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One-Electron Reduction Potential of the Neutral Guanyl Radical in the GC Base Pair of Duplex DNA

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Abstract: The one-electron oxidation of guanine in the GC base pair of DNA has been investigated using pulse radiolysis combined with DFT calculations. Reaction of benzotriazinyl radicals with DNA results in the formation of the neutral guanyl radical and redox equilibria. The one-electron reduction potential, E_7, of the neutral guanyl radical in the GC base pair is determined for the first time as 1.22 ± 0.02 V, from both absorption and kinetic data.

Introduction

The one-electron oxidation of guanine bases (G) is widely taken as giving rise to the prominent sites of oxidative damage (holes) in duplex DNA.[1,2] The full characterization of the energetics underpinning this phenomenon is of fundamental importance to the fields of health-related problems arising from oxidative damage to the genome, and molecular constructs in nanotechnology.[3,4] Purine bases are more easily oxidized than pyrimidine bases, as evidenced by gas phase ionization potentials[5] and the one-electron reduction potentials, E_7, of the base radicals in neutral aqueous solution.[6−9] The most widely quoted values of E_7 for the radicals of the individual DNA nucleosides, arise from the studies of Steenken and Jovanovic,[9] where pulse radiolysis was used to establish redox equilibria with reference standards within a few tens of μs, thereby avoiding radical losses and side reactions. The determined E_7 values of the guanyl radical (1.29 V) and adenyl radical (1.42 V) are compared to the recalculated values for the cytosyl radical ($\sim$1.6 V) and thymidyl radical ($\sim$1.7 V), based on earlier measurements of the relative redox differences between the base radicals at high pH.[10] The guanyl radical cation, $G^{\bullet+}$ (formed on the one-electron oxidation of deoxyguanosine), has a radical pK_a of 3.9,[11] and it has been postulated that, following formation of $G^{\bullet+}$ in the GC base pair, the proton is stabilized by its partnered cytosine base (C), which has a pK_a for its protonation of 4.3.[12] However, it is the uncharged guanyl radical, $G(-H^+)^{\bullet}$ which has been observed in duplex DNA, by EPR[13,14] and in pulse radiolysis and laser flash photolysis studies[15−17] in neutral aqueous solution, implying that a proton is lost rapidly from $G^{\bullet+}$. Also, kinetic isotope effect studies have provided evidence for a deprotonation reaction taking place upon one-electron oxidation of the GC pair.[18,19] Rapid formation of the $G^{\bullet+}$ species, upon reaction of the sulfate radical, $SO_4^{\bullet-}$ (a very strong oxidant, $E = 2.43$ V[20]) with a duplex oligonucleotide, followed by its deprotonation on the 50 ns time scale, has been reported to be observed.[15] Only partial absorption spectra, which must be compromised by the absorption of the $SO_4^{\bullet-}$ radical and its nonselectivity, are presented by these authors without extinction coefficients. We have proposed that the proton is lost from the amino group of its partnered C as this moiety is exposed to solvent water.[16] Our interpretation of this system is that the rapid loss of a proton from the duplex oligonucleotide is likely to be from the cytosyl radical following initial oxidation of C by its partnered guanyl radical cation.[16] Ab initio calculations indicate that the GC base pair is more easily oxidized than the adenine-thymine, AT, base pair[21] and in a GC pair the E_7 of the cytosyl radical is raised while that of the guanyl radical is lowered.[21−23] Voltammetric

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Chart 1. Oligonucleotides and Redox Indicators

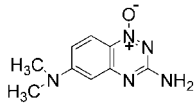
Duplex 1

5'-TGTGTTGTGT
ACACAACACA

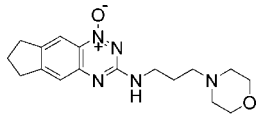
Duplex 2

5'-TGACTAGTCA
ACTGATCAGT

SN 28514



SN 29433



studies in non aqueous solvents on the effect of GC base pairing, report that there is a lowering of the oxidation potential of guanine (by 0.34 V),[24] however there are no reports of direct measurements in aqueous solution. An E_7 value of 1.39 V for the guanyl radical in plasmid DNA has been reported,[25] however this is not a directly measured value and relies on several assumptions and an enzymatic reaction. The value is far higher than that known for the guanyl radical of guanosine and goes against all theoretical predictions for the guanyl radical in the GC pair, which predict a decrease in potential.[21−23] To ensure selectivity in producing the guanyl radical over other base radicals in duplex DNA irradiated solution, we reason that (i) the radiolytically produced oxidizing radicals oxidize the redox indicators rather than the DNA, and (ii) the redox indicator radicals need to possess E_7 values lower than that of the guanyl radical. Our studies on the properties of benzotriazinyl radicals (BTZ)[26,27] have identified examples which are well suited for such studies, their E_7 values being in the region of $\leq$1.2 V. These redox indicators are used to establish redox equilibria with the guanyl radicals in the GC base pair of calf thymus (ct)DNA and two duplex 10-mer oligonucleotides.

Experimental Section

Materials. All chemicals and calf thymus DNA were obtained from Sigma Aldrich and used as supplied. The oligonucleotides obtained as desalted products from Alpha DNA, Montreal, Canada, were annealed into their duplex forms in 5 mM phosphate buffer, pH 7, by warming to 85 °C for 10 min and cooling to room temperature overnight. Melting curves for the 10-mer duplexes **1** and **2**, Chart 1, were obtained by measuring the changes in absorbance at 260 nm with temperature under the experimental conditions of high ionic strength solutions (see Supporting Information). Millipore Milli-Q water was used throughout. The redox indicator, Chart 1, SN 28514, *N*6,*N*6-dimethyl-1,2,4-benzotriazine-3,6-diamine 1-oxide, was prepared as described previously,[28] and the redox indicator SN 29433, *N*-[3-(4-morpholinyl)propyl]-7,8-dihydro-6*H*-indeno[5,6-*e*][1,2,4]triazin-3-amine 1-oxide was prepared as described in the Supporting Information.

Pulse Radiolysis. Time-resolved optical absorption and kinetic experiments were carried out using a 4 MeV linear accelerator which delivered 200 ns electron pulses of typically 3 Gy dose. The radical detection system and method of dosimetry have been described. [29] The radiolysis of water produces well-characterized primary radical species and molecular products (μM per absorbed dose of 1 Gy (J kg^{-1}) given in parenthesis).

$$H_2O \longrightarrow e_{aq}^- (0.28) + {}^\bullet OH (0.28) + H^\bullet (0.055) + H_2 (0.04) + H_2O_2 (0.07) + H^+ (0.28) \quad (1)$$

The selenite radical, $SeO_3^{\bullet -}$, was produced by the reaction of e_{aq}^- with selenate ions, while scavenging the other primary radical with 2-methylpropan-2-ol to form an inert radical. The $SeO_3^{\bullet -}$ radical reacted with the redox reference compounds, R (R-NH_2), to produce their benzotriazinyl radicals, (R-HN•).

$$e_{aq}^- + SeO_4^{2-} \rightarrow SeO_3^{\bullet -} + 2OH^- \quad (2)$$

$$^\bullet OH(H^\bullet) + (CH_3)_3COH \rightarrow {}^\bullet CH_2(CH_3)_2COH + H_2O(H_2) \quad (3)$$

$$SeO_3^{\bullet -} + R{-}NH_2 \rightarrow SeO_3^{2-} + (R{-}HN^\bullet, H^+) \quad (4)$$

Radical spectra are presented as the change in extinction coefficient vs wavelength, relative to the nonirradiated solution, by converting the changes in absorption due to the radical yield of the $SeO_3^{\bullet -}$ radical (0.28 μM Gy^{-1}). Redox equilibria were established to determine the one-electron reduction potentials. The ΔE values were calculated from the equilibrium constants, K, eq 5, using the Nernst equation and allowing for ionic strength effects.[30]

$$(R{-}HN^\bullet) + DNA(GC) \rightleftarrows R(R{-}NH_2) + DNA(G^\bullet C) \quad (5)$$

Density Functional Theory Calculations. Unrestricted DFT calculations were carried out using the Gaussian 03 revision C.02 software package.[31] The nonlocal UB3LYP functional hybrid method was employed with the standard 6-31G(d,p) basis set, which was previously reported to provide sufficient accuracy for DNA base molecules.[32−34]

Results and Discussion

Calibration of Redox Indicators. The reference compounds used in this study were standardized against the radical cation of 1,2,4-trimethoxybenzene (TMB). This redox indicator, which possesses a well-established E_7 value of 1.13 $\pm$ 0.01 V,[35] was also used in the seminal studies of Steenken and Jovanovic[9] to determine the E_7 of guanosine. (While TMB is an excellent redox indicator for determining the E_7 of guanosine, electron transfer reactions between it and the GC pair in duplex DNA

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Table 1. Equilibria, Kinetic Data, and One-Electron Reduction Potentials of Radicals in the GC Base Pair in Duplex DNA

duplex DNA	redox[a] indicator	K_{eq}[b]	$10^{-7}\ k_f$ / $M^{-1}s^{-1}$	$10^{-7}k_r$ /$M^{-1}\ s^{-1}$	K_{kin}[c]	ΔE /mV	$E_7(G^{\bullet},H^+/G)$ /V[d]
5′-TGTGTTGTGT ACACAACACA**1**	SN 28514	0.05 ± 0.01	0.51 ± 0.1	17.7 ± 4.8	0.04 ± 0.01	80 ± 3	1.24 ± 0.02
5′-TGACTAGTCA ACTGATCAGT **2**	SN 28514	0.12 ± 0.01	1.10 ± 0.1	13.5 ± 1.8	0.08 ± 0.01	56 ± 2	1.22 ± 0.02
ctDNA	SN 28514	0.15 ± 0.03	3.3 ± 0.2	26.6 ± 5.3	0.12 ± 0.01	51 ± 2	1.21 ± 0.02
	SN 29433	0.09 ± 0.04	2.10 ± 0.5	17.2 ± 4.6	0.12 ± 0.01	58 ± 7	1.20 ± 0.02

[a] SN 28514: *N*6,*N*6-dimethyl-1,2,4-benzotriazine-3,6-diamine-1-oxide; $E_7(R^{\bullet+}/R) = 1.16 \pm 0.01$ V. SN 29433: 3-[3-(4-morpholinyl)propyl]-7,8-dihydro-6*H*-indeno[5,6-*e*][1,2,4]triazine-1-oxide; $E_7(R^{\bullet+}/R) = 1.14 \pm 0.01$ V. [b] K_{eq} values were calculated using the percentage of GC present in each duplex DNA (42% in ctDNA and 40% in **1** and **2**). [c] K_{kin} is a ratio of the forward, k_f, and reverse, k_r, rate constants. [d] Data obtained at 20 °C.

were found to be too slow for accurate equilibria data to be obtained). The E_7 of the benzotriazinyl radical (BTZ) of SN 29433 (Table 1) was determined as a function of ionic strength (0.035−0.155 M) in solutions of Na_2SeO_4 (25−50 mM), $NaClO_4$ (0−0.1 M), 2-methyl-2-propanol (0.2 M) and sodium phosphate (5 mM, pH 7.0). The $SeO_3^{\bullet-}$ radical reacts with SN 29433 to generate the transient absorption spectrum of the benzotriazinyl radical with a λ_{max} at 360 nm and a molar extinction ∼ 9500 $M^{-1}cm^{-1}$, which was used to measure the equilibrium constant, K (Methods Section). Three ratios in concentration (75 μM − 200 μM) of SN 29433 and TMB were used to establish redox equilibria at different ionic strengths. The E_7 value of the radical was found to vary from 1.18 ± 0.01 V at ionic strength 0.155, which equals the ionic strength used in the redox studies with DNA, to an extrapolated value of 1.14 ± 0.01 V at zero ionic strength. The E_7 of the benzotriazinyl radical of SN 28514 (Table 1) has been similarly determined as 1.16 ± 0.01 V.[28]

Radical Absorption Spectra and Redox Equilibria. All experiments were performed in solutions of high ionic strength (0.155) at 20 °C, to ensure that the duplexes (and ctDNA) are in their duplex form.[36] The BTZ radicals were produced in solutions containing both the reference compounds, R (30−80 μM) and DNA (0.5−4.4 mM, in GC base pairs). The $SeO_3^{\bullet-}$ radicals react with a high rate constant of 5×10^9 M^{-1} s^{-1} with R, similar to that for the $SO_4^{\bullet-}$ radical,[26] but with a lower rate constant of 3×10^7 M^{-1} s^{-1} with DNA.[37] This means that the $SeO_3^{\bullet-}$ radicals initially react with both R and the G of GC pairs of DNA in the equilibrium mixtures, however by *ca*. 5 μs after the pulse we almost exclusively observe the full yield of the BTZ radicals. A possible reason for this observation is that the guanyl radical cation, transiently formed on the reaction of the $SeO_3^{\bullet-}$ radical with G or, as we have proposed, its rapidly formed partnered cytosyl radical,[16] quickly oxidizes R. Due to the time taken for the $SeO_3^{\bullet-}$ radical to react with DNA, we could not study these possible faster reactions using our experimental setup. Radical absorption spectra and oscilloscope traces obtained when using the BTZ radical of SN 28514 as the one-electron oxidant of ctDNA and the two duplexes are presented in Figures 1−3. The initial absorption spectrum of the reference BTZ radical decayed to composite spectra, monitored at *ca*. 50 μs after the pulse, which exhibit mixtures of the guanyl radical (peaks at 310 and 390 nm)[11,38] and the BTZ radical. This is understandable as the E_7 values of the reference radicals are lower than the radical potentials of the free DNA bases, strongly favoring their interaction with G over the other DNA bases. The resultant spectra in Figures 1−3 also display weak absorption in the >650 nm region indicating that

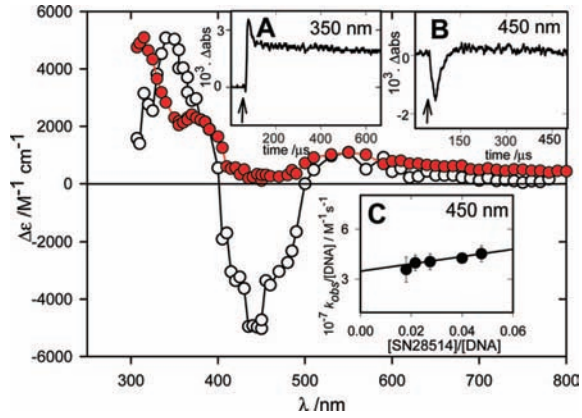

Figure 1. Spectral changes at (O) 5 μs and (●) 50 μs following pulse radiolysis (3 Gy, arrow) of N_2-saturated aqueous solution of ctDNA (0.84 mM in GC base pairs) and SN 28514 (30 μM) containing Na_2SeO_4 (25 mM), $NaClO_4$ (0.1 M), 2-methyl-2-propanol (0.2 M) and phosphate buffer (5 mM, pH 7.0). (A) and (B) Kinetic traces observed. (C) Kinetic analysis plot of radical transfer.

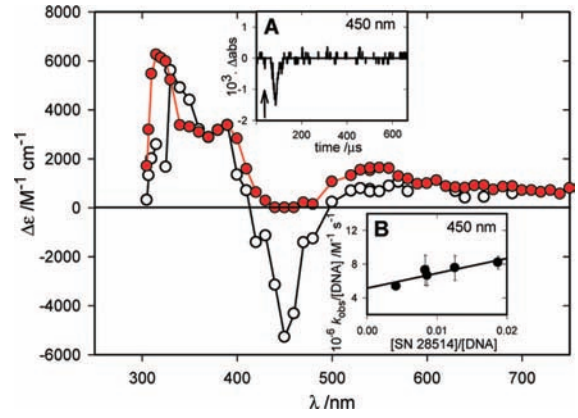

Figure 2. Spectral changes at (○) 5 μs and (●) 50 μs following pulse radiolysis (3 Gy, arrow) of N_2-saturated aqueous solution of Duplex **1** (4.4 mM in GC base pairs) and SN 28514 (40 μM) containing Na_2SeO_4 (25 mM), $NaClO_4$ (0.1 M), 2-methyl-2-propanol (0.2 M) and phosphate buffer (5 mM, pH 7.0). (A) Kinetic trace observed and (B) kinetic analysis plot of radical transfer.

the neutral guanyl radical is being formed as both experimental[11] and theoretical[39] evidence reveal that the guanyl radical cation does not absorb in this region whereas the neutral guanyl radical does. Redox equilibria were also established between the BTZ radical of SN 29433 (1.14 V) and ctDNA, but proved to be not practicable for the two duplexes (too high a concentration of the duplex is required for establishing redox equilibria) in comparison to the BTZ radical of SN 28514 (1.16 V). The redox equilibrium constants, K_{eq}, were determined from the absorbance at equilibrium for different ratios in concentration of each DNA

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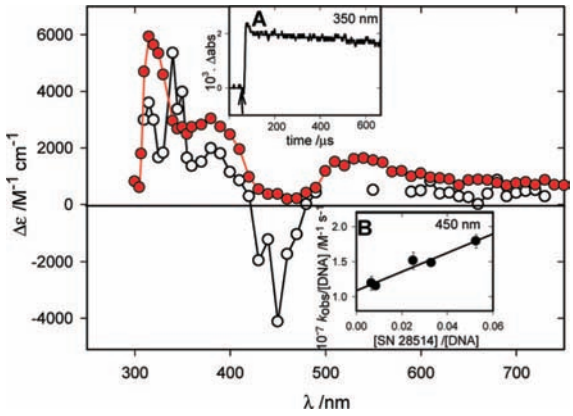


Figure 3. Spectral changes at (○) 5 μs and (●) 50 μs following pulse radiolysis (3 Gy, arrow) of N_2-saturated aqueous solution of Duplex **2** (4.4 mM in GC base pairs) and SN 28514 (30 μM) containing Na_2SeO_4 (25 mM), $NaClO_4$ (0.1 M), 2-methyl-2-propanol (0.2 M) and phosphate buffer (5 mM, pH 7.0). (A) Kinetic trace observed and (B) kinetic analysis plot of radical transfer.

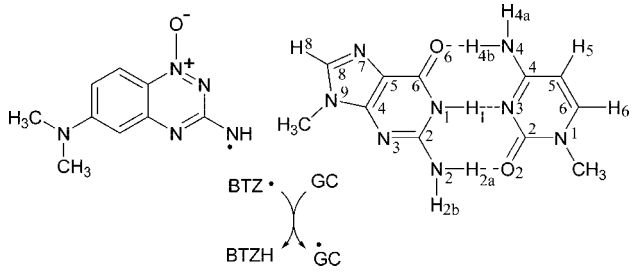


Figure 4. BTZ radical of SN 28514 and the model GC structure with numbering scheme, used for DFT calculations.

and R and corrected for ionic strength. (The concentrations of the GC pairs in each duplex and ctDNA were calculated from their percentages of composition). The obtained ΔE values and the E_7 of the guanyl radical in the GC base pair, are presented in Table 1. Kinetic equilibrium constants, K_{kin} (= intercept/slope), were also obtained from plots of the ratio of the rate of approach to equilibrium to concentration of DNA vs the ratio of concentrations, for each R and duplex DNA pair (Inserts to Figures 1−3). The K_{kin} data agree closely with the K_{eq} data with errors, mainly arising from extrapolation in the kinetic plots,

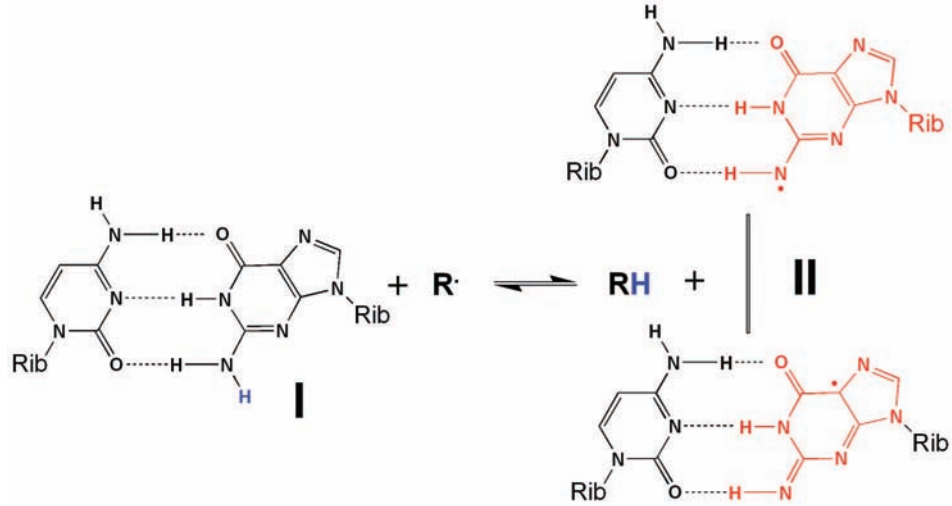
Table 2. Energetics for H Abstraction from Different Sites (as in Figure 4) in the GC Pair by Benzotriazinyl Radical, Calculated Using DFT UB3LYP/6-31G(d,p)

reaction site	$\Delta_rH°$(298 K)/kcal mol^{-1}	$\Delta_rG°$(298 K)/kcal mol^{-1}
H_{4a}	8.8	9
H_{4b}	19.7	19.5
H_1	20.6	22.3
H_{2a}	2.2	1.9
H_{2b}	−2.7	−1.9
H_{4a} (lone Cyt)	1	1
H_{2b} (lone Gua)	−9	−9.2

being of the same order as those for K_{eq}, Table 1. The obtained data, together with averaged E_7 values derived from K_{eq} and K_{kin} give a value for E_7(G$^{\bullet}$, H$^+$/G), in ctDNA of 1.21 V, Table 1. There are no significant differences in E_7 values measured for ctDNA and the two oligonucleotides. Hence, the small percentage of multi-G sites in ctDNA do not appear to have a large lowering effect on the E_7 value. Overall, the E_7 of the guanyl radical in the GC pair of 1.22 ± 0.02 V is lower than the value for the uncomplexed nucleoside of 1.29 V (or 1.26 V from data using only TMB as the reference indicator). [9]

DFT Calculations. In DNA, the moieties most likely to undergo reaction with oxidizing radicals are the amino groups of the bases, which are exposed to solvent in the DNA grooves. With this in mind, we have performed DFT calculations on the series of possible radicals formed upon H-atom abstraction from the GC pair. Abstraction of an H-atom from the amine substituent on guanine, k_f eq 5, proved feasible in thermodynamic terms, Figure 4 and Table 2. The resulting guanyl radical is stabilized (compared to single G(-H$^+$)$^{\bullet}$, see Supporting Information) and it has a lifetime long enough for redox equilibrium to be established with the reference compounds. Radical transformation resulting in concomitant bond rearrangement to remove the central hydrogen bond of the GC pair, is unlikely on energetic grounds (see Supporting Information). Calculations show that H-atom abstraction by the BTZ radical of SN 28514 is energetically favorable at the terminal H-atom of the amino group on G in the minor groove (H_{2b}, Figure 4), with the Gibbs free energy change of the reaction $\Delta_rG°$(298 K) = −1.9 kcal mol^{-1}. This leads to the formation of radical **II**, which is best represented by mesomeric structures (Scheme 1). This radical retains intramolecular hydrogen bonding of the GC pair. From Table 2 it is evident that abstraction of H_1 is unlikely,

Scheme 1. Proposed Radical Pathway and Redox Equilibrium for the Reaction of Benzotriazinyl Radicals (R•) and the GC of Duplex DNA



not only for steric reasons (stacked base pairs in DNA), but also for energetic reasons. Assuming that the radical formed with H_1 missing from the GC pair is a true transition state, we calculate a prohibitive energy of activation of 22.3 kcal mol^{-1}. Overall, for H_1 abstraction by the BTZ radical followed by slippage of the GC base pair,[40,41] we calculate a small $\Delta_r G°$(298 K) of +0.3 kcal mol^{-1}. Abstraction of H_{2a} cannot be excluded from consideration as the value $\Delta_r G°$(298 K) is +1.9 kcal mol^{-1}, which considering the typical 1−2 kcal mol^{-1} deviation from experiment in theoretical calculations, might well equate to zero or a small negative value in DNA. This site is still accessible from the DNA groove, and abstraction would remove one of the intramolecular hydrogen bonds, potentially leading to a different mechanism - guanine radical tautomerization[42] where H_1 takes the place of H_{2a} in the guanine amino group, whereas the spin distributes mainly on the ring system.

Conclusions

In the present study, the BTZ radicals, of $E(1)$ values 1.14 and 1.16 V, reacted with DNA to give the observable neutral guanyl radical on the short time scale (ca. 50 μs). Previously, only oxidizing radicals of considerably higher reduction potential ($\geq$ *ca.*1.6 V) have been found to have appreciable rate constants in oxidizing duplex DNA, [43] with the transient formation of the guanyl radical cation by electron transfer. [15] This raises the possibility that the BTZ radicals react with DNA by a different mechanism, and our DFT calculations point to H-atom abstraction from the solvent exposed amine substituent on the guanine. However, radical addition to guanine followed by a fast elimination reaction, or proton-assisted electron transfer between the species, cannot be ruled out. The redox equilibria obtained in this study give a value of 1.22 ± 0.02 V for the neutral guanyl radical in the GC pair, which is lower than that found for the uncomplexed nucleoside and is in line with the trend previously predicted by theoretical studies. [21−23]

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Supporting Information Available: Synthesis details for SN 29433, melting curves for duplex oligonucleotides **1** and **2**, DFT-calculated parameters, and the full citation for ref 31. This material is available free of charge via the Internet at http://pubs.acs.org.

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