Chemistry of the 8-nitroguanine DNA lesion- reactivity, labelling and repair

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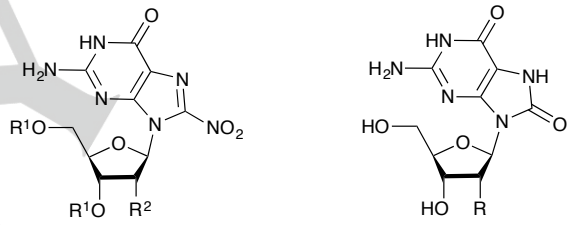
Abstract: The 8-nitroguanine lesion in DNA is increasingly associated with inflammation-related carcinogenesis, whilst the same modification on guanosine 3',5'-cyclic monophosphate generates a second messenger in NO-mediated signal transduction. Very little is known about the chemistry of 8-nitroguanine nucleotides, despite the fact that their biological effects are closely linked to their chemical properties. To this end, a selection of chemical reactions have been performed on 8-nitroguanine nucleosides and oligodeoxynucleotides. Reactions with alkylating reagents reveal how the 8-nitro substituent affects the reactivity of the purine ring, by significantly decreasing the reactivity of the *N*2 position, whilst the relative reactivity at *N*1 appears to be enhanced. Interestingly, the displacement of the nitro group with thiols results in an efficient and specific method of labelling this lesion and is demonstrated in oligodeoxynucleotides. Additionally, the repair of this lesion is also shown to be a chemically feasible reaction through a reductive denitration with a hydride source.

Introduction

The guanine base in DNA is particularly sensitive to chemical modification[1] by reactive oxygen species and reactive nitrogen species (RNS) and a number of mutagenic lesions, such as 8-nitro-2'-deoxyguanosine[2] (8-nitro-dG, **1a**, Figure 1) and the more widely studied 8-oxo-2'-deoxyguanosine[3] (8-oxo-dG, **2a**) are associated with these reactive species. A characteristic of 8-nitrodG is that the nitro group labilises the glycosidic bond to release 8-nitroguanine (8-nitroG), leaving an apurinic site in the DNA.[4] The mutagenic nature of 8-nitrodG results from both the generation of apurinic sites[5] in the DNA and the mispairing of 8-nitrodG[6-8] during DNA synthesis; both these events lead to G to T transversion mutations.

The RNS, which are responsible for the nitrative damage are generated particularly at sites of inflammation, due to over-expression of nitric oxide synthase and given the mutagenic nature of 8-nitro-dG, it is not surprising that this lesion is increasingly linked with inflammation-related carcinogenesis.[9] Specific antibodies raised against 8-nitroG have been used to show that 8-nitroG is present in surgically-removed human tissues from the sites of cancers that are caused by chronic inflammation[9] and the accumulation of 8-nitroG is also associated with a poor prognosis of inflammation-induced cancers.[9,10] Thus, 8-nitroG is progressively seen as a biochemical marker for DNA damage and as potential indicator of inflammation-induced cancers. Nitration of the guanine base also occurs in the ribonucleotide pool and 8-nitroguanosine-3',5'-cyclic phosphate[11] (8-nitro-cGMP) has attracted attention as an electrophilic second messenger that is involved in an unusual post translational modification of proteins. This process of protein *S*-guanylation,[11] results from the nucleophilic sulfhydryl group of a cysteine residue displacing the nitro group of 8-nitro cGMP. More recently H_2S, a proposed gaseous transmitter in mammals, has been shown to react with and sequester 8-nitro-cGMP, and provides a potential mechanism to shut down this electrophile-mediated signalling pathway.[12]



1a, $R^1 = R^2 = H$ = 8-nitro-dG
1b, $R^1 = H$, $R^2 = OH$, 8-nitro-rG
1c, $R^1 = CH_3CO$, $R^2 = OAc$
1d, $R^1 = H$, $R^2 = OMe$

2a, R = H = 8-oxo-dG
2b, R = OMe

Figure 1.

Collectively these diverse studies have shown that the chemical properties of 8-nitroguanine nucleotides are inherently related to their biological function and have encouraged us to look more closely at their chemistry. We now report our studies on some chemical reactions performed on 8-nitroguanine nucleosides and oligonucleotides. The results shed some light on how the 8-nitro substituent affects the reactivity of the purine ring, they demonstrate that the 8-nitroguanine lesion in DNA can be fluorescently labelled and raise the possibility of a repair mechanism for this lesion.

Results and Discussion

Alkylation reactions- Previous studies have demonstrated that some chemical properties of guanine nucleosides are significantly altered by introduction of the 8-nitro group. The nitro group greatly enhances the hydrolytic lability of the glycosidic bond[4] and also significantly lowers the pK_a of the proton on *N*1 (pK_a = 8.4 for 8-nitroguanosine compared to 9.4 for guanosine).[8]

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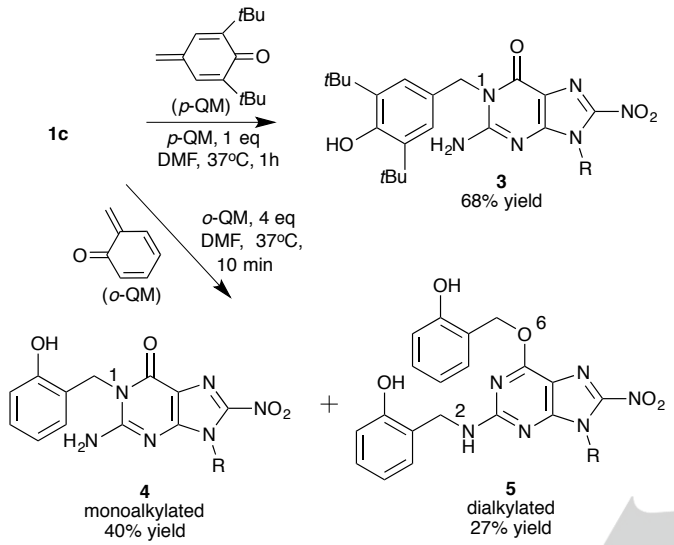
In addition, we have made attempts to introduce the usual acyl or formamidine protecting groups at the *N*2 position of 8-nitroG nucleosides, but were unable to prepare these derivatives using routine procedures.[8] This failure to derivatise the *N*2 position implied that the reactivity of the guanine base is significantly reduced because of the strongly electron withdrawing nitro group and suggested that the chemistry of 8-nitroguanine nucleosides required wider exploration.



Scheme 1. Reactions of 2',3',5'-tri-*O*-acetyl-8-nitroguanosine with quinone methides. Experimental details in the Supplementary Information.

We chose to investigate alkylation reactions on 2',3',5'-tri-*O*-acetyl-8-nitroguanosine (**1c**, Figure 1), as in this substrate the electron withdrawing acetyl groups, particularly on the 2'-OH, help to further stabilise the glycosidic bond. Given the seemingly inert nature of the 8-nitroguanine base, *ortho*- and *para*-quinone methides were selected as alkylating agents as they possess a benzylic carbon which is highly electron deficient and extremely susceptible to nucleophilic attack.[13,14] In addition, the products of the reactions of these quinone methides with 2'-deoxyguanosine have been well characterised.[13,14]

In initial experiments, acetylated 8-nitroguanosine (**1c**) was reacted with a molar equivalent of the *para*-quinone methide (*p*-QM) prepared from 2,6-bis(*tert*-butyl)-4-methylphenol by oxidation with silver (I) oxide (Scheme 1).[13] Under these conditions TLC and HPLC showed that a single nucleoside product (**3**) was formed and was subsequentlty isolated in 68% yield following column chromatography. Mass spectrometry ascertained that (**3**) was a monoalkylated product and *N*1 was established as the position of alkylation through NMR techniques. DEPT 135 established the presence of two CH_2 groups and through HSQC and COSY experiments benzylic protons from the quinone methide adduct were identified as a singlet at 5.11ppm. In the ^{13}C NMR spectrum the benzylic signal resonated at 45.70ppm, characteristic of a carbon-nitrogen bond; a chemical shift of approximately 70ppm would be expected should the benzylic carbon have been attached to an oxygen atom. From the ^{1}H NMR it was possible to determine which nitrogen atom was alkylated. Acquisition of the spectra in a non-protic solvent indicated the absence of the signal corresponding to the *N*1 proton which was present in the starting material at 11.34ppm. Additionally, the broad peak at 6.06ppm corresponding to the *N*2 signal integrated to 2 protons. These observations confirm that alkylation occurred at the *N*1 position. This assignment was further strengthened when compared to the *N*2 alkylated product previously characterised from the reaction between deoxyguanosine and a *para*-quinone methide,[13] in which these observations were reversed; the peak at 10.35ppm associated with the *N*1 proton was present and the signal associated with the *N*2 proton integrated as a single proton.

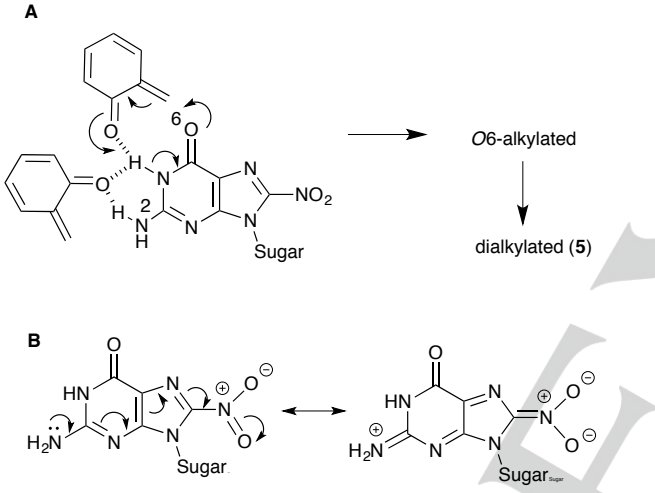
We also examined the alkylation of acetylated 8-nitroguanosine with an *ortho*-quinone methide (*o*-QM) produced from 2-bromomethyl-*O*-*tert*-butyldimethyl-silylphenol using potassium fluoride (Scheme 1).[15,16] In an initial experiment with one equivalent of the *o*-QM, two products were observed by HPLC; a more polar product (~40%) and a less polar product (~5%). When four molar equivalents of *o*-QM were used a more even distribution of the products was obtained and the more polar and less polar products were isolated in yields of 40% and 27%, respectively. Mass spectrometry revealed the more polar (**4**) and less polar (**5**) products to be mono-alkylated and di-alkylated species, respectively (Scheme 1). NMR techniques revealed the mono-alkylated product (**4**) to be entirely consistent with the *N1*-*ortho* quinone methide, based on similarities to the *N*1-*para* quinone methide product (**3**).

For the dialkylated product (**5**), the presence of two non-nucleosidic CH_2 groups with chemical shifts of 67.36ppm and 40.00ppm in the ^{13}C NMR spectrum, was established. These resonances are characteristic of a benzylic carbon-oxygen bond and a benzylic carbon-nitrogen bond, respectively and therefore indicated that *O*6 is one of the alkylation sites.[14,17] With regard to the second alkylation site on nitrogen, alkylation at the *N*1 position seemed unlikely. This was supported by ^{1}H NMR spectra recorded in a non-protic solvent revealed the presence of a single *NH* signal which integrated to one proton.

The results of alkylation reactions performed here with 2',3',5'-tri-*O*-acetyl-8-nitroguanosine (**1c**) show significant differences to those previously reported for 2'-deoxyguanosine (dG), both in terms of the products formed and their distribution. For example, the sole nucleoside product isolated from alkylation of (**1c**) with *p*-QM is the *N*1 adduct (**3**), whereas previous results obtained with dG show *N*2 to be the favoured site of alkylation.[14] That the 8-nitro group leads to preferential reaction at *N*1 rather than *N*2 is not so surprising given that the lone pair on *N*2 is directly conjugated through to the nitro group (Scheme 2) and that reaction at *N*1 of the nitrated nucleoside might be enhanced by deprotonation, due to its significantly lower pKa.

The reaction of excess *o*-QM with nitrated nucleoside (**1c**) once again gave the *N*1 adduct (**4**) as the major product (40%), together with the *O*6,*N*2-dialkylated product (**5**, 27%). Alkylation

at *N*1 was expected based on the result obtained with *p*-QM, particularly since the use of excess potassium fluoride to generate the *o*-QM in unbuffered conditions, would be expected to lead to mildly basic conditions that favour reaction at *N*1 due to deprotonation. The *O*6,*N*2-dialkylated product was unexpected, since no *O*6 or *N*2 mono-alkylated products were observed. The *ortho* geometry of the *o*-QM means that hydrogen-bonding of *NH*1 with the carbonyl group of the quinone methide could assist nucleophilic attack by either *O*6 or *N*2 (Scheme 2). Given, that *N*2 appears to be highly deactivated, attack by *O*6 is likely to be favoured from the hydrogen-bonded complex. In order to explain why no *O*6 mono-alkylated product is observed, the subsequent alkylation on *N*2, would have to be relatively fast and might be assisted by the alkylated and therefore electron donating *O*6. In contrast, studies on the reaction between dG and *o*-QM have been shown to give *N*2 alkylation as either the sole product[18] or to favour *N*2 alkylation over *N*1 by a factor of about 5.[14]

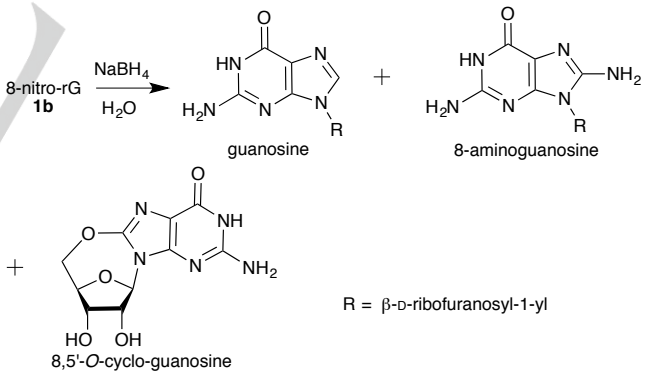


Scheme 2. Panel A shows *O*6 versus *N*2 hydrogen bond-directed alkylation, resulting in initial alkylation at the presumably more reactive *O*6 position and subsequent alkylation at *N*2, to give dialkylated **5**. Panel B shows reduced nucleophilicity of *N*2 because of conjugation through to the nitro group.

The alkylation reactions reported here have been performed on 2',3',5'-tri-*O*-acetyl-8-nitroguanosine in order to reduce depurination and were also optimised to generate good yields of the products to aid their characterisation, therefore the reaction outcomes cannot be directly compared with quantitative studies previously obtained with dG. However, given that the sites of alkylation for DNA bases with quinone methides remain constant in their deoxynucleoside, single-stranded oligodeoxynucleotide and duplex polydeoxynucleotide forms,[18] the presence of the acetyl protecting groups on the sugar is not expected to alter the site of alkylation. Thus, the results presented here indicate that the reactivity of the guanine base is significantly altered by the introduction of the 8-nitro group; the reactivity of the *N*2 position significantly decreases whilst *N*1 appears to be enhanced, presumably due to its considerably lower pKa.

Reductive denitration of 8-nitroguanosine- Aromatic nitro compounds such as nitroimidazoles have emerged as potent anti-TB pro-drugs that exert their activity through the release of nitric oxide (NO), which is the effective agent.[19] NO released from these drugs is initiated by nucleophilic attack of a hydride equivalent from a deazaflavin cofactor that is present in a deazaflavin-dependent nitroreductase and the overall reaction is a denitration in which NO_2 is replaced by H.[19] A related reaction performed on the 8-nitroG lesion in DNA, in which a hydride equivalent was delivered to the *C*8 position, could remove the nitro group through an addition-elimination reaction, thus suggesting a potential DNA repair mechanism by directly reversing the lesion. Relatively few DNA lesions are repaired by direct chemical reversal, but this route is well established for the repair of UV-induced pyrimidine dimer lesions[20] and the repair of guanine *O*6 alkylation products by *O*6-alkylguanine DNA alkyltransferase.[21] We were therefore interested in investigating the chemical feasibility of reducing 8-nitroguanosine back to guanosine as this could suggest a potential repair mechanism for this lesion by direct chemical reversal.

There are a few examples where borohydride has been used to effect aromatic denitration and these have been reported for both polysubstituted nitrobenzenes[22,23] and heterocyclic systems.[24] These reactions take place through an addition elimination mechanism in which nitrite is displaced by hydride.[23] Earlier studies[25] on the characterisation of the 8-nitroguanine base have shown that reducing agents such as: sodium hydrosulfite; zinc-HCl; hydrazine hydrate-Raney nickel, all yield the expected 8-aminoguanine, but to our knowledge the reaction has not been studied with hydride reducing agents.



Scheme 3. Reduction of 8-nitroG with sodium borohydride.

Prior to studying the reduction, 8-nitroguanosine (**1b**, Figure 1) was purified by reverse-phase (RP)-HPLC, although as can be seen in Figure 2, a small amount of the depurination product 8-nitroguanine was unavoidably introduced into the starting samples of 8-nitroguanosine during evaporation of the fractions. It was also necessary to develop RP-HPLC conditions that would separate the two most likely products; namely 8-aminoguanosine and guanosine. An extensive investigation of eluents revealed that 8-aminoguanosine and guanosine were readily separated using a gradient of acetonitrile in aqueous

acetic acid (0.1% AcOH) and these elution conditions were also compatible with HPLC-MS.

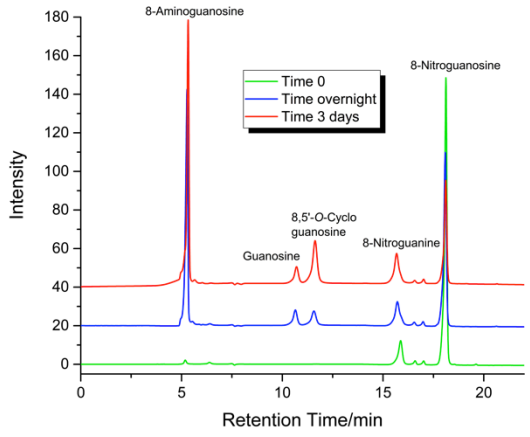


Figure. 2. RP-HPLC analysis of the reduction of 8-nitroguanosine with sodium borohydride. Details of the reaction conditions are described in the Experimental section. HPLC chromatograms were obtained using an elution gradient of 0-20% acetonitrile in 0.1% aqueous acetic acid over 20 min, with a flow rate of 0.5 ml/min. Chromatograms were recorded at 254 nm.

Table 1. High-resolution mass data for the borohydride (either $NaBH_4$ or $NaBD_4$) reduction of 8-nitroguanosine as shown in Scheme 3. Accurate mass measurements were obtained by LC-MS as described in the experimental section. Mass spectra for individual compounds are shown in the SI.

Nucleoside	Formula	Calculated exact mass for $[M-H]^-$	Measured accurate mass for $[M-H]^-$
Guanosine	$C_{10}H_{13}N_5O_5$	282.0844	282.0827
8-Deuteroguanosine	$C_{10}H_{12}DN_5O_5$	283.0907	283.0906
8,5'-*O*-Cycloguanosine	$C_{10}H_{11}N_5O_5$	280.0687	280.0693
8-Aminoguanosine	$C_{10}H_{14}N_6O_5$	297.0953	297.0963

Reduction reactions (Scheme 3) were generally performed in unbuffered distilled water using a very large excess of sodium borohydride that was repeatedly added at regular intervals to compensate for hydrolysis. Under these conditions a slow reduction occurred (Figure 2) that gave 8-aminoguanosine as the major product together with a small amount (8-10%) of guanosine (Scheme 3). The identity of both products was confirmed by co-elution with authentic samples of guanosine and 8-aminoguanosine and also by HPLC-MS analysis (see Figure 2, Table 1 and SI). In addition to the reduction products, an additional nucleoside product (~20%) was observed that was identified by HPLC-MS as 8,5'-*O*-cycloguanosine and presumably results from displacement of the nitro group by the 5'-hydroxyl group. Formation of the cyclo-nucleoside is probably facilitated by the rise in pH caused by hydrolysis of the borohydride.

Further proof for the direct displacement of the nitro group by hydride was obtained by performing the reaction using $NaBD_4$ in H_2O, and in this case HPLC-MS revealed the guanosine fraction to have the correct mass for the incorporation of one deuterium atom (see Table 1). As expected, the peak corresponding to 8-aminoguanosine showed no inclusion of deuterium as any incorporated during reduction would exchange through protonation.

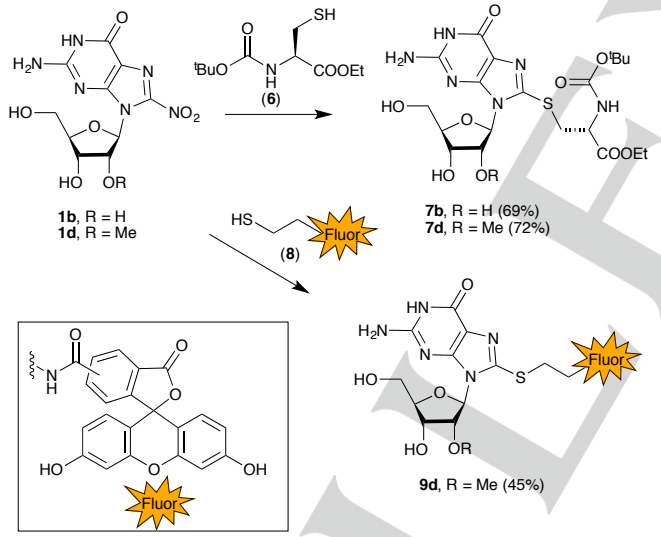
In an attempt to increase the proportion of guanosine, the reduction was investigated under a variety of conditions. Using buffered aqueous conditions between pHs 6.0 – 9.0 (0.1M ammonium acetate) there was little difference in terms of the product distribution from those performed in water, although the reaction at lower pH did progress slightly faster. It is not surprising that all the aqueous reduction reactions gave similar results as the very large excess of borohydride, which raises the pH as it is hydrolysed, resulted in all these reactions becoming alkaline as the reaction proceeded (pH ~ 9.6 at the end of the reaction). No reduction products were observed when the weaker reducing agent sodium cyanoborohydride was used at pH 6.0. The work of Lamson[23a] has established that the denitration of polysubstituted nitrobenzenes proceeded more rapidly in DMSO than in protic solvents. However, when the reaction was performed in DMSO we were unable to detect any reduction products, even during a prolonged reaction (>1 week) and only a trace amount of 8,5'-*O*-cycloguanosine was formed.

Whilst we have been unable to increase the proportion of guanosine above about 12%, its formation by displacement of the nitro group by a hydride equivalent, in aqueous solution, does clearly show that a direct repair of the 8-nitroguanine lesion is a chemically feasible reaction.

Detection of 8-nitroguanine through the displacement of the nitro group by thiols- Methods have been developed to detect the 8-nitroguanine base in tissues[9,10] and urine,[26] but these methods are only applicable once the 8-nitroguanine base has been hydrolytically released from DNA and they give no information as to the steady-state levels of this lesion in DNA samples. We have recently shown that surface-enhanced Raman spectroscopy is a very sensitive technique for detecting this lesion due to the nitro group and can distinguish between the 8-nitroguanine that is covalently bound to DNA or has been released by hydrolysis.[27]

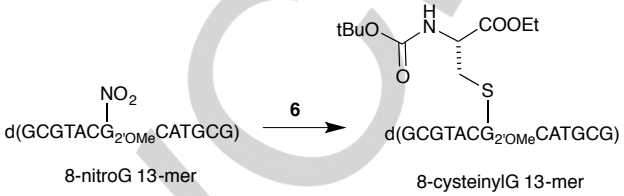
The displacement of the nitro group in 8-nitroguanine nucleosides by sulfur nucleophiles is well established and specific examples include the capture of 8-nitroguanosine by the nucleoside derivative nitroG-Grasp,[28] sulfhydration[12] and protein *S*-guanylation[29] of 8-nitro-cGMP. The mild conditions for these displacement reactions suggested that it might be possible to develop a detection procedure for this lesion based on displacement of the nitro group with a fluorescent thiol. In theory, each lesion site would be converted to a fluorescently labelled guanine base that would be stable to depurination (due to the loss of the strongly electron withdrawing nitro group) and thus create a permanent and quantifiable record of the lesion sites, which in theory could be identifiable through sequencing methods.

As 8-nitro-dG (**1a**), which is the naturally occurring lesion in DNA readily undergoes depurination, our initial experiments to explore this labelling technique were performed on 8-nitroguanosine (**1b**, Figure 1) and 8-nitro-2'-*O*-methylguanosine (**1d,** , Figure 1). Both of these ribonucleosides have a more stable glycosidic bond than their 2'-deoxy counter part, due to the electronegative 2'-oxygen. The 2'-*O*-methylriboside is also ideally suited as a means to incorporate this lesion into oligonucleotides, as no protection of the 2´-position is necessary during DNA synthesis and it has previously been used as a model for 8-nitro-dG in oligonucleotides.[8] We chose to use *N*-(*tert*-butoxycarbonyl)cysteine ethyl ester (**6**, Scheme 4) as a model thiol as its hydrophobicity was expected to significantly increase the retention time of the resulting nucleoside/oligonucleotide adducts on reverse-phase HPLC and its low extinction coefficient at 260 nm, was not expected to significantly interfere with the HPLC analysis. Thus, reaction of 8-nitroguanosine with a slight excess of cysteine thiol (**6**) in aqueous buffer (pH 7.5), gave the *S*-guanylated cysteine derivative (**7b**) in good yield (69%).[30] Analysis of ^{1}H-^{13}C HMBC contour map revealed a long range ^{1}H-^{13}C correlation between the C8 resonance and the protons adjacent to the sulfur atom, thus confirming that the sulfur atom had displaced the nitro group. A similar yield (72%) was obtained for the conversion 8-nitro-2'-*O*-methylguanosine (**1d**) to its corresponding 8-*S*-cysteinyl derivative (**7d**, Scheme 4).



Scheme 4. Reaction of thiols with 8-nitroguanosine nucleosides. Compound **8** is shown as a mixture of the 5(6)-regioisomers.

To apply this labelling technique to model DNA sequences, 8-nitro-2'-*O*-methylguanosine (**1d**) was incorporated into 13-mer, 27-mer and 37-mer oligodeoxynucleotides. These model sequences each contained a single 8-nitroguanine base and were prepared using previously reported procedures.[8] Reaction of the nitro 13-mer sequence (Scheme 5) with a 100-fold excess of cysteine thiol (**6**), in Tris-HCl buffer (pH 8.5) was monitored by RP-HPLC. A relatively smooth reaction was observed with greater than 90% conversion to the less polar cysteinyl 13-mer after 6 hours, together with formation of a small amount of the depurinated 13-mer, which runs slightly faster than the nitro-13-mer (Figure 3, Table 2 for retention times.). The cysteinyl 13-mer was isolated by RP-HPLC and was shown by negative mode electrospray mass spectrometry to have the expected mass (Table 3 in the Experimental section).



Scheme 5. Reaction of nitro-13-mer with cysteine nucleophile **6**.

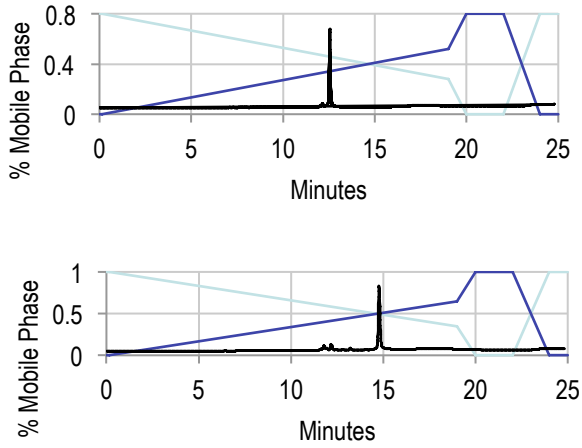


Figure 3. RP-HPLC chromatogram showing reaction of 8-nitro-13-mer with cysteine derivative **6**. Top panel shows the nitro-13-mer starting material and the bottom panel shows the reaction mixture after 6 hours. RP-HPLC was performed as described in the experimental section using a linear gradient of 0% to 65% eluent B (blue line) in eluent A, over 19 min, with a flow rate of 1.0 ml/min. Eluent A = aq TEAB (0.1M), eluent B = 40% CH_3CN in aq TEAB (0.1M). Chromatograms were recorded at 254 nm.

The cysteine nucleophile (**6**) was also used in displacement reactions on the longer 27-mer and 37-mer sequences, which are more representative of the naturally occurring 8-nitroG lesion in DNA. These reactions were carried out under the same conditions as those described for the nitro 13-mer and although they proceeded more slowly, >90% conversion was observed after 11 hours. Both the nitro 27-mer and nitro 37-mer were predominantly converted to a single product with the expected longer retention times when compared to the nitro-containing oligonucleotides (see Table 2 for retention times). As we were unable to obtain mass spectrometry data for the products from these longer oligomers,

the presence the 8-*S*-cysteinyl group in the 27-mer and 37-mer was confirmed by enzymatic digestion. Treatment of the product oligomers with phosphodiesterase and alkaline phosphatase (pH 8.5), followed by HPLC analysis showed the presence of **7d** in the digestion mix, together with the four naturally occurring 2'-deoxynucleosides. When the chromatogram from the digestion mix was analysed to obtain the composition ratios of the component nucleosides, the peak area for **7d** was lower than expected and an unidentified peak, with the UV absorption characteristics of a nucleoside (retention time 9.10 min) was also observed. It was initially thought that the unidentified peak might be a partially digested dinucleotide, but its proportion in the digestion mixture increased as the digestion time increased. Given that the original cysteine nucleophile (**6**, retention time = 19.10 min) could also be detected in the digestion mix, the most likely cause of this unidentified nucleoside was hydrolysis of the 8-*S*-cysteinyl derivative (**7d**) to give 8-oxo-2'-*O*-methylguanosine (**2b**, Figure 1) and releasing (**6**). A standard of 8-oxo-2'-*O*-methylguanosine was prepared[31] and it was shown to have the same retention time on HPLC as the unidentified nucleoside in the digestion mixture, thus implying that 8-*S*-cysteinyl derivative (**7d**) was breaking down in the digestion mixture.

Table 2 Reverse-phase HPLC retention times (min) for nitro oligomers and their reaction products with thiol nucleophiles. HPLC analysis conditions are as described in the legend to Figure 3. Nitro-13-mer = d(GCGTA C**G**CATGCG); nitro-27-mer = d(GTGACTGG**G**AAAACCCTGGCGTTACCC); nitro-37-mer = d(GCGCAACGCAATTAATGT**G**AGTTAGCTCACTCATTAG). **G** = position of 8-nitro-2'-*O*Me-guanosine.

Oligomer length	nitro-oligomers	cysteinyl-oligomers	8-fluoro oligomers
13-mer	12.55	14.78	18.63
27-mer	12.82	14.13	-
37-mer	12.52	13.74	-

Finally, we wanted to use a fluorescent thiol derivative in order to demonstrate the labelling of the 8-nitroG lesion with a readily quantifiable reporter group. We chose to use a cystamine derivative of carboxyfluorescein (**8**), which had previously been prepared as a mixture of regioisomers and used to label cell-penetrating oligoguanidinium compounds.[30] This fluorescent thiol (**8**) was subsequently used to displace the nitro group in 8-nitro-2'-*O*-methylguanosine (**1d**), using similar conditions to those described for the preparation of (**7d,** Scheme 4). As the fluorescent thiol (**8**) is a mixture of regioisomers, the resulting fluorescein adduct formed with 8-nitro-2'-*O*-methylguanosine (**9d**) was more difficult to isolate and characterise than the 8-*S*-cysteinyl derivatives, but the identity of the product was confirmed by high resolution mass spectrometry. The fluorescent thiol (**8**) was also used to label the nitro 13-mer and the resulting fluorescein-tagged 13-mer was isolated by HPLC and identified by electrospray mass spectrometry.

The mild conditions and clean reaction of the cysteine thiol (**6**) with the nitro 13-mer (Figure 3) and longer sequences, clearly indicates the potential for using this reaction to label the site of 8-nitroG lesions in DNA. However, whilst the hydrolysis of the 8-*S*-cysteinyl guanosine derivative (**7d**) during the enzymatic digestion was not evident when analysing and manipulating the 8-*S*-cysteinyl oligodeoxynucleotides, this reaction does suggest that the labelling conditions might have to be optimised to eliminate hydrolysis to the 8-oxo guanine nucleoside. The observed hydrolytic breakdown of *S*-guanylated nucleosides does suggest a potential additional source of 8-oxoguanosine nucleosides/nucleotides in biological systems.

Conclusions

These studies reveal that the 8-nitro group exerts a significant effect on the reactivity of the guanine base. In particular, the reactivity of the guanine NH_2 group towards electrophiles is significantly reduced, whilst the reactivity of the NH group appears to be enhanced, due to its lower pKa. The ease with which the nitro group is replaced by thiol nucleophiles under mild conditions and the high efficiency of this reaction show that sites of guanine nitration in DNA can be fluorescently labelled using suitably derivatised thiols. The derivatisation process leaves a permanent marker of the original lesion that can be quantified. By using the appropriate thiol (e.g. a thiol derivatised crown ether) it should be relatively easy to adapt this labelling technique so that the 8-nitroguanine base is converted into a current-modulating marker that can be read through nanopore sequencing.[32]

Finally, we have shown that reduction of 8-nitroguanosine with sodium borohydride in aqueous solution gives 8-aminoguanosine as the major product, but is also accompanied by a denitration reaction to give guanosine. The formation of guanosine, in the presence of a hydride equivalent, demonstrates that the direct repair of the 8-nitroguanine lesion is a chemically feasible reaction.

These results significantly enhance our knowledge of the chemical properties of this base modification and provide new methods to detect and study this lesion.

Experimental Section

8-Nitroguanosine (**1b**) and 8-nitro-2'-*O*-methylguanosine (**1d**) were prepared as previously described.[8] 2'3'5'-Tri-*O*-acetyl-8-nitroguanosine (**1c**) was prepared as described by Saito *et al.*[29(b)] *N*-(*tert*-butoxycarbonyl)cysteine ethyl ester (**6**) was prepared by esterification of *N*-(*tert*-butoxycarbonyl)cystine.[33] 8-Oxo-2'-*O*-methylguanosine was prepared from 8-bromo-2'-*O*-methylguanosine[8] following the procedure of Nampalli and Kumar.[31] 5(6)-Carboxyfluorescein was purchased from Sigma-Aldrich as a mixture of 5- and 6-carboxy regioisomers.

All RP-HPLC was performed using Gemini™ C18 columns (110 Å, 250 mm x 4.6 mm) purchased from Phenomenex. RP-HPLC analysis of oligonucleotides was performed on an automated Gilson HPLC system equipped with an autoinjector, a photodiode array detector and dual hydraulic pump, operating with a flow rate 1 ml/min. Chromatograms were recorded at 254 nm. Chromatographic data were controlled and processed using UniPoint© Version 3.0. All oligonucleotides were purified

by RP-HPLC using a triethylammonium bicarbonate (TEAB) buffer system. A 1M stock solution of TEAB was prepared by bubbling CO_2 through a 1M aqueous solution of Et_3N for 6 – 8 hours 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.

RP-HPLC analysis of 8-nitroguanosine reduction reactions were performed on an Agilent 1260 Infinity system equipped with an autoinjector, a photodiode array detector and quaternary pump. Chromatographic data were controlled and processed using Agilent Open LAB Chemstation software. An elution gradient of 0-20% acetonitrile in 0.1% aqueous acetic acid over 20 min, with a flow rate of 0.5 ml/min. Chromatograms were recorded at 254 nm.

LC-MS was performed on an Agilent 6530B accurate mass Q-TOF G6530B mass spectrometer connected to an Agilent 1260 Infinity HPLC system as described above, with a flow rate of 0.5 ml/min. LC-MS data was obtained using a multimode ion source and processed using Agilent Masshunter software. Spectra were recorded in negative ionisation mode.

All NMR spectra were recorded on a Bruker 400 MHz spectrometer; ^{1}H (400 MHz) and ^{13}C (100 MHz). All chemical shifts are reported in p.p.m. and coupling constants (J) in Hz. ^{13}C spectra were ^{1}H decoupled. ^{1}H and ^{13}C chemical shifts are relative to an internal standard of tetramethylsilane. Mass spectra of nucleosides and chemically synthesised ODNs were recorded on a MicroMass LCT mass spectrometer using electrospray ionisation (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 ionisation.

Table 3. Mass data for all the 13-mer oligonucleotides, obtained by ES ionisation as described above. 13-mer sequence = d(GCGTAC**G**CATGCG), **G** = position of modification.

Oligonucleotide	Calculated exact mass	Measured accurate mass
8-NitroG-13mer	4050.7025	4050.6133
Cysteinyl 13-mer	4250.7996	4250.6850
Fluoroscein 13-mer	4438.7810	4438.7090

Oligodeoxynucleotide synthesis and purification

Synthesis and purification of 13- and 27- and 37-mer oligodeoxynucleotides containing 8-nitro-2'-*O*-methyl-guanosine was performed as previously described.[8] All oligodeoxynucleotides containing the 8-nitroguanine modification showed the characteristic absorption at 395 nm.

Borohydride reduction of 8-nitroguanosine

8-Nitroguanosine (1.0 mg, 3.05 µmoles) was dissolved in distilled water (1 ml) with stirring. Sodium borohydride (5.8 mg, 0.153 mmoles, 50 equiv) was added, with additional portions (50 equiv) subsequently added every 30 mins for 90 mins. Reaction monitored by HPLC, with samples taken every 30 mins..

8-cysteinyl labelled oligomers

8-cysteinyl 13-mer = d(GCGTAC$\underline{G}^{(tBu\text{-}Cys)}$CATGCG)
8-cysteinyl 27-mer = d(GTGACTGG$\underline{G}^{(tBu\text{-}Cys)}$AAAACCCTGGCG-TTACCC)
8-cysteinyl 37-mer = d(GCGCAACGCAATTAATGT$\underline{G}^{(tBu\text{-}Cys)}$AGT-TAGCTCACTCATTAG)

To a solution of the appropriate 8-nitro oligomer (3.0 OD (260nm) units) dissolved in distilled H_2O (100µl) was added 1M TRIS.HCl (pH 8.5,10µl) and ethyl *N*-(*tert*-butoxycarbonyl)-cysteinate (6, 0.80mg, >100eq). The resulting mixture was stirred at 37°C for 6 hours during which time the progress of the reaction was monitored by RP-HPLC using a gradient of 0-26% acetonitrile in TEAB (0.1 M). Retention times for the oligonucleotide substrates and products are presented in Table 2. Oligonucleotide products were purified using the same gradient and the fractions containing the products were evaporated 3 times with distilled water in a vacuum centrifuge.

8-Fluoroscein labelled oligomer

8-Fluoroscein 13-mer = d(GCGTAC$\underline{G}^{(Fluro)}$CATGCG). To a solution of the 8-nitro 13-mer (3.1 OD units) dissolved in distilled H_2O (100µl) was added 1M TRIS.HCl (pH 8.5, 10µl) and 3',6'-dihydroxy-3-oxo-*N*-(2-sulfanylethyl)-3*H*-spiro[2-benzofuran-1,9'-xanthene]-5(6)-carboxamide (8, 1.7mg). The resulting mixture was stirred at 37°C for 24 hours during which time the progress of the reaction was monitored by RP-HPLC using a gradient of 0-26% acetonitrile in TEAB (0.1 M). Retention times for the oligonucleotide substrates and products are presented in Table 2. Oligonucleotide products were purified using the same gradient and the fractions containing the products were evaporated 3 times with distilled water in a vacuum centrifuge.

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