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Efficient Synthesis of DNA Containing the Guanine Oxidation-Nitration Product 5-Guanidino-4-nitroimidazole: Generation by a Postsynthetic Substitution Reaction

William L. Neeley, Paul T. Henderson,[†] and John M. Essigmann*

Department of Chemistry and Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

jessig@mit.edu

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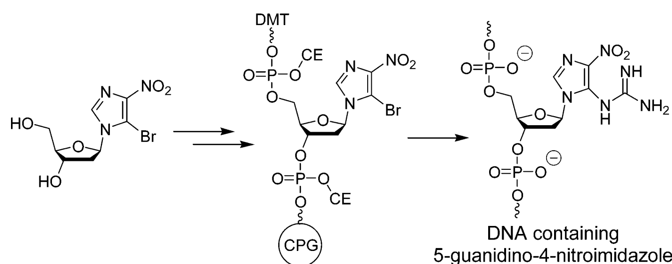
ABSTRACT



A convertible nucleoside was synthesized and used to prepare the 2′-deoxynucleoside of 5-guanidino-4-nitroimidazole, a putative in vivo product of the reaction of peroxynitrite with guanine. The convertible nucleoside was incorporated into an oligodeoxynucleotide by the phosphoramidite method and converted postsynthetically to yield an oligodeoxynucleotide containing 5-guanidino-4-nitroimidazole at a specific site. The oligodeoxynucleotide was inserted into a viral genome. Melting temperature analysis revealed that duplexes containing 5-guanidino-4-nitroimidazole were greatly destabilized relative to unmodified duplexes.

Of the four nucleobases of DNA, guanine has the lowest oxidation potential, and thus considerable attention has been given to the study of the oxidation of this base.[1] The DNA lesions 7,8-dihydro-8-oxoguanine (8-oxoG) and 8-nitroguanine (8-nitroG) are two major guanine (G) oxidation products that are formed by reaction with the endogenous oxidant peroxynitrite ($ONOO^-$).[2] These lesions are also labile to oxidation and form a variety of secondary oxidation products.[3−5] These products may result from in vivo oxidation by oxidants other than $ONOO^-$,[1,6] and many are susceptible to hydrolysis or further oxidation. In contrast, 5-guanidino-4-nitroimidazole (NI) forms directly from the oxidation of G by $ONOO^-$ and is both chemically stable and resistant to tested forms of enzymatic repair in vitro.[7−9] Additionally,

[†] Present address: Lawrence Livermore National Laboratory, Biology and Biotechnology Research Program, L-411, Livermore, CA 94551

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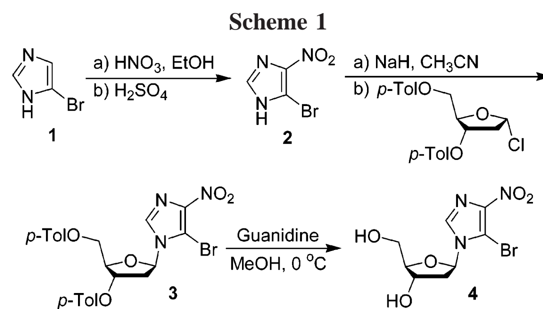
this lesion may be well-bypassed by polymerases and mutagenic, as suggested by in vitro primer extension assays.[7] NI therefore may contribute to the mutagenic spectrum of DNA damage induced by $ONOO^-$, and its measurement may be useful as a specific biomarker of $ONOO^-$-mediated oxidation.

Evaluation of the chemical and biological properties of oxidative DNA lesions is facilitated by the availability of oligodeoxynucleotides (ODN) and, eventually, genomes containing these lesions site-specifically.[1] Most commonly, oxidative lesions are prepared site-specifically by damaging a specific base within an ODN.[10−12] This approach facilitates and accelerates the initial characterization of these lesions while circumventing the common problem of lesion instability, a major obstacle to incorporation of these damaged bases by automated DNA synthesis. However, low yields and the formation of multiple products that are often difficult to separate limit the use of ODN sequences to those containing only one G residue. The present methods for preparing NI-containing ODNs are hindered by both of these characteristics.[7,8] In advance of biological evaluation of NI mutagenicity and genotoxicity, we sought to develop a synthetic method for the preparation of NI lesions site-specifically within ODNs that is high-yielding and independent of base sequence.

A convertible nucleoside phosphoramidite offers a convenient route to incorporating an unnatural nucleoside at a defined site within an ODN.[13] This approach has been used successfully to prepare ODNs containing nucleosides that are unstable to DNA synthesis conditions,[14,15] containing structural analogues of a given base, and with additional functionality.[16−22] In the present case, the use of the convertible nucleoside approach would avoid potential protection-deprotection schemes with the guanidino functionality of NI, enabling an efficient synthesis of a phosphoramidite building block. Herein, we describe the synthesis of a convertible nucleoside phosphoramidite for the site-specific incorporation of an NI lesion into an ODN by postsynthetic substitution and the effect of this lesion on duplex stability. Although we only present the synthesis of the biologically relevant NI lesion, the method allows access to other potentially useful alternative structures.

The convertible nucleoside **4** was synthesized in three steps beginning with 5(4)-bromo-1*H*-imidazole (**1**) (Scheme 1).

Scheme 1



5(4)-Bromo-4(5)-nitroimidazole (**2**) was prepared in 91% yield by nitrating **1**.[23] Treatment of **2** with NaH in CH_3CN, followed by condensation with 3,5-di-*O*-toluoyl-α-1-chloro-2-deoxy-D-ribofuranose gave exclusively two *β*-nucleosides, the 5-bromo-4-nitroimidazole isomer **3** in 50% yield and the 4-bromo-5-nitroimidazole isomer in 35% yield. The structures of these isomers were assigned by comparison of the UV spectra with those of literature analogues.[24,25] Interestingly, 4-halo-5-nitroimidazoles have been reported to be resistant to nucleophilic displacement of the halo substituent.[26,27] In the present study, the 5-bromo-4-nitroimidazole isomer readily underwent reaction at the site of the bromo substituent, whereas the 4-bromo-5-nitroimidazole isomer was resistant to reaction at the bromo position under the same conditions.

Saponification of the toluoyl esters of **3** was achieved in 90% yield by treatment with guanidine in MeOH for 3.75 h at 0 °C. Initial attempts to deprotect **3** using reagents such as NaOMe in MeOH, NH_4OH, and K_2CO_3 in MeOH were unsuccessful because of the decomposition of **3**, presumably as a result of displacement of the bromo substituent. The *β* conformation of **4** was confirmed by NOE NMR spectroscopy. Irradiating at the 1′ proton, we observed an NOE enhancement at the 4′ proton, and by irradiating the imidazolic proton NOE enhancements were observed at the 3′ proton and 5′ protons, thus confirming the structure. It is anticipated that **4** will serve as a branching point for generating libraries of 5-substituted-4-nitroimidazole nucleosides and, when incorporated into DNA, 5-substituted-4-nitroimidazole-containing ODNs, since previous studies have shown the bromo substituent of 5-bromo-4-nitroimidazoles to be displaced by carbon,[25] sulfur and oxygen,[27] and nitrogen[9] nucleophiles.

For later use as an analytical standard, the 2′-deoxynucleoside of NI (dNI, **5**) was synthesized from **4** in nearly

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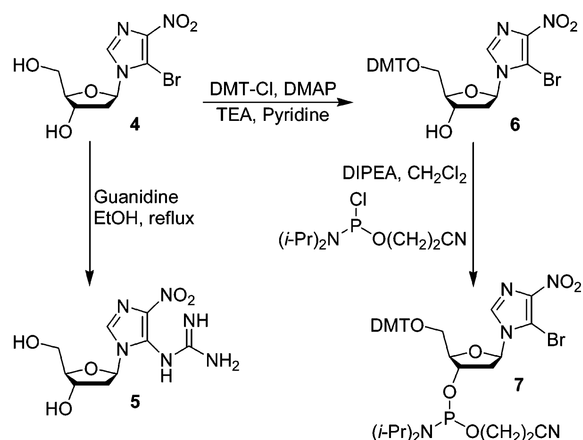
Scheme 2



quantitative yield by treatment with guanidine in refluxing EtOH (Scheme 2). Having prepared **4** and **5**, we next determined the stability of these compounds to DNA synthesis conditions. The half-life of **4** was found to be less than 15 min in 3% trichloroacetic acid in CH_2Cl_2 but greater than 120 min in 2% dichloroacetic acid in CH_2Cl_2. Compound **4** was also stable for at least 24 h under standard oxidizing conditions used for DNA synthesis (0.02 M I_2 in pyridine/H_2O) and therefore was determined to have the stability necessary for automated DNA synthesis. Compound **5** was stable to treatment with concentrated NH_4OH for at least 4 h at room temperature. Under these conditions, phenoxyacetyl-protected ODNs can be cleaved from the solid support and deprotected.

Compound **4** was protected as the 5′-*O*-dimethoxytrityl ether using DMT-Cl, triethylamine, and a catalytic amount of DMAP in pyridine (Scheme 2). Two rounds of purification of the tritylated product by flash chromatography were necessary and gave the desired compound **6** in 76% yield. Compound **6** was subsequently treated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite and diisopropylethylamine to produce the phosphoramidite **7** in 44% yield. Alumina chromatography was necessary for the purification of **6** and **7**, as both were unstable to purification on silica, even in the presence of triethylamine to reduce the acidity of the silica.

Compound **7** was incorporated into a 19mer ODN of mixed base sequence (**8X**, see Table 1 for structure and nomenclature) using standard automated procedures, with the exception that 3% dichloroacetic acid in CH_2Cl_2 was substituted for the deblocking reagent to minimize abasic site formation. The coupling efficiency after 20 min was greater than 95% as judged by trityl monitoring. Postsynthetic treatment of the DNA with 0.5 M guanidine in THF at room temperature caused a rapid color change of the solid support from white to bright yellow, indicating the production of NI. Deprotection of the DNA and cleavage from the support was achieved with concentrated NH_4OH for 4 h at room temperature. HPLC analysis of the crude **8NI** (Figure 1) showed a single major peak with absorbance maxima at 260 and 380 nm. The purified **8NI** was characterized by nuclease

Table 1. Melting Temperatures and Free Energies of Duplexes[a]

8X 5′-GCGAAGACC G**X**A GCG TCC G-3′
10Y 3′-CGC TTC TGG C**Y**T CGC AGG C-5′

X	Y	T_m (± 0.5 °C)	$-\Delta G_{37°C}$ (kcal/mol)
NI	G	57.0	16.8 ± 0.1
NI	A	56.4	16.7 ± 0.0
NI	C	55.8	16.7 ± 0.1
NI	T	54.8	16.0 ± 0.0
G	G	61.0	19.3 ± 0.0
G	A	62.4	20.4 ± 0.0
G	C	66.9	23.0 ± 0.1
G	T	60.6	19.4 ± 0.0

[a] Conditions: 50 mM sodium chloride, 5 mM sodium phosphate, pH 7.0. T_m and $-\Delta G_{37°C}$ values are the average of three melts.

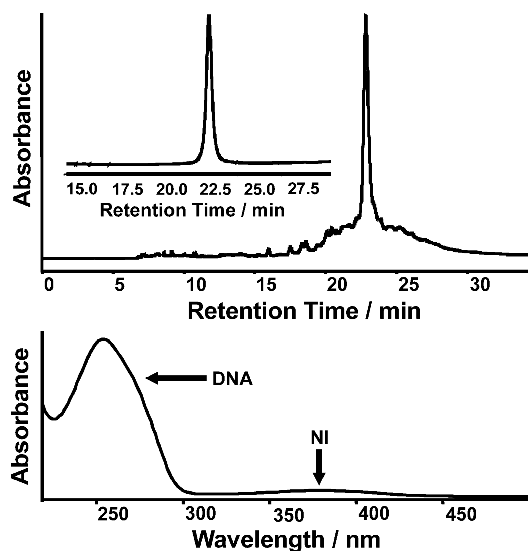


Figure 1. (Top) HPLC trace at 254 nm of the converted, deprotected **8NI** and purified **8NI** (inset). (Bottom) UV−vis spectrum of purified **8NI**.

and phosphatase digestion followed by HPLC analysis and by MALDI-TOF and ESI mass spectrometry.

Digestion of **8NI** with snake venom phosphodiesterase (SVPD) and alkaline phosphatase yielded the five expected nucleosides, with the dNI peak eluting with the same retention time and having the same UV−vis spectrum as the authentic standard **5**. It has been reported that SVPD is unable to cleave the phosphodiester bond in the sequence context 5′-C[NI]-3′.[8] We did not observe this effect in our sequence context; however, in the sequence 5′-TT[NI]TTTTTTT-3′ (**9NI**), whereas SVPD could completely digest the ODN, nuclease P1 could not and left an NI-containing dinucleotide. As previously reported, MALDI mass spectrometry allowed detection of two degradation products, presumably of the nitro substituent induced by the MALDI laser.[8] MALDI-induced degradation has been previously observed during MALDI analysis of aromatic nitro compounds.[28,29] Analysis

by ESI mass spectrometry did not show these degradation products.

Thermal denaturation studies were performed to determine the stability of duplexes containing NI paired with each of the four natural bases (**8NI·10Y**) relative to the stability of unmodified duplexes (**8G·10Y**) (see Table 1, which also defines nomenclature for **8** and **10**). We found the NI lesion greatly destabilizes the duplex. The presence of the NI lesion results in a 10−12 °C decrease in T_m relative to an unmodified duplex containing a G·C base pair at the same position and a 4−6 °C decrease in T_m relative to the least thermally stable mismatch of the unmodified duplex (G·T). A comparison of all T_m values and free energies shows a greater difference in magnitude among the unmodified duplexes than among the NI-containing duplexes, demonstrating that the presence of the lesion generally reduces the effect of the opposing base on duplex stability.

Given that a large reduction in thermal stability does not imply a large change in duplex structure and similarity among thermal stabilities does not necessitate structural similarities,[30] it is not possible to draw conclusions from the thermal stabilities alone about the effect of the NI lesion on duplex DNA structure. In an effort to probe the origins of the observed duplex destabilization, a simple AM1 semiempirical method[31] was used to optimize the geometry of **5** (see Supporting Information). The calculation predicts that the guanidino group will be rotated out of plane in relation to the imidazole ring and extend roughly 3 Å perpendicularly in either direction from the plane of the ring. Accommodation of such a structure into the helix would require at minimum a local distortion of the duplex and a disruption of the base π-stacking interactions.

We have site-specifically inserted **8NI** into an M13 bacteriophage genome for mutagenesis and genotoxicity studies (see Supporting Information).[32] The ligation efficiency of the genome construction was similar to that observed for **8G**. Mutagenesis and repair studies using this construct will be reported elsewhere.

In summary, we have developed a synthetic route for the preparation of **5** and provided a method for the incorporation of NI lesions into DNA by the phosphoramidite method utilizing the convertible nucleoside approach. Ready access to **5** will be beneficial for use as an analytical standard in future studies aimed at detecting the NI lesion in vivo, whereas the convertible nucleoside phosphoramidite will allow for the rapid preparation of NI-containing ODNs for mutagenesis and DNA repair studies, creation of libraries of 5-substituted-4-nitro analogues of NI (which may be useful as structural probes), and preparation of NI-containing ODNs in sufficient quantities for NMR and X-ray structure studies.

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Supporting Information Available: Experimental procedures; NMR, UV−vis, and mass spectra; HPLC traces; melting curves; AM1 structure image of **5**; figure of stained agarose gel from genome construction. This material is available free of charge via the Internet at http://pubs.acs.org.

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