNA damage pathway.²⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Beckman KB, Ames BN. *J Biol Chem* 1997;272:19633–19636. [PubMed: 9289489] (b) Burrows CM, Muller J. *Chem Rev* 1998;98:1109–1152. [PubMed: 11848927] (c) Bjelland S, Seeberg EM. *Mutat Res* 2003;531:37–80. [PubMed: 14637246] (d) Cadet J, Douki T, Gasparutto D, Ravanat JL. *Mutat Res* 2003;531:5–23. [PubMed: 14637244] (e) Greenberg MM. *Biochem Soc Trans* 2004;32:46–50. [PubMed: 14748710] (f) Neeley WL, Essigmann JM. *Chem Res Toxicol* 2006;19:491–505. [PubMed: 16608160] (g) Cooke MS, Evans MD. *Proc Natl Acad Sci USA* 2007;104:13535–13536. [PubMed: 17699620]
2. (a) Ames BN, Shigenaga MK, Hagen TM. *Proc Natl Acad Sci USA* 1993;90:7915–7922. [PubMed: 8367443] (b) Loft S, Poulsen HE. *J Molec Medicine* 1996;74:297–312. (c) Wiseman H, Halliwell B. *Biochem J* 1996;313:17–29. [PubMed: 8546679] (d) Klaunig JE, Kamendulis LM. *Annu Rev Pharmacol Toxicol* 2004;44:239–267. [PubMed: 14744246] (e) Hirano T. *J Radiat Res* 2008;49:329–340. [PubMed: 18596371]
3. Leonard GA, Guy A, Brown T, Teoule R, Hunter WN. *Biochemistry* 1992;31:8415–8420. [PubMed: 1390625]
4. Wood ML, Dizdaroglu M, Gajewski E, Essigmann JM. *Biochemistry* 1990;29:7024–7032. [PubMed: 2223758]
5. For recent reviews, see: (a) Fromme JC, Verdine GL. *Adv Protein Chem* 2004;69:1–41. [PubMed: 15588838] (b) David SS, O’Shea VL, Kundu S. *Nature* 2007;447:941–950. [PubMed: 17581577]
6. 8-oxoG’s low cellular abundance, complex localization and chemical degradation pathways make the development of small probes for its intracellular detection a formidable task.
7. (a) Shigenaga MK, Ames BA. *Free Radic Biol Med* 1991;10:211–216. [PubMed: 1650737] (b) Muller JG, Duarte V, Hickerson RP, Burrows CJ. *Nucleic Acids Res* 1998;26:2247–2249. [PubMed: 9547288] (c) Ropp PA, Thorp HH. *Chem Biol* 1999;6:599–605. [PubMed: 10467130] (d) Pouget JP, Douki T, Richard MJ, Cadet J. *Chem Res Toxicol* 2000;13:541–549. [PubMed: 10898585] (e) Soutanakis RP, Melamede RJ, Bessalov IA, Wallace SS, Beckman KB, Ames BN, Taatjes DJ, Janssen-Heininger YM. *Free Radic Biol Med* 2000;28:987–998. [PubMed: 10802231] (f) Persinger RL, Melamede R, Bessalov I, Wallace S, Taatjes DJ, Janssen-Heininger Y. *Exp Gerontology* 2001;36:1483–1494. (g) Gielazyn ML, Ringwoodb AH, Piegorsc WW, Stancyka SE. *Mutat Res* 2003;542:15–22. [PubMed: 14644349] (h) Gore MR, Szalai VA, Ropp PA, Yang IV, Silverman JS, Thorp HH. *Anal Chem* 2003;75:6586–6592. [PubMed: 14640732] (i) Dennany L, Forster RJ, White B, Smyth M, Rusling JF. *J Am Chem Soc* 2004;126:8835–8841. [PubMed: 15250737]
8. Fluorescence-based kinetic analysis of 8-oxoG DNA glycosylase using tryptophan and 2-aminopurine as fluorophores has been reported; see: Kuznetsov NA, Koval VV, Nevinsky GA, Douglas KT, Zharkov DO, Fedorova OS. *J Biol Chem* 2007;282:1029–1038. [PubMed: 17090545]
9. (a) Oliveira Brett AM, Piedade JAP, Serrano SHP. *Electroanal* 2000;12:969–973. (b) Diculescu VC, Chiorcea Paquim A-M, Oliveira Brett A-M. *Sensors* 2005;5:377–393.
10. (a) Berger M, Anselmino C, Mouret JF, Cadet J. *J Liq Chromatogr* 1990;13:929–940. (b) Goyal RN, Dryhurst G. *J Electroanal Chem* 1992;135:75–91. (c) Yanagawa H, Ogawa Y, Ueno M. *J Biol Chem* 1992;267:13320–13326. [PubMed: 1618833]
11. (a) Xue L, Greenberg MM. *J Am Chem Soc* 2007;129:7010–7011. [PubMed: 17497789] (b) Xue L, Greenberg MM. *Angew Chem Int Ed* 2007;46:561–564.
12. Torimura M, Kurata S, Yamada K, Yokomaku T, Kamagata Y, Kanagawa T, Kurane R. *Anal Sci* 2001;17:155–160. [PubMed: 11993654]
13. A modified G-clamp has indeed been reported to be quenched by 8-oxoG. Its ability to detect the damaged base was limited to chloroform and micellar solutions, and no utility has been demonstrated in oligonucleotides. See: Nakagawa O, Ono S, Li Z, Tsujimoto A, Sasaki S. *Angew Chem Int Ed* 2007;46:4500–4503.
14. For other emissive C analogs, see: (a) Godde F, Toulme JJ, Moreau S. *Nucleic Acids Res* 2000;28:2977–2985. [PubMed: 10908362] (b) Wilhelmsson LM, Holmén A, Lincoln P, Nielsen PE, Nordén B. *J Am Chem Soc* 2001;123:2434–2435. [PubMed: 11456897] (c) Liu CH, Martin CT. *J Biol Chem* 2002;277:2725–2731. [PubMed: 11694519] (d) Okamoto A, Tainaka K, Saito I. *J Am Chem Soc* 2003;125:4972–4973. [PubMed: 12708835] (e) Berry DA, Jung KY, Wise DS, Serce AD, Pearson WH, Mackie H, Randolph JB, Somers RL. *Tetrahedron Lett* 2004;45:2457–2461. (f)

- Dash C, Rausch JW, Le Grice SFJ. *Nucleic Acids Res* 2004;32:1539–1547. [PubMed: 15004241]
- (g) Engman KC, Sandin P, Osborne S, Brown T, Billeter M, Lincoln P, Nordén B, Albinsson B, Wilhelmsson LM. *Nucleic Acids Res* 2004;32:5087–5095. [PubMed: 15452275]
- (h) Tinsley RA, Walter NG. *RNA* 2006;12:522–529. [PubMed: 16431979]
- (i) Marti AA, Jockusch S, Li ZM, Ju JY, Turro NJ. *Nucleic Acids Res* 2006;34:e50. [PubMed: 16595796]
- (j) Wojciechowski F, Hudson RHE. *J Am Chem Soc* 2008;130:12574–12575. [PubMed: 18761442]
15. Conjugating furan to a dU core has generated an emissive analog that positively responds to the presence of abasic sites in DNA; see: Greco NJ, Tor Y. *J Am Chem Soc* 2005;127:10784–10785. [PubMed: 16076156] Greco NJ, Tor Y. *Nature Protocols* 2007;2:305–316.
16. Srivatsan SG, Greco NJ, Tor Y. *Angew Chem Int Ed* 2008;47:6661–6665.
17. (a) Greco NJ, Tor Y. *Tetrahedron* 2007;63:3515–3527. [PubMed: 18431439] (b) Tor Y, Del Valle S, Jaramillo D, Srivatsan SG, Rios A, Weizman H. *Tetrahedron* 2007;63:3608–3614.
18. (a) Tor YSG. *J Am Chem Soc* 2007;129:2044–2053. [PubMed: 17256858] Srivatsan SG, Tor Y. *Tetrahedron* 2007;63:3601–3607. [PubMed: 18431440] (b) Srivatsan SG, Tor Y. *Nature Protocols* 2007;2:1547–1555.
19. Li SN, Piccirilli JA. *J Org Chem* 2004;69:4751–4759. [PubMed: 15230598]
20. See Supporting Information for experimental details.
21. Note that, although relatively low, this is easily detected by standard benchtop fluorimeters with excellent signal to noise.
22. Reichardt C. *Chem Rev* 1994;94:2319–2358.
23. Fluorescence spectra were corrected according to: $I(v) = \lambda^2 I(\lambda)$. See Lakowicz, J. R. *Principles of fluorescence spectroscopy*, 2nd ed., Kluwer Academic/Plenum, New York, 1999.
24. (a) Aboulela F, Koh D, Tinoco I Jr. *Nucleic Acids Res* 1985;13:4811–4824. [PubMed: 4022774] (b) Allawi HT, SantaLucia J Jr. *Nucleic Acids Res* 1998;26:2694–2701. [PubMed: 9592156] (c) Hobza P, Sponer J. *Chem Rev* 1999;99:3247–3276. [PubMed: 11749516]
25. While individual steps (e.g., G to 8oxoG, or 8OxoG to T) can be monitored using appropriate calibration, to globally follow this pathway, an independent method, estimating the relative damaged/ mutant population, might be needed.

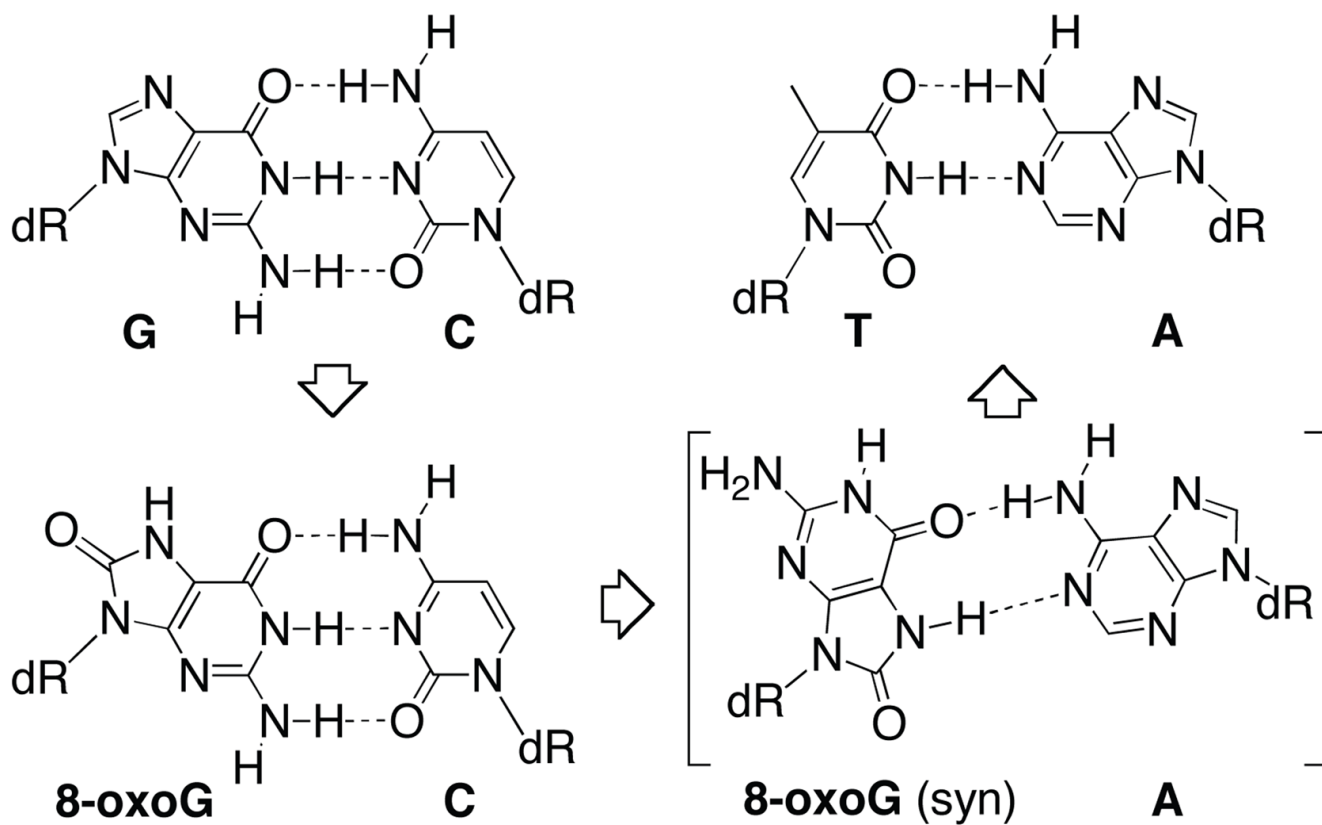


Figure 1.
Base pairing along the DNA damage pathway from G•C to T•A, (its transverse mutation product) via 8-oxoG.

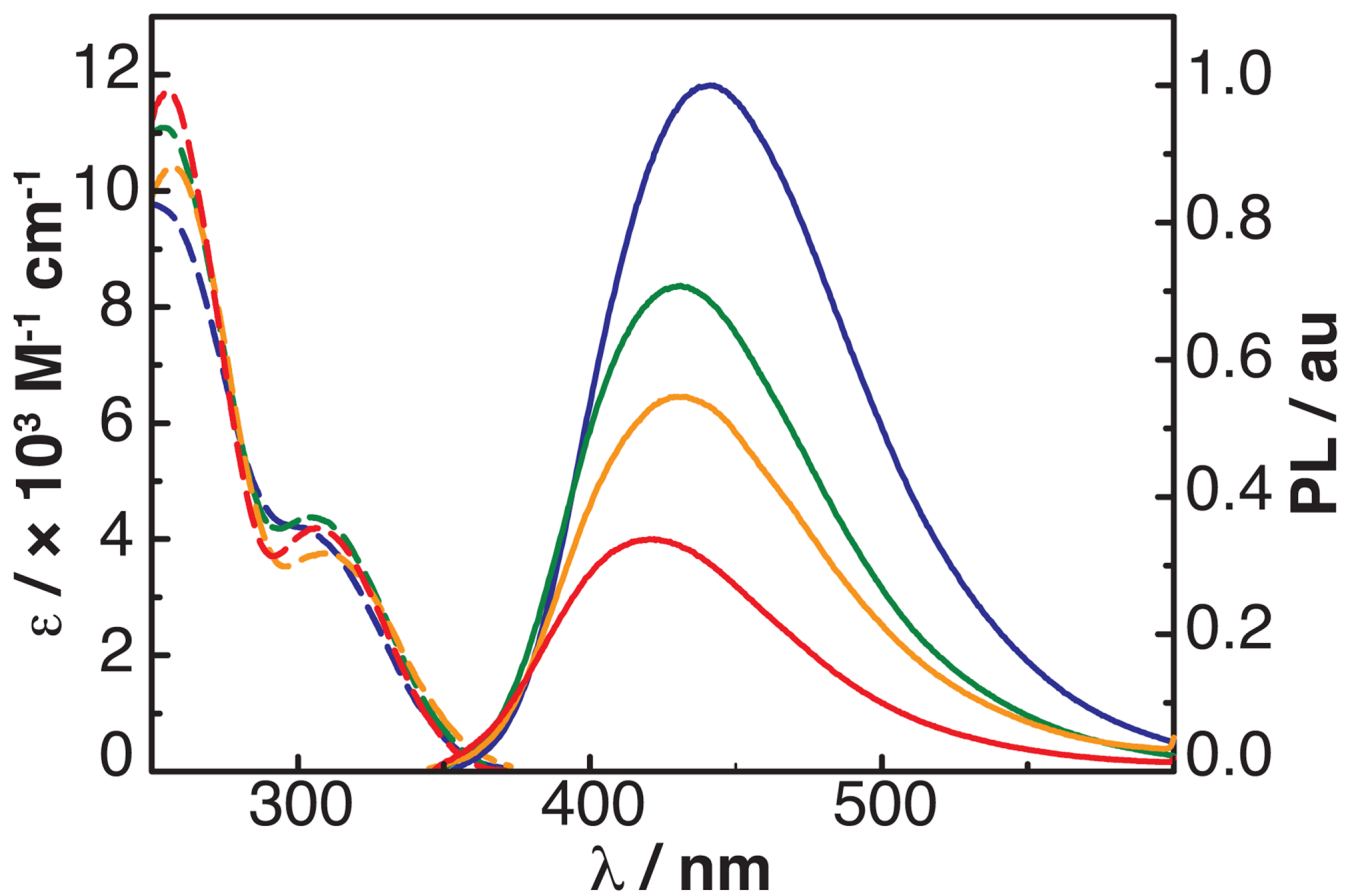


Figure 2. Absorption (dashed) and emission (solid) spectra of **5** in water (blue), methanol (green), dichloromethane (orange) and dioxane (red) at 2.4×10^{-5} M.

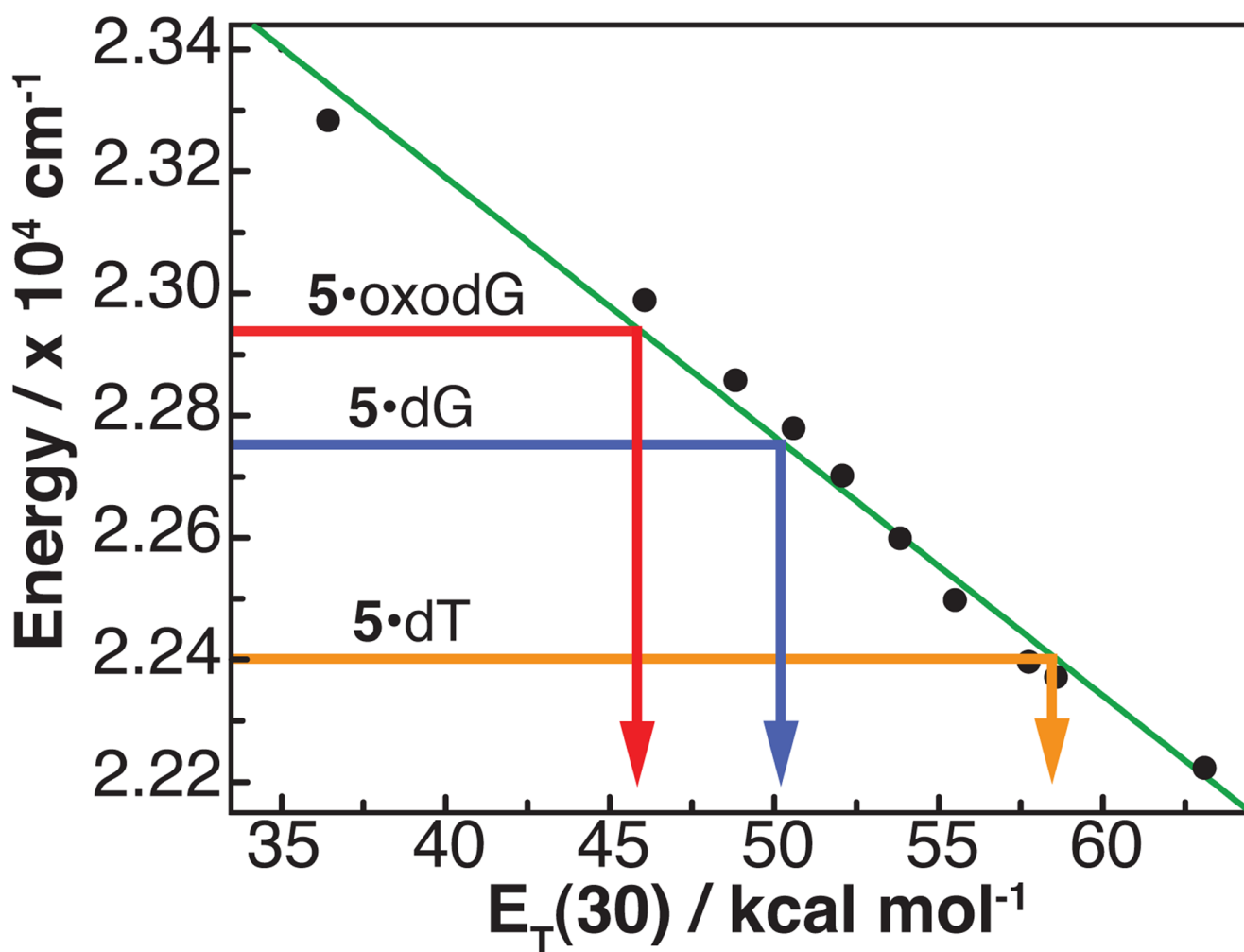


Figure 3. Correlating emission wavelengths with microscopic polarity $E_T(30)$ ²² for nucleoside **5** (filled circles and green line)²⁰ and interpolation of corrected emission maxima²³ of duplexes containing **5** (arrows).

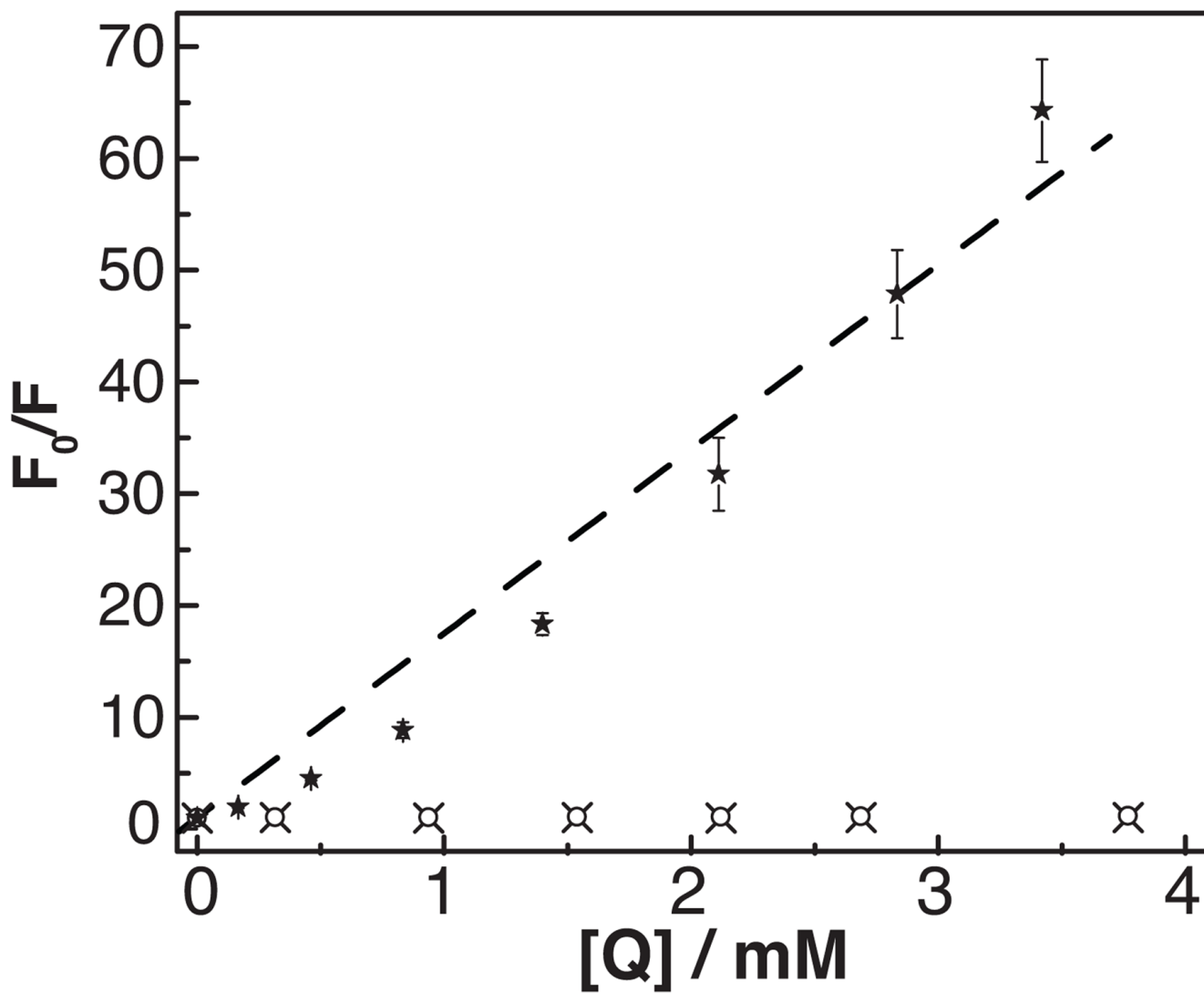


Figure 4. Steady-state Stern-Volmer plot for the titration of **5** with 8-oxo-2'-deoxyguanosine (stars, black dashed line), dGMP (X) and TMP (open circles). Error bars (dGMP and TMP) and data (dAMP and dCMP) have been omitted for clarity (Figure S3.1, Table S3.1).²⁰

11 5' – GCG – ATG – **5GT** – AGC – G – 3'
12 5' – CGC – TAC – **GCA** – TCG – C – 3'
13 5' – CGC – TAC – **YCA** – TCG – C – 3'
14 5' – CGC – TAC – **TCA** – TCG – C – 3'
15 5' – GCG – ATG – **CGT** – AGC – G – 3'

Figure 5.
Oligonucleotide sequences where Y = 8-oxoG.

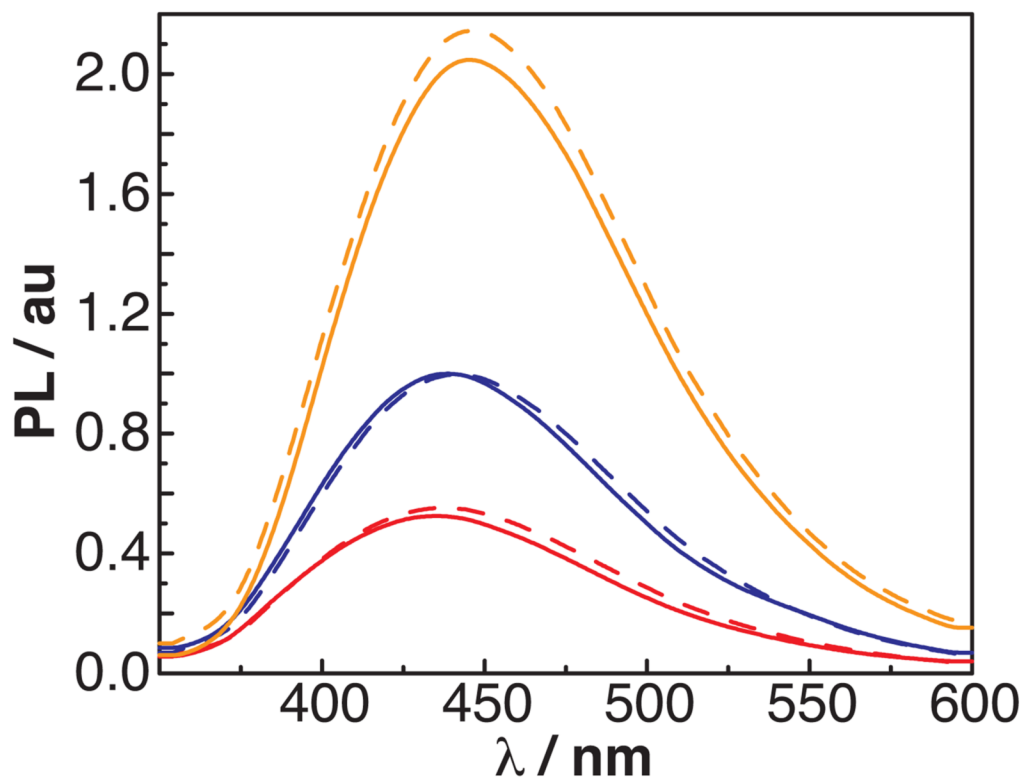
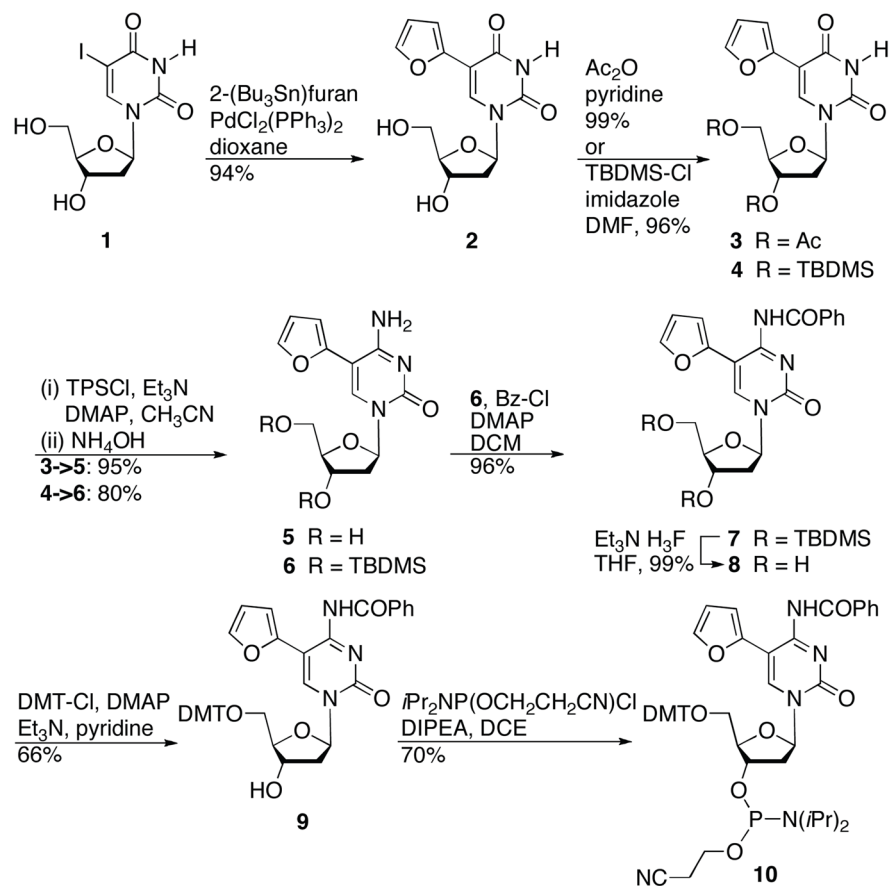


Figure 6. Steady state emission spectra of oligonucleotides **11•12** (5•G - blue), **11•13** (5•8-oxoG - red) and **11•14** (5•T - orange) at 5.0×10^{-6} M in 1.0×10^{-2} M phosphate aqueous buffer pH = 7.0 containing 1.0×10^{-1} M NaCl (solid) and at 4.6×10^{-6} M in 1.0×10^{-2} M phosphate aqueous buffer pH = 7.0 containing 5.0×10^{-1} M NaCl (dashed).²⁰



Scheme 1.
Synthesis of furan dC analog **5** and its corresponding amidite.

Table 1
Photophysical properties of nucleoside **5** in various solvents.²⁰

Solvent	λ_{abs} (nm)	λ_{em} (nm)	Φ	<i>I</i> normalized
Water	310	443	0.020	1.00
Methanol	305	439	0.011	0.70
Dichloromethane	309	439	0.009	0.54
Dioxane	309	421	0.006	0.33

Table 2
Thermal denaturation of control and modified oligonucleotides.

Duplex ^a	$T_m(\Delta T_m^b)$ (°C) 100 mM NaCl	$T_m(\Delta T_m^b)$ (°C) 500 mM NaCl
15•12	51.8	59.2
15•13	47.7	55.2
15•14	34.5	41.2
11•12	51.5 (-0.3)	59.0 (-0.2)
11•13	47.1 (-0.6)	54.5 (-0.7)
11•14	34.7 (+0.2)	42.0 (+0.8)

^a 1.0×10^{-6} M duplex DNA aqueous buffer pH = 7.0.

^b ΔT_m = modified = unmodified.

Table 3

Emission maxima of modified duplexes in phosphate buffers at different NaCl concentrations.

Duplex	λ_{em}/nm (cm^{-1}) 100 mM NaCl	Intensity at λ_{em}/au	λ_{em}/nm (cm^{-1}) 500 mM NaCl	Intensity at λ_{em}/au
11•12	439 (22,779)	1.00	440 (22,727)	1.00
11•13	435 (22,989)	0.52	437 (22,883)	0.55
11•14	446 (22,422)	2.05	447 (22,371)	2.15