Base-pairing preferences, physicochemical properties and mutational behaviour of the DNA lesion 8-nitroguanine[†]

Inder Bhamra[1], Patricia Compagnone-Post[2], Ian A. O'Neil[1], Lesley A. Iwanejko[3], Andrew D. Bates[4],* and Richard Cosstick[1],*

[1]Department of Chemistry, University of Liverpool, Crown Street, Liverpool L69 7ZD, UK, [2]Department of Biology and Chemistry, Albertus Magnus College, New Haven, CT 06511-1189, USA, [3]Institute of Ageing and Chronic Disease, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK and [4]Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK

ABSTRACT

8-Nitro-2′-deoxyguanosine (8-nitrodG) is a relatively unstable, mutagenic lesion of DNA that is increasingly believed to be associated with tissue inflammation. Due to the lability of the glycosidic bond, 8-nitrodG cannot be incorporated into oligodeoxynucleotides (ODNs) by chemical DNA synthesis and thus very little is known about its physicochemical properties and base-pairing preferences. Here we describe the synthesis of 8-nitro-2′-*O*-methylguanosine, a ribonucleoside analogue of this lesion, which is sufficiently stable to be incorporated into ODNs. Physicochemical studies demonstrated that 8-nitro-2′-*O*-methylguanosine adopts a *syn* conformation about the glycosidic bond; thermal melting studies and molecular modelling suggest a relatively stable *syn*-8-nitroG·*anti*-G base pair. Interestingly, when this lesion analogue was placed in a primer-template system, extension of the primer by either avian myeloblastosis virus reverse transcriptase (AMV-RT) or human DNA polymerase β (pol β), was significantly impaired, but where incorporation opposite 8-nitroguanine did occur, pol β showed a 2:1 preference to insert dA over dC, while AMV-RT incorporated predominantly dC. The fact that no 8-nitroG·G base pairing is seen in the primer extension products suggests that the polymerases may discriminate against this pairing system on the basis of its poor geometric match to a Watson–Crick pair.

INTRODUCTION

The cause and effect relationship between inflammation and cancer is now well established, with evidence suggesting that more than 20% of human cancers are attributable to various types of chronic inflammation (1–3). Specific examples include: liver cancer, which is often the consequence of inflammation caused by chronic viral hepatitis; lung cancer, which results from inflammation caused by years of inhaled tobacco smoke, and the association between inflammatory stomach infections (such as *Helicobacter pylori*) and gastric cancer (4,5). The proposed molecular basis for this link is through over-expression of nitric oxide synthase in inflamed tissues, which leads to increased levels of reactive oxygen and nitrogen species (RNS) that damage cellular macromolecules (6,7). The guanine base in DNA, with its low oxidation potential, is particularly sensitive and a large number of guanine lesions, particularly 8-oxodeoxyguanosine (8-oxodG), have been characterized and studied in some detail (8,9). One such modification readily identified is 8-nitro-2′-deoxyguanosine (8-nitrodG); this lesion labilizes the glycosidic bond to release 8-nitroguanine, which can be detected in urine, and leaves an abasic site in the DNA (Scheme 1) (10,11).

Our understanding of how chemical modification of DNA generates specific mutations has advanced rapidly, largely due to the synthesis and study of oligonucleotides containing site-specific DNA lesions. However, the sensitivity of the 8-nitrodG glycosidic bond means that the synthesis of oligodeoxynucleotides (ODNs) containing 8-nitroguanine by a conventional chemical approach has not been achieved. However, there is growing evidence to suggest that this lesion is highly mutagenic and thus
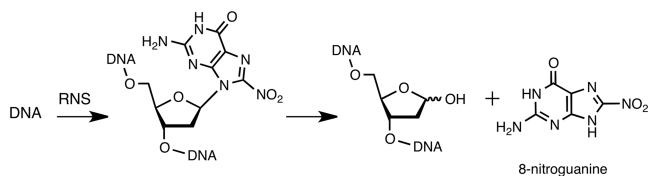
Scheme 1. Nitration of the guanine base in DNA by reactive nitrogen species and release of 8-nitroguanine to leave an abasic site in DNA.

warrants a detailed study of its base pairing and other physicochemical properties. Thus, in primer extension reactions with a template containing 8-nitrodG produced photochemically, Suzuki *et al.* showed that while DNA synthesis catalyzed by DNA polymerases α and β was partially blocked at the 8-nitrodG lesion, dA was incorporated opposite the lesion (equivalent to G·C to T·A transversion mutation) to a significant extent and a possible 8-nitroG·A base pairing scheme was suggested to explain this observation (12). However, these experiments were conducted with a template-primer system that readily releases 8-nitroguanine to leave abasic sites, which are known cause the same transversion mutation (13,14), although control experiments suggested that depurination was minimal during the experiment. Kaneko *et al.* also observed the same G·C to T·A mutation, together with increased levels of abasic sites, when a hamster cell line was treated with 8-nitroguanosine (15). Their results were consistent with mutations arising from the incorporation of 8-nitroguanine into DNA (through the salvage pathway), although in this case, the mutational effect was largely attributed to the formation of abasic sites. Despite the documented mutagenicity of this lesion (12,15), there is currently no information on how the physicochemical properties, such as pK_a, nucleoside conformation and base pairing preferences of dG are affected by introduction of the 8-nitro group. To obtain a better understanding of the mutagenic effect of this lesion, we now describe the synthesis of ODNs containing 8-nitro-2′-*O*-methylguanosine, in which the glycosidic bond is stabilized by a ribose sugar (16). The physicochemical properties of this lesion are reported and the data suggest that the most stable base pairing arrangement is an 8-nitroguanine—guanine pair. The mutational behaviour of this lesion has also been investigated in template-directed DNA synthesis using a template containing a stable 8-nitroguanine base, and DNA polymerase β prefers to insert dA opposite the lesion.

MATERIALS AND METHODS

2′-*O*-Methylguanosine was purchased from ChemGenes Corporation (Wilmington, USA), all reagents and standard nucleoside phosphoramidites for oligonucleotide synthesis were purchased from Link Technologies (Bellshill, Scotland). Unless otherwise stated, all other reagents and solvents for chemical synthesis were purchased from Sigma, TCI or Fisher Scientific and used as supplied.

Purified human DNA polymerase β was the kind gift of Dr Joann B. Sweasy, Yale University School of Medicine. Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) and T4 polynucleotide kinase were purchased from New England Biolabs (UK). [γ-^{32}P]ATP (6000 Ci mmol^{-1}) was supplied by Perkin Elmer. Deoxyribonucleotide triphosphates were supplied by Pharmacia. G-25 Sephadex mini Quick Spin Oligo columns were purchased from Roche Applied Science. ZipTip$_{C18}$ pipette tips were purchased from Millipore. Four 20-mer ODNs serving as DNA sequencing standards (sequence 5′–ATGTCGACTCCCAATG*N*TGA–3′, containing either A, T, C or G at position *N*) were synthesized and purified by Eurofins MWG Operon (UK).

Solid-phase oligonucleotide synthesis was carried out on a BioAutomation Corporation MerMade 4® synthesizer equipped with MerMade 12© v2.3.2 software. All syntheses were undertaken using 1.0 μmol, 500 Å controlled-pore glass (CPG) columns purchased from Link Technologies. HPLC was performed on an automated Gilson HPLC system equipped with an autoinjector, a photodiode array detector and dual hydraulic pump. Chromatographic data were controlled and processed using UniPoint© Version 3.0. All oligonucleotides were purified by reverse-phase using a Gemini™ C18 column (110 Å, 250 mm × 4.6 mm) purchased from Phenomenex using a triethylammonium bicarbonate (TEAB) buffer system. A 1 M stock solution of TEAB was prepared by bubbling CO_2 through a 1 M aqueous solution of Et_3N for 6–8 h until the pH measured 7.5–7.6. Stock solutions of TEAB were refrigerated until needed. All UV measurements were taken on a PerkinElmer Lambda 25 spectrophotometer equipped with a Peltier temperature controller and water circulator using standard quartz cuvettes with a 1 cm path length. TempLab 2.0 software was used to control heating and record absorbencies for thermal melting studies.

All NMR spectra were recorded on a Bruker 400 MHz spectrometer; ^{1}H (400 MHz), ^{13}C (100 MHz) and ^{31}P (162 MHz). All samples were run in deuterated chloroform (CDCl$_3$) or deuterated dimethyl sulfoxide (D$_6$-DMSO) as specified under Preparation of Nucleosides section. All chemical shifts are reported in p.p.m. and coupling constants (J) in Hz. ^{13}C and ^{31}P spectra were ^{1}H decoupled. ^{1}H and ^{13}C chemical shifts are relative to an internal standard of tetramethylsilane, ^{31}P spectra are relative to an external standard of 85% H_3PO_4. Mass spectra of nucleosides and chemically synthesized ODNs were recorded on a MicroMass LCT™ mass spectrometer using electrospray ionization (ES) and direct infusion syringe pump sampling. All small molecules were dissolved in methanol. Oligonucleotides were dissolved in water and analysed using negative mode ES ionization (ES-). Theoretical masses for oligonucleotides were calculated using a spreadsheet created in Microsoft® Excel. Mass spectra of primer extension assays were collected using a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, UK). The instrument was operated in a reflectron, positive ion mode. A panel of six oligonucleotides, in the M_r range 4400–7100, was used to calibrate the mass spectrometer. Baseline subtraction and peak detection were performed using Bruker Daltonics flexAnalysis software.

Preparation of nucleosides

2′,3′,5′-Tri-*O*-acetylguanosine (**2a**) and 8-bromo-2′,3′,5′-tri-*O*-acetylguanosine (**3a**) were prepared as previously described (17,18).

3′,5′-Di-*O*-acetyl-2′-*O*-methylguanosine (2b)

To a stirred suspension of 2′-*O*-methylguanosine (4.00 g, 13.50 mmol) in MeCN (50 ml) was added Et$_3$N (4.95 ml, 35.50 mmol) and DMAP (0.16 g, 1.35 mmol). The resulting suspension was cooled in an ice bath prior to addition of Ac$_2$O (3.19 ml, 33.75 mmol). Once the addition was complete the mixture was allowed to reach room temperature and was stirred for a further 2 h. The reaction was quenched by the addition of MeOH (5 ml) and concentrated *in vacuo* to a white semi-solid which was triturated with boiling ^{i}PrOH to afford the title product as a white solid (4.80 g, 93%). ^{1}H NMR (400 MHz, D$_6$-DMSO): δ (ppm) 10.70–10.80 (1H, bs, H1); 8.00 (1H, s, H8); 6.55–6.70 (2H, bs, C2 NH$_2$); 5.80–5.85 (1H, d, J = 7.6 Hz, H1′); 5.35–5.40 (1H, d, J = 5.0 Hz, H2′); 4.60–4.65 (1H, dd, J = 7.6 and 5.0 Hz, H3′); 4.20–4.35 (3H, m, H4′, H5′ and H5″); 3.25–3.27 (3H, s, 2′-OMe); 2.15–2.16 (3H, s, 3′-OAc); 2.05–2.06 (3H, s, 5′-OAc). ^{13}C NMR (100 MHz, D$_6$-DMSO): δ (ppm) 170.56, 169.99, 157.05, 154.28, 151.76, 135.50, 117.04, 84.48, 80.14, 80.11, 71.05, 63.74, 58.61, 21.01, 20.96. HRMS (ES+) (*m/z*): Found, 404.1181 [M+Na]$^+$; C$_{15}$H$_{19}$N$_5$O$_7$Na requires 404.1182.

8-Bromo-3′,5′-diacetyl-2′-*O*-methylguanosine (3b)

3′,5′-Di-*O*-acetyl-2′-*O*-methylguanosine (**2b**) (4.75 g, 12.48 mmol) was suspended in distilled water (30 ml). Saturated bromine water (ca. 20 ml) was added with vigorous stirring at room temperature at such a rate that the yellow colour dissipated between additions. Once the yellow colour persisted the mixture was allowed to stir for a further 30 min before filtering. The filter cake was washed with the liquors followed by ice cold ^{i}PrOH before drying under vacuum for 12 h to yield the title product as an off-white amorphous solid (5.20 g, 90%). R_f = 0.31 (15% MeOH in DCM). ^{1}H NMR (400 MHz, D$_6$-DMSO): δ (ppm) 10.90–10.95 (1H, s, H1); 6.50–6.65 (2H, bs, C2 NH$_2$); 5.75–5.80 (1H, d, J = 5.5 Hz, H1′); 5.50–5.55 (1H, m, H2′); 5.00–5.05 (1H, dd, J = 5.5 and 5.7 Hz, H3′); 4.35–4.40 (1H, dd, J = 3.4 and 10.9 Hz, H5′) 4.20–4.34 (2H, m, H4′ and H5″); 3.25 (3H, s, 2′-OMe); 2.15–2.16 (3H, s, 3′-OAc); 2.00–2.01 (3H, s, 5′-OAc). ^{13}C NMR (100 MHz, D$_6$-DMSO): δ (ppm) 170.54, 169.99, 155.81, 154.07, 152.45, 120.53, 117.62, 88.19, 79.95, 78.50, 71.18, 63.49, 58.66, 21.01, 20.94. HRMS (ES+) (*m/z*): Found, 482.0282 and 484.0267 [M+Na]$^+$; C$_{15}$H$_{18}$N$_5$O$_7$BrNa requires 482.0287 and 484.0267.

N2-Dimethoxytrityl-8-bromo-3′,5′-di-*O*-acetyl-2′-*O*-methylguanosine (4b) and N2-dimethoxytrityl-8-bromo-2′,3′,5′-tri-*O*-acetylguanosine (4a)

8-Bromo-3′,5′-di-*O*-acetyl-2′-*O*-methylguanosine (**3b**) (5.18 g, 11.30 mmol) was co-evaporated to dryness with

anhydrous pyridine before re-dissolving in anhydrous pyridine (35 ml). The resultant solution was stirred at room temperature under an atmosphere of N$_2$ and DMTCl (4.20 g, 12.40 mmol) was added in ca. 0.5 g portions over 5 min. The mixture was stirred for a further 8 h at room temperature before removing most of the pyridine under reduced pressure. The isolated sticky solid was partitioned between DCM (50 ml) and water (50 ml), the DCM layer was further washed with water (2 × 50 ml) and saturated aqueous NaHCO$_3$ (30 ml) before drying over Na$_2$SO$_4$, filtering and evaporating to a yellow solid. Flash column chromatography on silica gel (DCM, MeOH and Et$_3$N 19:1:0.1) led to isolation of product **4b** as a pale yellow solid (6 g, 70%). R_f = 0.21 (4:6 EtOAc and DCM). ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 11.10–11.45 (1H, bs, H1); 7.65–7.80 (1H, bs, C2 NH); 7.05–7.45 (9H, m, DMT Ar-H); 6.75–6.90 (4H, m, DMT Ar-H); 5.55–5.60 (1H, d, J = 6.4 Hz, H1′); 5.05–5.10 (1H, m, H2′); 4.15–4.20 (1H, m, H3′); 4.05–4.10 (1H, m, H4′); 4.00–4.10 (1H, m, H5′); 3.78–3.80 (6H, s, 2 x Ar-OMe); 3.70–3.78 (1H, m, H5″); 2.80–2.82 (3H, s, 2′-OMe); 2.15–2.16 (3H, s, 3′-OAc); 2.05–2.06 (3H, s, 5′-OAc). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.75, 170.27, 158.88, 158.83, 152.32, 152.11, 130.34, 128.81, 128.38, 127.49, 122.57, 119.05, 113.77, 113.67, 88.87, 80.03, 71.46, 70.50, 63.23, 58.86, 55.54, 21.47, 21.21. MS (ES+) (*m/z*): Found, 784/786 [M+Na]$^+$ and 863/865 [M+Et$_3$NH]$^+$.

Compound (**4a**) was prepared in a similar fashion to compound (**4b**) using (**3a**) (1 g, 2.05 mmol), DMTCl (0.83 g, 2.49 mmol) and pyridine (10 ml). The (**4a**) was isolated as a pale yellow foam after flash column chromatography (CHCl$_3$ to 98:2 CHCl$_3$ and MeOH), (1.53 g, 94%). R_f = 0.48 (1:9 MeOH and DCM). ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 8.21–8.70 (1H, bs, H1); 7.21–7.35 (9H, m, DMT Ar-H); 6.86–6.90 (1H, bs, C2 NH) 6.79–6.87 (4H, m, DMT Ar-H); 6.03–6.08 (1H, m, H1′); 5.84–5.89 (2H, m, H2′ and H3′); 4.26–4.37 (2H, m, H4′ and H5′); 4.16–4.22 (1H, dd, J = 5.9 and 11.4 Hz, H5″); 3.78–3.80 (6H, s, 2 x Ar-OMe); 2.11–2.12, 2.05–2.06 and 1.98–1.99 (9H, 3 x s, 2′,3′,5′-OAc). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.91, 169.93, 169.71, 159.37, 152.21, 151.77, 135.48, 130.16, 128.99, 128.59, 128.12, 118.70, 114.35, 88.12, 79.35, 72.08, 70.70, 70.69, 63.28, 55.67, 21.08, 20.99, 20.87. HRMS (ES+) (*m/z*): Found, 812.1512 and 814.1514 [M+Na]$^+$ C$_{37}$H$_{36}$N$_5$O$_{10}$BrNa requires 812.1543 and 814.1514.

N2-Dimethoxytrityl-8-nitro-3′,5′-di-*O*-acetyl-2′-*O*-methylguanosine (5b) and N2-dimethoxytrityl-8-nitro-2′,3′,5′-tri-*O*-acetylguanosine (5a)

Protected bromoguanosine (**4b**) (2.00 g, 3.58 mmol), was dissolved in a mixture of DMF (5 ml) and 18-crown-6 (9.40 g, 35.80 mmol) heated to 60°C. KNO$_2$ (3.00 g, 35.80 mmol) was added and the resultant mixture was stirred at 100°C for 6 h under an inert atmosphere before cooling to room temperature and pouring directly into water (50 ml). The precipitate that formed was extracted with EtOAc (50 ml) and the aqueous solution was further extracted with EtOAc (2 × 50 ml). The

combined organic solutions were washed with saturated aqueous $NaHCO_3$ (50 ml), dried (Na_2SO_4), filtered and concentrated to a yellow foam. Flash column chromatography on silica gel (1:1 EtOAc and hexane) afforded the title product as a yellow amorphous solid (1.12 g, 43%). R_f = 0.44 (4:6 EtOAc and DCM). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 11.25–11.55 (1H, bs, H1); 8.05–8.20 (1H, bs, C2 NH); 7.05–7.45 (9H, m, DMT Ar-H); 6.75–6.90 (4H, m, DMT Ar-H); 5.55–5.60 (1H, m, H1′); 5.05–5.10 (1H, m, H2′); 4.20–4.25 (1H, m, H3′); 4.14–4.20 (1H, dd, J = 3.3 and 12.3 Hz, H5′); 4.00–4.08 (1H, dd, J = 6.0 and 9.7 Hz, H4′); 3.90–3.96 (1H, dd, J = 6.0 and 12.3 Hz H5″); 3.78–3.80 (6H, s, 2 x Ar-OMe); 2.80–2.82 (3H, s, 2′-OMe); 2.15–2.16 (3H, s, 3′-OAc); 2.05–2.07 (3H, s, 5′-OAc). ^{13}C NMR (100MHz, $CDCl_3$): δ (ppm) 170.54, 169.99, 159.14, 158.90, 158.87, 153.39, 152.29, 145.03, 139.38, 136.02, 130.63, 130.42, 129.55, 128.94, 128.19, 127.40, 115.38, 113.65, 113.52, 90.33 79.53, 79.03, 71.29, 70.66, 62.51, 58.60, 55.61, 21.47, 21.21. HRMS (ES+) (m/z): Found, 751.2346 [M+Na]$^+$; $C_{36}H_{36}N_6O_{11}Na$ requires 751.2340.

Compound (**5a**) was prepared from compound (**4a**) (1 g, 1.32 mmol), KNO_2 (1.11 g, 13.2 mmol), 18-Crown-6 (3.49 g, 13.2 mmol) and DMF (20 ml) in an identical manner to compound (**5b**) affording a yellow amorphous solid (0.36 g, 36%). R_f = 0.49 (4:6 EtOAc and DCM). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 11.06–11.50 (1H, bs, H1); 7.80–7.99 (1H, bs, C2 NH); 7.20–7.34 (9H, m, DMT Ar-H); 6.82–6.87 (4H, m, DMT Ar-H); 6.01–6.06 (1H, m, H1′); 5.83–5.90 (2H, m, H2′ and H3′); 4.26–4.39 (2H, m, H4′ and H5′); 4.16–4.24 (1H, dd, J = 5.9 and 11.5 Hz, H5″); 3.78–3.80 (6H, s, 2 x Ar-OMe); 2.11–2.12, 2.05–2.06 and 1.98–1.99 (9H, 3 x s, 2′,3′,5′-OAc). ^{13}C NMR (100MHz, $CDCl_3$): δ (ppm) 170.96, 169.75, 169.46, 158.96, 153.58, 152.41, 143.84, 130.50, 130.45, 129.54, 128.90, 128.37, 127.62, 115.52, 113.80, 88.81, 78.98, 72.37, 71.45, 69.47, 62.44, 55.62, 21.48, 21.02, 20.83. HRMS (ES+) (m/z): Found, 779.2292 [M+Na]$^+$; $C_{37}H_{36}N_6O_{12}Na$ requires 779.2289.

N2-Dimethoxytrityl-2′-O-methyl-8-nitroguanosine (7)

Protected 2′-O-methylnitroguanosine (**5b**) (0.43 g, 0.59 mmol) was dissolved in 7M NH_3 (anhydrous) in MeOH (5 ml) and stirred in a sealed vessel for 48 h. The solvent was removed *in vacuo* and residue purified by flash column chromatography on silica gel (1:1 EtOAc, DCM), yielding the deacylated nucleoside as a yellow amorphous solid (0.36 g, 94%). R_f = 0.19 (40% EtOAc in DCM). ^{1}H NMR (400 MHz, MeOD): δ (ppm) 7.17–7.30 (9H, m, DMT Ar-H); 6.83–6.87 (4H, m, DMT Ar-H); 5.81–5.82 (1H, d, J = 3.0 Hz, H1′); 3.72–3.81 (m, 8H, 2 x Ar-OMe, H2′, H3′); 3.58–3.64 (1H, dt, J = 7.2 and 2.9 Hz, H4′); 3.51–3.56 (1H, dd, J = 12.2 and 2.9 Hz, H5′); 3.36–3.42 (1H, dd, J = 12.2 and 7.2 Hz, H5″); 3.00–3.01 (3H, s, 2′-OMe). ^{13}C NMR (100 MHz, MeOD): δ (ppm) 160.26, 159.13, 154.57, 152.81, 146.25, 145.38, 138.05, 137.45, 131.39, 131.29, 129.98, 129.37, 129.28, 116.69, 114.57, 91.32, 85.60, 81.46, 72.07, 70.75, 63.66, 58.69, 55.96. HRMS (ES+) (m/z): Found, 667.2154 [M+Na]$^+$; $C_{32}H_{32}N_6O_9Na$ requires 667.2128.

5′-O,N2-bis-dimethoxytrityl-2′-O-methyl-8-nitroguanosine (8)

*N*2-Dimethoxytrityl-2′-*O*-methyl-8-nitroguanosine (**7**) (0.29 g, 0.45 mmol) was co-evaporated with anhydrous pyridine before re-dissolving in pyridine (3 ml) and adding DMTCl (0.20 g, 0.60 mmol). The solution was stirred for 16 h under an inert atmosphere at room temperature. The pyridine was removed *in vacuo* and the residue subject to flash column chromatography on silica gel (1:4 EtOAc, hexane) affording the title compound as a yellow solid (0.34 g, 80%). R_f = 0.67 (40% EtOAc in DCM). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 11.30–11.55 (1H, bs, H1); 7.90–8.00 (1H, bs, C2 NH); 6.95–7.30 (18H, m, DMT Ar-H); 6.64–6.89 (8H, m, DMT Ar-H); 5.50–5.55 (1H, m, H1′); 3.74–3.84 (2H, m, H2′ and H3′); 3.41–3.70 (13H, m, H4′ and 4 x Ar-OMe); 3.05–3.20 (2H, m, H5′ and H5″); 2.91–2.95 (3H, s, 2′-OMe); 2.41–2.55 (1H, bs, 3′-OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 159.24, 153.38, 152.21, 145.24, 136.57, 130.98, 130.60, 129.57, 128.91, 128.51, 127.39, 127.11, 126.96, 115.42, 113.74, 111.75, 90.14, 86.54, 83.43, 80.61, 71.34, 69.72, 60.88, 58.21, 55.67. HRMS (ES+) (m/z): Found, 969.3481 [M+Na]$^+$; $C_{53}H_{50}N_6O_{11}Na$ requires 969.3435.

Phosphoramidite of 5′-O,N2-bis-dimethoxytrityl-2′-O-methyl-8-nitroguanosine (9)

5′-*O*,*N*2-bis-dimethoxytrityl-2′-*O*-methyl-8-nitroguanosine (**8**) (0.51 g, 0.53 mmol) was dissolved in anhydrous DCM (5 ml) and cooled to 0°C under an inert atmosphere. Diisopropyl ethylamine (1.15 ml, 6.61 mmol) was added followed by drop-wise addition of 2-cyanoethyl diisopropylchlorophosphoramidite (0.15 ml, 0.66 mmol). The solution was allowed to warm to room temperature and stir for 3 h before adding water (10 ml) and DCM (20 ml). The phases were separated and the organic solution dried (Na_2SO_4), filtered and concentrated to a yellow solid prior to flash column chromatography (1:4 EtOAc, hexane). Phosphoramidite 9 was isolated as a yellow foam containing both diastereoisomers (0.41 g, 67%). R_f = 0.40 (20% EtOAc in DCM). ^{31}P NMR (160 MHz, $CDCl_3$): δ (ppm) 150.81, 149.25. HRMS (ES-) (m/z): Found, 1145.4517 [M-H]$^-$; $C_{62}H_{66}N_8O_{12}P$ requires 1145.4538.

8-Nitroguanosine (6a)

Compound (**5a**) (40 mg, 0.05 mmol) was dissolved in $CHCl_3$ before adding a solution of TsOH (10 mg, 0.06 mmol) in MeOH (0.5 ml). After 10 min the resulting orange solution was evaporated to dryness and triturated with cold Et_2O before dissolving the residue in 7 M NH_3 in MeOH, in a sealed vessel and stirring at room temperature for 48 h. The solvent and NH_3 were removed *in vacuo* leaving an orange solid which was washed with Et_2O and identified as the deprotected ribonucleoside (12 mg, 73%). ^{1}H NMR (400 MHz, D_6-DMSO): δ (ppm) 9.73–9.98 (1H, bs, H1); 7.12–7.30 (2H, bs, C2 NH_2); 6.24–6.26 (1H, d, J = 5.2 Hz, H1′); 5.35–5.40 (1H, bs, OH); 5.01–5.07 (1H, bs, OH); 4.86–4.90 (1H, m, H2′); 4.17–

4.21 (1H, m, H3′); 3.81–3.86 (1H, dd, J = 4.8 and 10.2 Hz, H4′); 3.64–3.69 (1H, dd, J = 11.9 and 3.0 Hz, H5′); 3.47–3.53 (1H, dd, J = 11.9 and 5.5 Hz, H5″). ^{13}C NMR (100 MHz, D_6-DMSO): δ (ppm) 158.11, 157.09, 153.60, 143.10, 115.54, 88.71, 85.24, 80.50, 70.64, 60.31. HRMS (ES-) (m/z): Found, 327.0698 [M-H]$^-$; $C_{11}H_{13}N_6O_7$ requires 327.0689. λ_{max} (10 mM Tris-HCl pH 7.4) 231 nm, ε = 9798 M^{-1}; 398 nm, ε = 8109 M^{-1}

8-Nitro-2′-*O*-methylguanosine (6b)

Compound (**5b**) (0.12 g, 0.16 mmol) was dissolved in CHCl$_3$ (2 ml) before adding a solution of TsOH (32 mg, 0.17 mmol) in 5:1 CHCl$_3$ and MeOH (1 ml). After 15 min the solvent was removed *in vacuo* leaving an orange gum, which was triturated with Et$_2$O to afford the crude base deprotected nucleoside as an orange solid. This was dissolved in 7 M NH$_3$ in MeOH (2 ml) and stirred in a sealed vessel at room temperature for 48 h. The precipitated solid was isolated by drawing off the solution with a syringe and was triturated with Et$_2$O three times to afford compound 6b as an orange solid (44 mg, 81%). ^{1}H NMR (400 MHz, D_6-DMSO): δ (ppm) 9.90–10.1 (1H, bs, H1); 7.15–7.39 (2H, bs, C2 NH$_2$); 6.32–6.34 (1H, d, J = 4.8 Hz, H1′); 5.02–5.20 (1H, bs, 3′-OH); 4.86–4.97 (1H, bs, 5′-OH); 4.63–4.67 (1H, dd, J = 5.8 and 4.8 Hz, H2′); 4.35–4.40 (1H, dd, J = 5.9 and 5.8 Hz, H3′); 3.79–3.84 (1H, ddd, J = 6.0, 5.9 and 3.9 Hz, H4′); 3.64–3.69 (1H, dd, J = 11.8 and 3.9 Hz, H5′); 3.47–3.53 (1H, dd, J = 11.8 and 6.0 Hz, H5″); 3.58–3.60 (3H, s, 2′-OMe). ^{13}C NMR (100 MHz, D_6-DMSO): δ (ppm) 157.81, 156.33, 152.60, 143.10, 115.29, 88.74, 85.64, 80.50, 69.14, 61.64, 57.86. HRMS (ES-) (m/z): Found, 341.0843 [M-H]$^-$; $C_{11}H_{13}N_6O_7$ requires 341.0843. λ_{max} (10 mM Tris-HCl pH 7.4) 231 nm, ε = 9874 M^{-1}; 396 nm, ε = 8147 M^{-1}

2′-*O*-Methyl-8-bromoguanosine

Compound (**3b**) (0.1 g, 0.21 mmol) was dissolved in 7 M NH$_3$ (anhydrous) in MeOH (3 ml) and stirred in a sealed vessel for 48 h. The resulting precipitate was isolated by drawing off the solution with a syringe and was triturated with Et$_2$O three times to afford compound 9 as a white solid (76 mg, 90%). ^{1}H NMR (400 MHz, D_6-DMSO): δ (ppm) 9.70–9.85 (1H, bs, H1); 6.59–6.71 (2H, bs, C2 NH$_2$); 5.76–5.79 (1H, d, J = 6.4 Hz, H1′); 5.07–5.24 (1H, bs, 3′-OH); 4.90–5.05 (1H, bs, 5′-OH); 4.73–4.78 (1H, dd, J = 6.4 and 5.7 Hz, H2′); 4.35–4.40 (1H, dd, J = 5.7 and 3.6 Hz, H3′); 3.86–3.91 (1H, m, H4′); 3.63–3.69 (1H, dd, J = 11.9 and 4.9 Hz, H5′); 3.50–3.56 (1H, dd, J = 11.9 and 5.4 Hz, H5″); 3.29–3.31 (3H, s, 2′-OMe). ^{13}C NMR (100 MHz, D_6-DMSO): δ (ppm) 156.23, 154.23, 152.43, 120.99, 117.87, 88.01, 86.67, 79.79, 69.27, 62.21, 57.91. HRMS (ES+) (m/z): Found, 398.0091 and 400.0061 [M+Na]$^+$; $C_{11}H_{14}N_5O_5$Br requires 398.0076 and 400.0056.

Oligonucleotide synthesis and purification

Standard 3′–5′ synthesis procedures were employed with extended coupling times (3 min) for 2′-*O*-Me phosphoramidites. Cleavage of the oligonucleotides from the linker and removal of base labile protecting groups was achieved by heating the support-bound oligomers at 55°C in 33.3% ammonium hydroxide for 8 h. The 5′-DMT group was retained to facilitate purification by preparative reverse-phase HPLC prior to lyophilization and DMT removal by treatment with 20% aqueous AcOH for 15 min at room temperature. Removal of the AcOH solution *in vacuo* was followed by partitioning between water and EtOAc to remove the DMT residues. Purity of the oligonucleotides was measured by HPLC with UV detection at 254 nm, quantification was achieved by determination of the concentration of aqueous solutions of the oligos from their UV absorbencies at 260 nm.

A list of all the ODNs prepared together with their retention times on HPLC and analysis by electrospray mass spectrometry is presented in the Supplementary Material.

PAGE analysis of primer extension reactions

Primer extension reactions were performed in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl$_2$, 10 mM DTT, 250 µg ml^{-1} BSA. For gel-based analyses, the 16-mer primer (5′–ATGTCGACT CCCAATG–3′) was labelled at the 5′-end with [γ-^{32}P]ATP (6000 Ci mmol^{-1}) and T4 polynucleotide kinase. Unincorporated radionucleotide was removed by size-exclusion chromatography using G-25 Sephadex mini Quick Spin columns. 10 µl primer extension reactions were initiated by mixing 5 µl each of two solutions. One solution was prepared by incubating purified [γ-^{32}P]-labelled primer and template DNA (5′-TCA*N*CATTGG GAGTCGACATAGCGC–3′, *N* = 2′-*O*-methylguanosine or 8-nitro-2′-*O*-methylguanosine) in a 1:2 molar ratio in reaction buffer at 70°C for 20 min followed by cooling at room temperature for 15 min. AMV-RT or human DNA polymerase β was then added to the annealed primer-template solution. The second solution was made by heating various concentrations of single dNTPs or all four dNTPs in reaction buffer at 37°C for 10 min. The final concentrations of primer extension components were 20 nM primer, 40 nM template, and 2 U µl^{-1} AMV-RT or 750 nM human DNA polymerase β. Reactions were incubated at 37°C for 60 min then quenched with the addition of 10 µl gel loading dye (98% (v/v) formamide, 10 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol). Primer extension products were separated on an 8 M urea/18% (w/v) (acrylamide to bisacrylamide ratio 29:1) polyacrylamide gel (20 cm × 47 cm × 0.04 cm) that was electrophoresed at 2200 V until the bromophenol blue had migrated 35 cm. Gel bands were visualized by phosphorimaging and band intensities quantified with ImageQuant^{TM} software (GE Healthcare). Tabulated band intensities derived from Figures 5 and 6, calculated as percentage of total band intensities in a gel lane, are presented in the Supplementary Material.

MALDI-TOF mass spectrometric analysis of primer extension reactions

Samples were prepared by carrying out primer extension reactions as described above with the following exceptions: (i) 10 pmol of unlabelled primer was annealed to 20 pmol template; (ii) reactions contained 1 mM each of

the four deoxynucleoside triphosphates; and (iii) reactions were terminated by heating samples at 90°C for 5 min. To remove salt contaminants and concentrate extension products, reactions were processed through ZipTip$_{C18}$ pipette tips. The desalting agent triethylammonium acetate, pH 7.0 was added to each primer extension reaction to give a final concentration of 0.1 M. Chromatographic tips were washed with 50% acetonitrile and equilibrated with 0.1 M triethylammonium acetate, pH 7.0. Primer extension samples were bound to the tip resin, and then washed sequentially with 0.1 M triethylammonium acetate, pH 7.0 and Milli-Q water. Oligonucleotides were eluted with 2 µl of the MALDI-TOF matrix 3-hydroxypicolinic acid (3-HPA) and spotted directly on stainless steel target plates. The matrix was prepared by vortexing a solution of 50 mg ml^{-1} 3-HPA in 50% acetonitrile containing 10 mg ml^{-1} ammonium citrate for 1 min, followed by sonication for 15 min. Undissolved matrix was separated from the solution by centrifugation in a microcentrifuge at 13 000 rpm for 10 min.

Other techniques

Experimental details and data relating to the depurination studies, pK_a measurements and thermal melting studies are all presented in the Supplementary Material.

RESULTS and DISCUSSION

Synthesis and hydrolytic stability of 8-nitro-2′-O-methylguanosine

8-Nitroguanine has previously been incorporated into ODNs in a site-specific manner through photochemical nitration of a single dG residue present in the sequence (12,19). This procedure gave extremely low yields (~3% based on the starting ODNs) of the hydrolytically unstable nitrated oligonucleotides and the products were only available in nanomole quantities, which was insufficient to perform any physicochemical studies on this lesion (19). In addition, this method is not suited to the preparation of oligonucleotides containing multiple guanine bases. As described above, the approach adopted here was to prepare hydrolytically stable analogues of 8-nitrodG that could be incorporated into chemically synthesized ODNs.

Saito *et al.* (20) have previously described the synthesis of 8-nitroguanosine (**6a**, Scheme 2), in addition to its 2′-fluoro and 5′-azido derivatives, and established that these nucleosides are relatively stable to depurination (half-life of 72 and 4.5 days for the 2′-fluoro and 5′-azido derivatives, respectively, at pH 7.4). These results suggested that in order to stabilize the glycosidic bond, 8-nitroguanine could be incorporated into ODNs as its 2′-O-methyl riboside as this electronegative substituent stabilizes the glycosidic bond (21). The 2′-O-methyl nucleosides are widely available and have been extensively investigated with regard to antisense oligonucleotides and in comparison to the ribonucleoside, has the advantage that no protection of the 2′-position is necessary (22). While the 2′-fluoro modification could also be considered
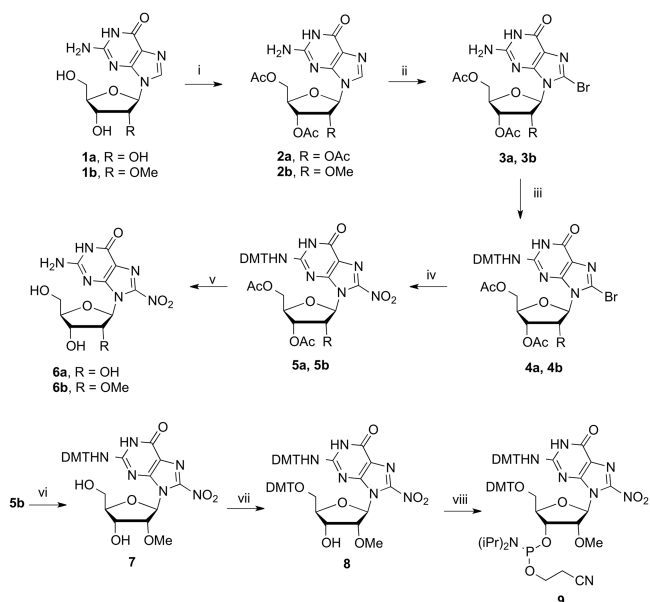
Scheme 2. Synthesis of 8-nitroguanosine nucleosides. Reagents and conditions: (i) Ac$_2$O (2.5 eq), Et$_3$N (2.6 eq), DMAP (0.1 eq), MeCN 0°C, r.t. 2 h (94%, **2b**) (ii) Sat. bromine water (excess), H$_2$O, r.t. (90%, **3b**) (iii) DMTCl (1.2 eq), pyridine, r.t., 6 h (94% **4a**, 70% **4b**); (iv) KNO$_2$ (10 eq), 18-C-6 (10 eq), DMF, 100°C, 6 h (36% **5a**, 43% **5b**); (v) TsOH (1.1 eq), CHCl$_3$, r.t. 1 h then 7 M NH$_3$ in MeOH, r.t., 48 h (73% **6a**, 81% **6b**); (vi) 7 M NH$_3$ in MeOH, r.t., 48 h (94%) (vii) DMTCl (1.2 eq), pyridine, r.t., 16 h (80%) (viii) DIPEA (12.5 eq), 2-cyanoethyldiisopropylchlorophosphoramidite (1.2 eq), DCM, 0°C to r.t. 3 h (67%).

for this purpose, the greater electronegativity of the fluorine atom causes these analogues to adopt a sugar conformation that is even further removed from that of 2′-deoxynucleosides, when compared with ribonucleosides (23). Thus, Saito's synthesis of 8-nitroguanosine was repeated as shown in Scheme 2 and also applied to the synthesis of 8-nitro-2′-O-methylguanosine (**6b**). This reaction scheme was also repeated starting with 2′-deoxyguanosine, but in this case only depurinated products were detected after nitration, confirming the extreme lability of 8-nitrodG.

In preparation for oligonucleotide synthesis, efforts were made to introduce the usual acyl or formamidine protection at the *N*2 position (24) of the nitrated guanine nucleosides. However the *N*2 amino group proved stubbornly unreactive and these *N*2 protected derivatives could not be prepared by routine procedures. In an alternative approach to *N*2 protection, the 8-bromo nucleoside (**3a**) for example, could be protected at the *N*2 position as the isobutyryl amide or at *O*6 as the 2-(4-nitrophenyl)ethyl ether (24), however these compounds could not then be converted through to the 8-nitro derivative and, so far, acceptable nitration yields have only been achieved with dimethoxytrityl (DMT) protection at *N*2. This failure to derivatize the *N*2 position demonstrates that the reactivity of the guanine base is significantly reduced by the nitro group, suggesting that it should be possible to introduce 8-nitro-2′-O-methylguanosine (**6b**) into DNA without permanent protection of the nucleobase. Thus, through manipulation of the protecting

Table 1. Half-lives for the hydrolysis of 8-nitroguanosine nucleosides at pHs, 2.1, 5.0 and 7.1 and at 37°C

	Half-life (h)		
	pH 2.1	pH 5.0	pH 7.1
8-NO$_2$-G (**6a**)	5.35	15.1	22.1
8-NO$_2$-2′-*O*-Me-G (**6b**)	11.0	26.4	66.7

Experimental details and the plotted data are presented in the Supplementary Material.

Figure 1. Top trace shows the nucleoside composition analysis of GCGTACG**CATGCG obtained by enzymatic digestion with SVPD and alkaline phosphatase, followed by analysis by RP-HPLC at 260 nm. The bottom trace was recorded at 350 nm, where only 8-nitroguanine derivatives have a significant absorbance. The elution time for 8-nitroguanine is marked on the chromatogram. (G** = 8-nitro-2′-*O*-methylguanosine).

groups, nucleoside (**6b**) was converted through to its phosphoramidite (**9**) (Scheme 2). The *N*2-DMT group was retained, in order to aid solubility and purification of the intermediate products, even though it would undoubtedly be removed along with the 5′-*O*-DMT group during oligonucleotide synthesis.

Prior to incorporation into oligonucleotides it was important to establish that 8-nitro-2′-*O*-methylguanosine was sufficiently resistant to depurination in order to withstand the conditions of chemical DNA synthesis and to allow unambiguous biological studies. The half-lives of both 8-nitroguanosine (**6a**) and 8-nitro-2′-*O*-methylguanosine (**6b**) were measured over a range of pHs and are reported in Table 1. As can be seen, (**6b**) had a half-life of 66.7 h (pH 7.0 and 37°C), which dropped to 11.0 h at pH 2.0. Interestingly, the ribose derivative (**6a**) had a significantly shorter half-life at both pHs (22.1 h and 5.35 h at pHs 7.1 and 2.0, respectively). Prior to this work there was one published study on the hydrolytic stability of 8-nitroguanosine (**6a**), which reported a shorter half-life of 5 h (pH 7 and 37°C). The results presented here show a half-life for the 8-nitro-2′-*O*-Me-guanosine, which is closer to that recently published for the related 8-nitro-5′-azidoguanosine [half-life of 108 h at a slightly higher pH (pH 7.4 and 37°C)] (20).

It is useful to compare these results to studies previously conducted on the depurination of 2′-deoxy-8-nitroguanosine either in DNA or ODNs. For example, the half-life for the release of 8-nitroguanine from a 13-mer ODN was measured (25) to be 1 h at 37°C (pH 7.2), while for an 11-mer ODN the depurination half-life (19) was 20 h at 23°C (pH 7), which was extrapolated to a half-life of 2 h at 37°C. Interestingly, in double-stranded calf-thymus DNA, 8-nitro-dG appears to be more stable (half-life ~4 h, 37°C, pH 7.4), (10) indicating that secondary structure and other factors such as sequence context are also likely to influence the rate of depurination. It is clear that the 2′-*O*-methyl group exploited here considerably stabilizes the glycosidic bond, as compared with 8-nitrodG.

Synthesis and physiochemical studies on ODNs containing 8-NO$_2$-2′-*O*-Me-G

ODNs containing 8-nitro-2′-*O*-methylguanosine were prepared using phosphoramidite (**9**) (Scheme 1) in a standard coupling procedure. ODNs were purified as their 5′-DMT derivatives by reverse-phase HPLC and the DMT group finally removed by treatment with acetic acid:water (20:80, 15 min). This deprotection step was deliberately kept short
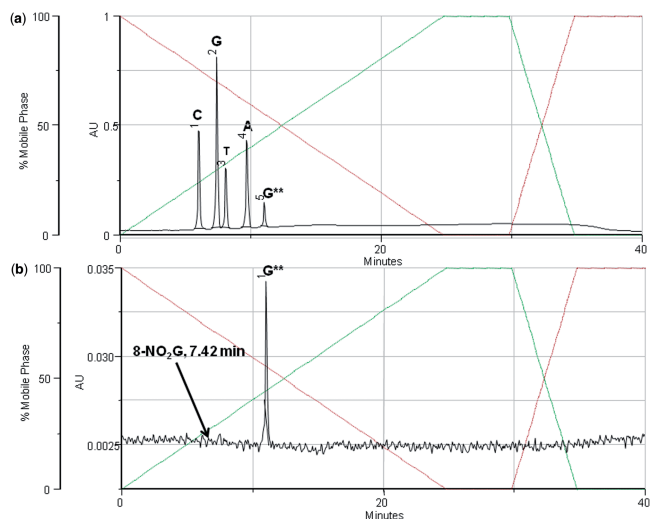
to minimize the possibility of depurination. If necessary oligonucleotides were repurified to ~98–99% purity, as determined by HPLC and characterized by mass spectrometry (Supplementary Material). The presence of 8-nitro-2′-*O*-methylguanosine within the oligomer could also be established by enzymatic hydrolysis to the constituent nucleosides and analysis of the hydrolysate by HPLC (Figure 1). The HPLC analysis was also monitored at 350 nm (Figure 1), which specifically reveals 8-nitro-2′-*O*-methylguanosine. The chromatogram shows no observable peak corresponding to the depurination product 8-nitroguanine, which also absorbs at this wavelength.

To analyse the ODNs for contamination with abasic sites resulting from loss of 8-nitroguanine, GCGTACG**CATGCG (G** = 8-nitro-2′-*O*-methylguanosine) was treated at pH 5 and analysed by HPLC. The chromatogram (Supplementary Figure S2b) revealed a new peak with the expected slightly shorter retention time corresponding to the ODN product containing the abasic site (26). The 8-nitro-2′-*O*-methylguanosine appeared to be considerably more resistant to hydrolysis when incorporated into ODNs and the HPLC peak attributed to depurination was only observed in significant quantities at pH 2. However, at this pH the ODNs also began to decompose further and thus the depurinated ODN could not be isolated and characterized and the half-life of depurination within the ODN could not be measured. Prior to treatment at pH 5 the extent of depurination in the purified ODN sample was estimated to be less than 2%.

pK_a is an important property of nucleosides influencing their base pairing properties, since the strength of the hydrogen-bonding interactions is correlated with the relative pK_a values of the donor and acceptor nucleobases (27,28). 8-Nitroguanine nucleosides have previously been shown to have a maximum absorption at around 390 nm

(29). The pH-dependent shift in the absorption spectrum was used to determine the pK_a for deprotonation at *N*1 (Figure 2). Thus, 8-nitroguanosine (**6a**) and 8-nitro-2′-*O*-methylguanosine (**6b**) were shown to have p*K*a values of 8.4 and 8.3, respectively, and hence are more acidic than guanosine (30) ($pK_a = 9.4$) and 2′-*O*-methylguanosine ($pK_a = 9.5$), due to the electron withdrawing nitro group. Due to the wavelength of absorption, the pK_a of 8-nitro-2′-*O*-methylguanosine could also determined within the single-stranded oligonucleotide (GCGTACG **CATGCG, where G** = 8-nitro-2′-*O*-methylguanosine) and in this situation the p*K*a was increased to 9.1. The decrease in acidity of 8-nitro-2′-*O*-methylguanosine within the ODN is probably due to deprotonation being suppressed by the negatively charged phosphodiester backbone; the pK_a will also be modulated by the sequence context (31). However, it should be noted that the conformational studies presented below largely rule out base pairing schemes involving the *N*1 position of the 8-nitroguanine base.

To assess the thermal stability of DNA containing the 8-nitroguanine lesion, ODN duplexes were prepared in which either 2′-*O*-methylguanosine or its 8-nitro derivative was paired in turn with each of the DNA nucleosides and spectrophotometric thermal melting curves were recorded; the melting temperatures (T_m) are shown in Table 2. The DNA sequences used were based on those previously
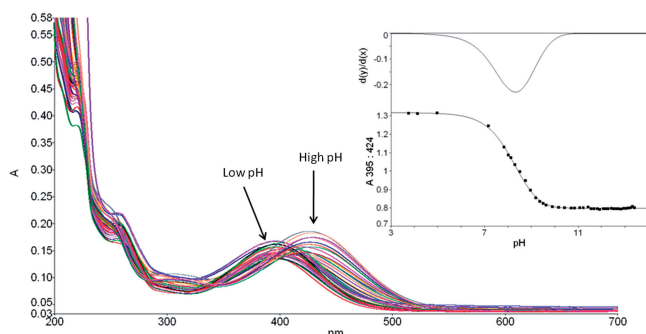

Figure 2. pH-dependent shift in spectrophotometric absorption of 8-nitro-2′-*O*-methylguanosine. Insert shows the plot of pH versus absorption at 395 nm for 8-nitro-2′-*O*-methylguanosine and the first derivative of the curve.

Table 2. Melting temperatures (average of three determinations) of a family of DNA duplexes with and without the 8-nitroG modification

	5′-GCGTACXCATGCG 3′-CGCATGYGTACGC		
Y		X	
	dG (°C)	2′-OMeG (°C)	8-NO$_2$-2′-OMeG (°C)
C	63.6	61.0	52.4
T	55.3[a]	54.2	48.7
A	51.5[a]	50.0	49.9
G	54.9[a]	51.0	54.2

Conditions: 1 M NaCl, 0.1 M MgCl$_2$, 60 mM sodium cacodylate (pH 7), 3µM duplex. [a]Taken from reference (31).

studied in the analysis of the base pairing preferences of 8-oxo-2′-deoxyguanosine (32). Duplexes containing the control 2′-*O*-methylguanosine all showed a slight destabilization (∼1-3°C) when compared with the T_m data for the corresponding duplexes containing dG (32), but importantly revealed the same trends for the melting temperatures (C >> T > G > A). These data established that 2′-*O*-methyl ribose is a sufficiently good mimic of 2′-deoxyribose for the purpose of these thermal melting studies. T_m data obtained for the duplexes containing 8-nitro-2′-*O*-methylguanosine showed a considerable lowering of the T_m for the 8-nitroG·C pairing, relative to the 2′-*O*-methylguanosine control (52.4°C versus 61.0°C, Table 2). While the nitro group also significantly destabilized the G·T pair, it had no effect on G·A, and it actually stabilized the G·G pair (54.2°C versus 51.0°C). Comparing the thermal stabilities of the 8-nitroG·C and 8-nitroG·G base pairings, (52.4°C and 54.2°C) the data are consistent with a greater stability for the G.G pairing (see Supplementary Material for statistical analysis).

To provide further insight to the base pairing behaviour of this lesion we examined the conformation about the glycosidic bond for 8-nitro-2′-*O*-methylguanosine, as it determines which hydrogen bonding face is presented to the complementary nucleobase (Figure 3). It is well known that the introduction of sterically demanding groups at the 8-position of purine nucleosides favours a conformational change from *anti* to *syn* (33). NMR shifts are diagnostic of conformation and a change from *anti* to *syn* is characterized by a downfield shift of the H2′, C1′, C3′ and C4′ signals and an upfield shift of C2′ (34,35). A comparison of the NMR spectra for 2′-*O*-methylguanosine and its 8-nitro derivative (Table 3) showed that all of the chemical shifts indicative of a syn conformation were observed for the nitrated nucleoside. Data are also presented for
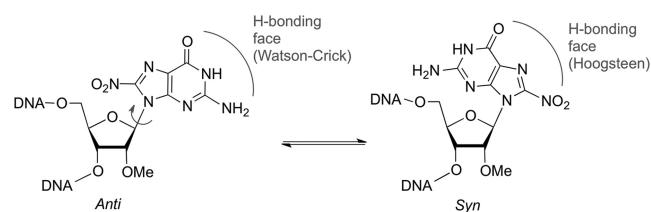

Figure 3. The *anti* and *syn* conformations of 8-nitro-2′-*O*-methylguanosine showing the change in hydrogen bonding face.

Table 3. Selected NMR data for studies on the *syn*/*anti* conformation

Nucleoside	H1′	H2′	C1′	C2′	C3′	C4′
2′-OMeG	5.82	4.28	85.85	82.73	68.66	84.32
8-Br-2′-OMeG	5.79	4.78	88.01	79.79	69.27	86.67
8-NO$_2$-2′-OMeG	6.32	4.68	89.12	80.88	69.55	86.02

^{1}H and ^{13}C NMR shifts of sugar protons and carbons of the 2′-*O*-methyl derivatives of guanosine (2′-OMeG), 8-bromoguanosine (8-Br-2′-OMeG) and 8-nitroguanosine (8-NO$_2$-2′-OMeG), measured in D6-DMSO. Downfield and upfield chemical shifts for the *syn* conformation are indicated by turquoise and yellow shading, respectively.

8-Br-2′-OMeG as 8-bromoguanosine nucleosides are established to adopt the *syn* conformation (33). Interestingly, a large downfield shift is observed for H1′ of 8-nitro-2′-*O*-methylguanosine and is probably due to the close proximity of the nitro group to H1′ in the *syn* conformation.

The NMR data, taken in conjunction with the results of the thermal melting studies are consistent with the 8-nitroG·C base pair being destabilized by a change from the *anti* to the *syn* conformation on introduction of the nitro group. This conformational change presents the Hoogsteen face of 8-nitroG, which can form two hydrogen bonds to a paired guanine base (Figure 4). Molecular modelling calculations performed on the *N*9 methylated bases, show that the *syn*-8-nitroG·G base pair, with its two hydrogen bonds is considerably weaker than the regular G·C pair. *Anti*-G·*syn*-A and *anti*-G·*anti*-A base pairs are both known from crystal structures (36) of oligonucleotides and *anti*-8-nitroG·*anti*-A base pairing was also proposed by Suzuki *et al.* (12) to explain their observed incorporation of dA opposite this lesion in their primer extension reactions. While analogous structures with 8-nitroG may form, they would be inconsistent with the preferred *syn* conformation suggested here by NMR. It is also of interest to compare 8-nitroG to the behaviour of the notionally similar 8-oxoG, which is established to adopt a *syn*-8-oxoG·*anti*-A base pair in crystal structures (37). However, the equivalent *syn*-8-nitroG·A structure is less plausible, since formation of two hydrogen bonds requires protonation (Figure 4c) and the calculated energy for this protonated *syn*-8-nitroG·A pair was considerably greater ($-166 \, \mathrm{kJ\,mol^{-1}}$) than the aforementioned *syn*-8-nitroG·G pairing.

DNA synthesis directed by a template containing 8-nitroG

As discussed previously, Suzuki *et al.* have investigated the mutagenic behaviour of 8-nitroguanine in primer extension reactions performed using an ODN template containing the natural 8-nitrodG lesion, which was produced photochemically. Despite being derived from a ribonucleoside, 8-nitro-2′-*O*-methylguanosine has potential advantages over the natural lesion in that it can be incorporated into ODNs by conventional chemical synthesis and the 2′-*O*-methyl substituent stabilizes the glycosidic bond. It was therefore of interest to compare the results of Suzuki's primer extension studies to those obtained with our stabilized template. To conduct these studies, 8-nitro-2′-*O*-methylguanosine was incorporated into an ODN template, and primer extension experiments were carried out (Scheme 3). A 16-mer primer was designed to anneal to a template such that the first nucleotide to be added would be paired with 8-nitroG, with the unmodified 2′-*O*-methylguanosine as a control. Two polymerases were investigated: firstly the DNA polymerase AMV-RT, which naturally uses both DNA and RNA as a template and is particularly suited to our lesion analogue as it is known to extend past 2′-*O*-methyl nucleotides on the template (38,39). Secondly, and to allow direct comparison with Suzuki's work, the higher fidelity human DNA polymerase β (pol β), was also selected (40).
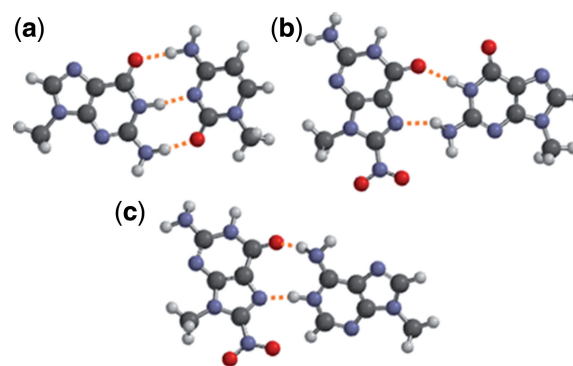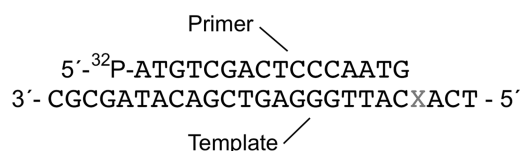

Figure 4. Computational modelling of *N*9-methylated bases. Calculated using Spartan 08 v1.2.0 at AM1 level: (**a**) Watson–Crick G·C base pair ($-701 \, \mathrm{kJ\,mol^{-1}}$) (**b**) *syn*-8-nitroG·G ($-354 \, \mathrm{kJ\,mol^{-1}}$) (**c**) protonated *syn*-8-nitroG·A base pair ($-166 \, \mathrm{kJ\,mol^{-1}}$).

Primer

5′-³²P-ATGTCGACTCCCAATG
3′- CGCGATACAGCTGAGGGTTAC**X**ACT - 5′

Template

Scheme 3. Oligonucleotides used in primer extension. The X indicates the position of 2′-*O*-methyl G or 8-nitro-2′-*O*-methyl G in the template.

Primer extension reactions using single deoxynucleoside triphosphates as well as mixtures of the four dNTPs were analyzed by polyacrylamide gel electrophoresis (Figure 5 and 6). In reactions containing single nucleoside triphosphates and the control 2′-*O*-methyl G template, AMV-RT showed selectivity in pairing dCTP opposite the target site at low nucleotide concentrations (50–100 µM), showing that fidelity was not seriously compromised by the 2′-O-methyl modification. At significantly higher concentrations (1 mM), the enzyme incorporated each of the other three dNTPs with high efficiency (Figure 5a). The predominant products in Figure 5a are consistent with the addition of any single nucleotide after the last correctly templated addition at high dNTP concentration. With dCTP, a second nucleotide is added, and with dTTP, a third, after the 'correct' T in the second position (Scheme 3). In contrast, pol β efficiently incorporated only dCTP, even at high concentrations (Figure 5b), again suggesting that the 2′-*O*-methyl analogue is appropriately discriminated by the enzyme. With a mixture of all four dNTPs, both enzymes could efficiently extend past 2′-*O*-methylG to give 20-mer products corresponding to extension to the end of the template (Scheme 3). Pol β also gave a significant fraction of 19-mer product (Figure 5b).

In contrast, a template containing 8-nitro-2′-*O*-methylguanosine resulted in a substantial reduction in both the level and specificity of nucleotide insertion opposite this lesion (Figure 6). AMV-RT inserted single dNTPs at levels 10–60% of that measured for 2′-*O*-methylG, with dATP apparently most preferentially inserted, whereas pol β incorporated dCTP at a level <20% of the control, although dTTP incorporation was actually increased around 2-fold (from a low level).
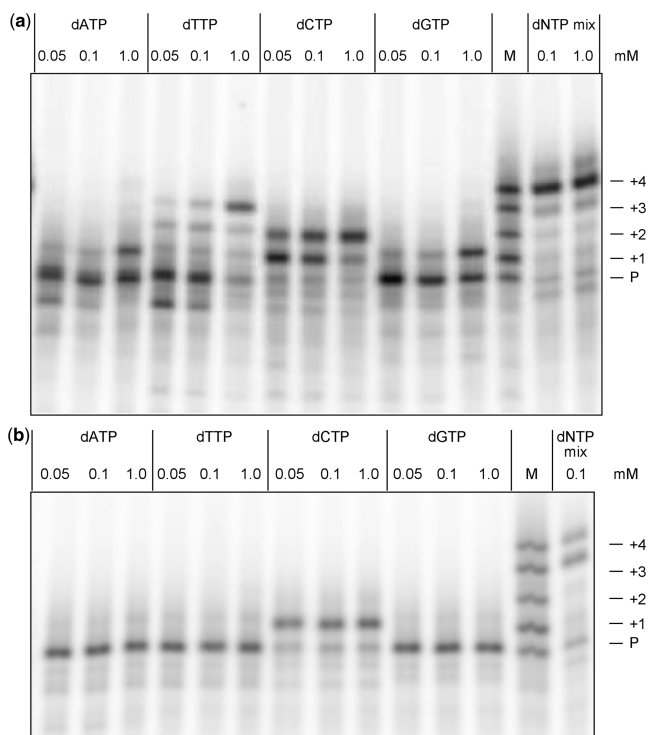
Figure 5. Gel analysis of primer extension products synthesized by (**a**) AMV-RT and (**b**) human DNA polymerase β, from the control template containing 2′-*O*-methylguanosine. Varying concentrations of single deoxynucleoside triphosphates or an eqimolar mixture of all four dNTPs (dNTP mix) served as substrates for reactions as indicated. Lane M, 5′-[^{32}P]-labelled oligonucleotide size standards consisting of primer (P; 16-mer) and synthetic oligonucleotides generated by incremental addition of nucleotides, forming a 17-mer (+1), 18-mer (+2), 19-mer (+3) and 20-mer (+4). The +4 band corresponds to a primer that has been extended to the end of the template. Some 21-mer is visible in the dNTP mix reactions of AMV-RT, and is likely to result from non-templated terminal transferase activity of that enzyme (41). Band intensities are reported in the Supplementary Material.

Figure 6. Gel analysis of primer extension products synthesized by (**a**) AMV-RT and (**b**) human DNA polymerase β, from template containing 8-nitro-2′-*O*-methylguanosine. The experimental details are as described in Figure 5. The tracks marked –E and –T correspond to control reactions carried out without the addition of enzyme and template, respectively. Band intensities are reported in the Supplementary Material.

Consistent with this, the formation of extended 19- and 20-mer products in the presence of all four dNTPs was reduced by 70–90% with AMV-RT and over 90% with pol β, even at high (1 mM) dNTP concentrations.

The composition of these extended products was analysed by MALDI-TOF mass spectrometry (Figure 7), using synthetic 20-mer products with the 4 alternative nucleotides at position 17 as standards. MALDI-TOF is widely used for the detection and quantitation of single nucleotide polymorphisms and allele frequencies using similar primer extension reactions; see for example (42). Consistent with the gel results, AMV-RT gave 20-mer products containing predominantly dC (with minor fractions containing dA and dG) with the control template, whereas pol β incorporated only dC into 19-mer and 20-mer products. With the 8-nitroG template, AMV-RT incorporated predominantly dC (with a small fraction of dA) into fully-extended product, whereas pol β preferentially incorporated dA over dC in a ratio of 2:1—only 19-mers were detected in this experiment. There was no evidence for incorporation of dTTP in the extended 19-mer products with pol β. The products of single
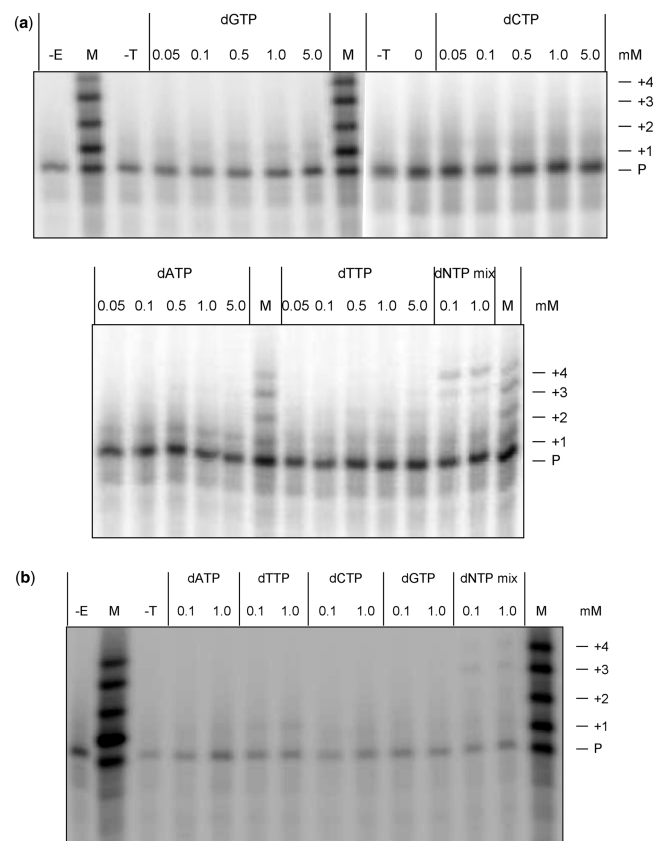
dTTP incorporation visible in Figure 6b may not serve as a competent substrate for further extension.

The experiments with DNA polymerase β show a strong tendency towards the incorporation of dA opposite the lesion and in this respect they agree qualitatively, but not quantitatively with those of Suzuki *et al.* performed using a related 'running start' primer extension reaction with a template containing an unstable 8-nitrodG produced photochemically (a 1:18 dA:dC ratio) (12). There are possible reasons for this difference from the 2:1 dA:dC ratio that we see. Our use of the more stable 2′-*O*-methyl derivative may have perturbed the incorporations, although our control experiments suggest that the non-nitrated 2′-*O*-methyl G template does not significantly affect the specificity of the enzyme. Another possible cause is a pH difference. Suzuki *et al.* (12) carried out their pol β primer extensions at pH 8.8, which we now know from our experiments is rather close to the pK_a of 8-nitroG in an oligonucleotide (9.1); deprotonation of the 8-nitroG may have a significant effect on specificity. In contrast, we used pH 8.0 based on previous literature, specifically the work of Kosa and Sweasy (43), from whom we obtained our enzyme. In
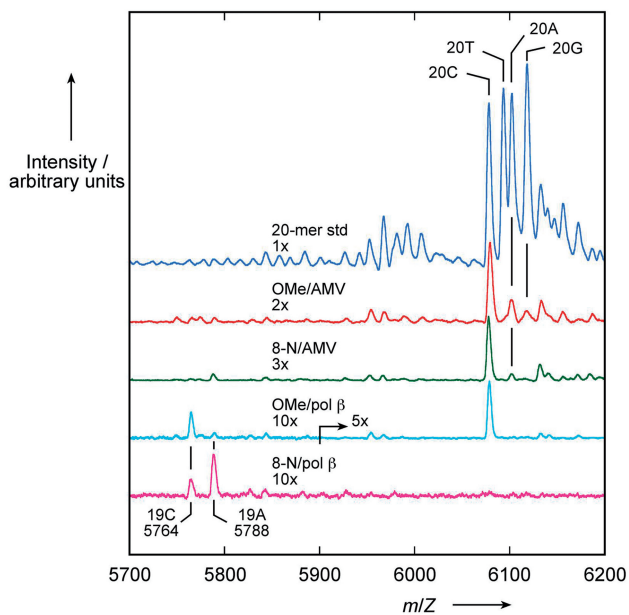
Figure 8. Overlay of *N*9-methylated base pairs modelled using Spartan. Watson–Crick G–C (light grey) and *syn*-8-nitroG·G (dark grey).

Figure 7. MALDI-TOF mass spectrometric analysis of primer extension reactions. Reactions contained 1 mM each of dATP, dTTP, dCTP and dGTP and either a 2′-*O*-methylguanosine (OMe) or 8-nitro-2′-*O*-methylguanosine (8-N) template with AMV reverse transcriptase (AMV) or human DNA polymerase β (pol β). The mass spectra of these reactions are aligned with the spectrum of a mixture of oligonucleotides (20-mer std) representative of the fully-extended primers that would result from incorporation of one of the four dNTPs opposite 2′-OMeG or 8-nitro-2′-OMeG (20C, 20T etc.). 19-mer pol β products incorporating a C or an A opposite the modified nucleotide (19C, 19A) are identified by their calculated m/Z values. For clarity, the spectra have been expanded along the arbitrary *y*-axis by the factors indicated (2x, 3x *etc.*).

addition, we did not observe a significant level of incorporation of nucleotides opposite the 8-nitroG lesion, whereas Suzuki *et al.* report retardation of the extension one base prior to and opposite the lesion, with human pol α and β (12). The lower level of nucleotide incorporation opposite the 8-nitroG lesion seen in the current study could be due to the 2′-*O*-methyl derivative or the pH used, as discussed above, or may be due to the difference in the primer template systems; the construct used here does not provide a 'running start' (44).

Once again it is of interest to compare the behaviour of 8-nitroG to that of 8-oxoG, where primer extension is not markedly impaired, but incorporation of dA through pairing with the *syn* conformation of the modified base is common (45,46). With human pol β, there is a reported 2:1–4:1 preference for incorporation of dC over dA opposite 8-oxodG (46,47), compared with the 1:2 preference seen here opposite 8-nitro-2′-*O*-methylG. Thus based on the present experiments, the 8-nitroG lesion appears to have more mutagenic potential than 8-oxoG.

With regard to the 8-nitroG·G base pairing, there is little evidence for this arrangement in the presence of either of the polymerases. This is not altogether unexpected; for example, *O*6-methylguanine forms its most stable base pair with cytosine, but DNA polymerase ι prefers to incorporate T opposite this lesion because of
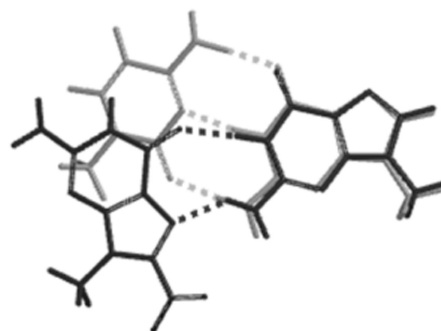
better shape complementarity to a Watson–Crick pair (48). Related to this, studies on non-polar isosteres of DNA bases, has also revealed that shape complementarity between the incoming nucleotide and template is an important factor (49). A comparison of the G·C base pair with the *syn*-8-nitroG·G base pair shows that the G·G pairing has a poor match to the Watson–Crick G·C pair, particularly with regard to the position of the methyl carbon atoms, which represent C1′ of the sugar (Figure 8). Thus, while the *syn*-8-nitroG·G base pair emerges as the most stable pairing in the duplex, this arrangement shows a poor superpositional fit to a G·C base pair and the polymerases are likely to discriminate against it on this basis. Molecular dynamics studies of conformational changes in the pol β active site both before and after the incorporation of mismatched nucleotides have suggested that G·G mispairs are particular disfavoured both in initial incorporation and subsequent extension (50,51). These results are also consistent with kinetic studies on the extension of mutagenic intermediates by pol β (52).

CONCLUSIONS

8-Nitro-2′-*O*-methylguanosine has been incorporated into DNA as a stabilized, ribose derivative of 8-nitrodG. Thermal melting studies conducted on DNA duplexes containing this modification show that this lesion forms an apparently more stable base pair with dG than with dC. This is also consistent with 8-nitro-2′-*O*-methylguanosine preferentially adopting the *syn* conformation in which it can form a *syn*-8-nitroG·G base pair with two hydrogen bonds. In studies with DNA polymerases, the 8-nitroG lesion induces very significant stalling with both a reverse transcriptase and a high fidelity DNA polymerase, with a tendency towards the incorporation of dA opposite the lesion in the latter case, leading to the possibility of G-T transversion mutations. It is possible that the polymerases select against a *syn*-8-nitroG·G due to a poor geometric match with the natural Watson–Crick pairings.

As previously discussed, the 8-nitroG lesion has been proposed to cause G to T mutations through two mechanisms; either mispairing with A or through depurination to yield apurinic sites. It is clear from these studies that this mutational effect cannot entirely be attributed to

apurinic sites and in this respect our results support the work of Suzuki (12). Which of these two mutational mechanisms are more important *in vivo* is unclear, as there is little relevant information such as the steady state levels of this lesion in cellular DNA and if or how the lesion is repaired. The use of this and other stabilized analogues of this lesion will enable us to perform structural studies on the 8-nitroG·G and 8-nitroG·A base pairs in order to understand why polymerases may discriminate between these pairings and also enable the study of potential repair pathways that might remove the lesion. Of particular interest for future studies will be the use of 8-nitroguanine nucleoside analogues in which the sugar moiety is a better mimic of deoxyribose, such as the cyclohexenyl nucleosides (53). To date the synthesis of these analogues has eluded us due to the difficulties in preparing suitable derivatives/precursors of the 8-nitroguanine nucleobase.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–8, Supplementary Figures 1–7, Supplementary Methods and Supplementary Data.

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