JOC*Note*

Synthesis and Characterization of the Oxidized dGTP Lesions Spiroiminodihydantoin-2′-deoxynucleoside-5′-triphosphate and Guanidinohydantoin-2′-deoxynucleoside-5′-triphosphate

Yu Ye, James G. Muller, and Cynthia J. Burrows*

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

burrows@chem.utah.edu

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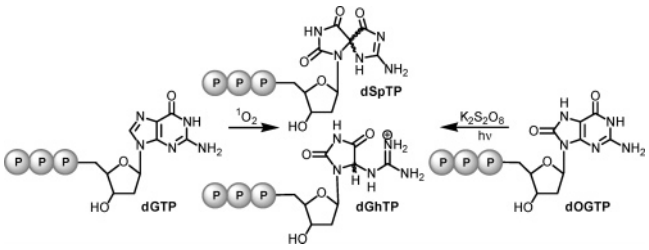

Two convenient synthetic routes to the oxidized guanosine triphosphate lesions spiroiminodihydantoin-2′-deoxynucleoside-5′-triphosphate (dSpTP) and guanidinohydantoin-2′-deoxynucleoside-5′-triphosphate (dGhTP) are reported. Both two-electron oxidation of 2′-deoxy-7,8-dihydro-8-oxoguanosine-5′-triphosphate (dOGTP) using $SO_4^{\bullet-}$ generated photolytically from $K_2S_2O_8$ or four-electron oxidation of 2′-deoxyguanosine-5′-triphosphate (dGTP) from singlet oxygen provide either dSpTP or dGhTP at pH 8.0 or 4.4, respectively. Highly purified triphosphates are obtained by ion pair reversed-phase HPLC.

Oxidative damage occurring at DNA bases has been the focus of considerable interest during the past decade owing to its implications in mutagenesis, carcinogenesis, and aging.[1−3] DNA and the nucleotide triphosphate (dNTP) pool are subject to damage by both endogenous and exogenous agents, resulting in DNA strand breaks, abasic sites, DNA−protein cross-links, and oxidized bases that require DNA repair enzymes to maintain the integrity of the genome.[2] Recent studies demonstrated that key intermediates in the oxidation of guanosine (G) as well as its common oxidation product 7,8-dihydro-8-oxoguanosine (OG), which is sensitive to further oxidation, are susceptible to adduction by nucleophiles including water,[4] alcohols,[5] peroxynitrite,[6] polyamines,[7] amino acids, and proteins.[8] When H_2O serves as the nucleophile, the products spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) have been unambiguously identified from both G and OG oxidation (Scheme 1).[4,9−17] Our previous work has demonstrated that Sp is the thermodynamic product in nucleosides under neutral or slightly basic conditions, while Gh is preferred at pH < 6 in nucleosides or near neutral pH in oligomers.[9,10,12,18] Both Sp and Gh are proposed to derive from the common intermediate 5-OH-OG, an analogue of which has been characterized at low temperature by Foote and coworkers.[19] Rearrangement of 5-OH-OG to Sp requires a 1,2-acyl shift that is favorable for the unencumbered nucleoside, but is hindered in base-stacked oligomers. Formation of Gh follows the same pathway as uric acid oxidation to allantoin;[20] in this mechanism, hydration at C6 and cleavage of the C6−N1 bond is facilitated by protonation of the guanidine group at lower pH. Decarboxylation then leads to Gh.

Previous in vivo[21] and in vitro[22,23] mutagenesis assays indicate that both Sp and Gh are >98% mutagenic and cause G to C and G to T transversions, whereas OG is only about 5% mutagenic, leading to G to T transversions. The mutagenicity of 2′-deoxy-7,8-dihydro-8-oxoguanosine-5′-triphosphate (dOGTP) originates with its incorporation into duplex DNA by various polymerases,[24,25] although this process can be prevented by repair enzymes such as *Escherichia coli* MutT and its human homologue hMTH, which hydrolyze dOGTP to dOGMP.[26,27] However, it has been recently argued by Tassotto and Mathews

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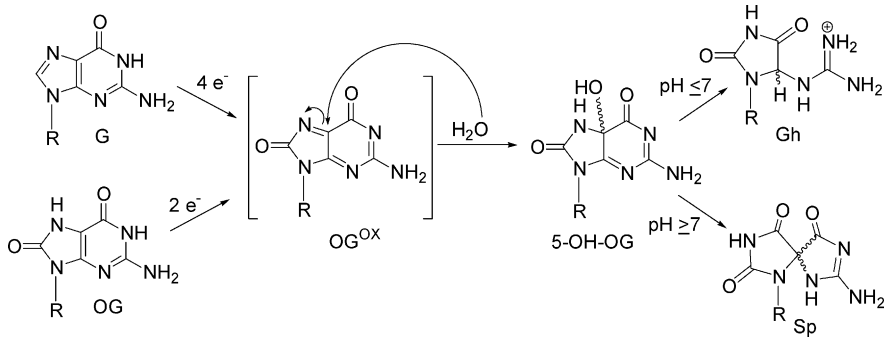
SCHEME 1. Oxidation Pathways from Guanosine and 8-Oxoguanosine



that dOGTP may not be the best substrate of MutT.[28] Due to the lower redox potential of OG (0.5 V lower than G),[29] dOGTP may be oxidized to spiroiminodihydantoin-2′-deoxynucleoside-5′-triphosphate (dSpTP) and to a lesser extent guanidinohydantoin-2′-deoxynucleoside-5′-triphosphate (dGhTP) under physiological conditions. Therefore, it is of current interest to study how cells process dSpTP and dGhTP including their incorporation into replicating DNA by polymerases and their hydrolysis by the DNA repair enzymes MutT and hMTH. These studies depend on the synthesis of dSpTP and dGhTP, which we report herein.

A common synthetic method for modified nucleoside triphosphates involves phosphorylation of the modified nucleoside by chemical or enzymatic methods.[30−32] Chemical phosphorylation typically involves treatment with phosphoryl chloride and subsequent displacement of the chloride with pyrophosphate, introducing reagents that may not be compatible with the sensitive glycosidic bonds of the Sp and Gh nucleosides. In addition, efficient enzymatic phosphorylation generally requires strong binding between the enzyme and substrate; this interaction has been shown to be sensitive to the nature of the purine or pyrimidine base. For example, OGMP cannot be phosphorylated enzymatically by guanylate kinase.[32] Furthermore, each of the above synthetic routes requires multiple purification steps that complicate the synthesis and reduce the overall yield. On the basis of the above concerns, we chose to start the synthesis of dSpTP and dGhTP with the triphosphate groups already present using commercially available 2′-deoxyguanosine-5′-triphosphate (dGTP) and dOGTP.

The major difficulty associated with the synthesis of dSpTP and dGhTP from dGTP or dOGTP was anticipated to be the removal of salts from low molecular weight organic polyanions. In addition, a small amount of contamination from the starting dNTP could result in misleading polymerase data, so we devised synthetic routes that would generate dSpTP and dGhTP from either dOGTP or dGTP. Synthesis of dSpTP and dGhTP from both triphosphate precursors allows one to test for erroneous enzymatic results caused by trace contaminants that are better enzyme substrates. In this Note, we describe the first synthesis and characterization of dSpTP and dGhTP.

Previous work in our lab documented the formation of Gh and Sp by the oxidation of OG nucleoside with Na_2IrCl_6.[4,9,10,22] However, this method cannot be applied to the Gh and Sp triphosphate synthesis due to the difficulties of removing iridium salts, and the pathway is low-yielding when conducted with G as the starting material. Alternatively, Sp and Gh nucleosides can be synthesized by singlet oxygen oxidation of guanosine under controlled pH conditions.[12]

In the present work, synthesis of dSpTP from commercially available dOGTP proved to be the most efficient method. While numerous oxidants can be used to convert OG to Sp or Gh, we found that potassium persulfate, generating sulfate radical under UV photolysis, is the most advantageous route since it produces potassium sulfate as a benign side product. The pH-dependent results obtained (Scheme 2) were consistent with those previously observed for the oxidation of *O*-protected OG or G nucleosides by either Na_2IrCl_6[9] or singlet oxygen.[12] With *O*-protected guanosine as substrate, singlet oxygen as well as hypochlorous acid both yield Sp at pH > 6 and Gh at pH < 6.[16] Similar pH effects were also observed previously for the oxidation of *O*-protected OG nucleoside by peroxynitrite or $CoCl_2/KHSO_5$.[13] However, Henderson and co-workers recently reported that Gh was the major product for the oxidation of dOG at all pH values ranging from 4.6 to 8.4 at 22 °C, while Sp is the main product only at higher temperatures (pH 7.2, 65 °C),[33] suggesting that there could be an effect of an unprotected 5′-OH on the partitioning between Gh and Sp.

Purification of dGhTP and dSpTP is accomplished by a combination of size exclusion and ion pair chromatography. Since dSpTP and dGhTP do not have strong UV absorptions at 260 nm, traditional ion exchange chromatography using high salt could not be employed for the purification of dSpTP and dGhTP. Therefore, we turned to ion pair chromatography[34] to analyze and purify dSpTP and dGhTP. The use of the ion pair reagents, tetrabutylammonium sulfate (TBS) or tetrabutylammonium acetate (TBAA), in conjunction with a C18 reversed-phase column, allows the separation of anions with little background absorption at 220 nm. The two diastereomers of dSpTP were separated using ion pair chromatography, although their absolute configurations have not yet been assigned. The UV−vis spectra of dSpTP shown on ion pair chromatography were consistent with what was previously obtained for the Sp

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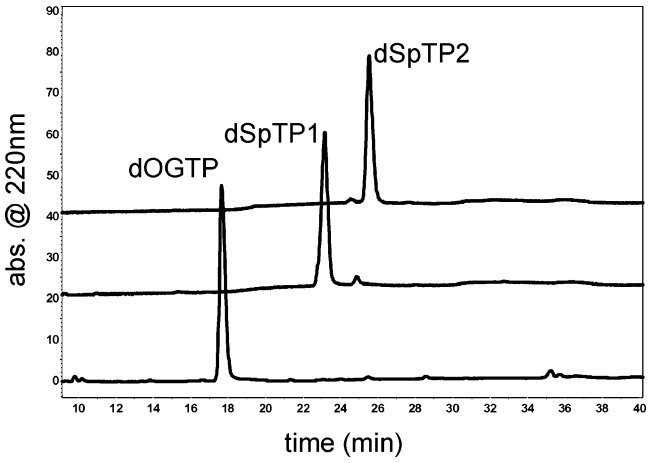


FIGURE 1. HPLC analysis of purified diastereomers of dSpTP.

nucleoside.[4] After initial desalting by size exclusion chromatography, the purity was observed to be approximately 90% for dGhTP and 95% for dSpTP. After ion pair HPLC purification, dSpTP was 99% pure (see Figure 1). The reaction yield of dSpTP was 75% prior to chromatography, and the final purified yield of dSpTP was 50%. Note that dGhTP is also formed as a mixture of two diastereomers, but we have previously shown that these interconvert over a period of hours via enolization of the hydantoin ring.[9,12]

An alternate synthetic approach from dGTP involved its oxidation by $^1O_2$ at various pH values. Under acidic conditions (pH 4.4), dGhTP was formed preferentially while dSpTP was generated at slightly basic conditions (pH 8.0). The purity of dSpTP was approximately 90% after purification by size exclusion chromatography, and the overall purified yield was estimated to be 35%.

The purified dSpTP was analyzed by various MS experiments in comparison to prior studies with protected nucleosides.[4] ESI/MS results showed masses corresponding to dSpTP, dSpDP, and dSpMP (Figure 2). We speculated that dSpTP was undergoing fragmentation, generating dSpDP and dSpMP during ESI/MS analysis. To test this theory, a parent ion ESI/MS experiment was preformed, which unambiguously demonstrated that the dSpDP ion was derived from dSpTP. Furthermore, the identity of dSpTP was confirmed by an MS fragmentation experiment. This experiment consisted of selectively cleaving the glycosidic bond and subsequently forming the free nucleobase. The fragmentation pattern was consistent with the results formerly obtained for Sp nucleoside.[4] In addition, an LC-ESI/MS experiment of dSpTP indicated that the two diasteromers of dSpTP eluted at 21 min with a corresponding mass of 538, along with masses associated with dSpDP and dSpMP. Similarly, the LC-ESI/MS results associated with a crude dGhTP sample revealed the masses of dGhTP, dGhDP, and dGhMP at 19 min. From these studies, we conclude that the triphosphate moiety remains intact during synthesis and purification and that

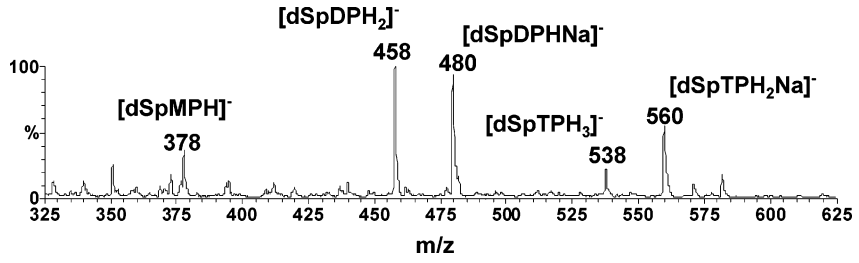
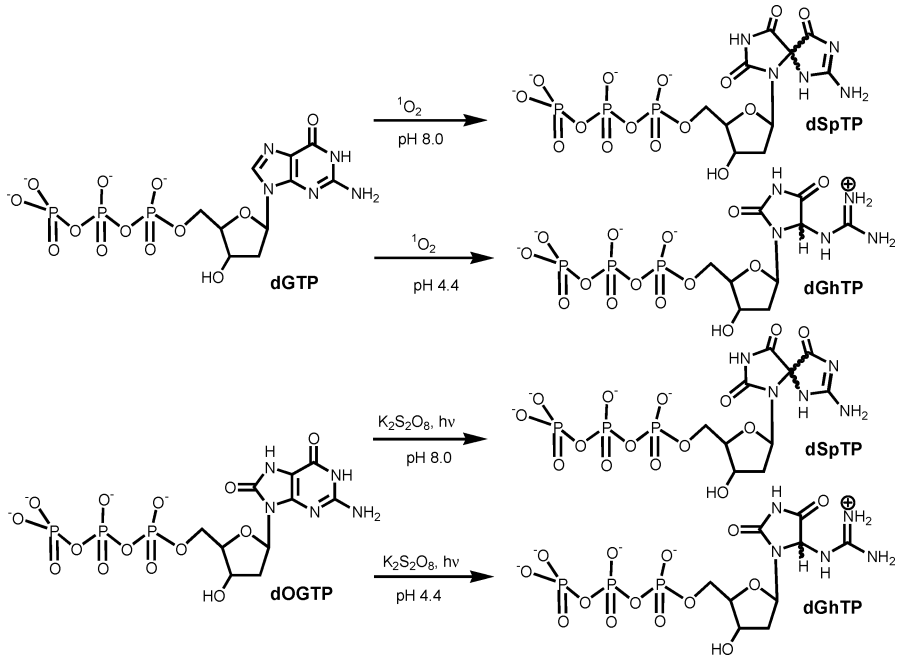


FIGURE 2. Negative ion ESI-MS analysis of purified dSpTP.

SCHEME 2. Oxidation Routes from dGTP and dOGTP



fragmentation to diphosphates and monophosphates only occurs in the mass spectrometer during analysis.

NMR experiments were also conducted, and the ^{31}P NMR of dSpTP showed three clusters of peaks corresponding to α, β, and γ phosphorus atoms. The splitting pattern suggested that the two doublets at −4.6 ppm were derived from the two α ^{31}P of the diastereomers in contrast to the doublet observed for ATP.[35] The γ P at the terminus is expected to be the least perturbed by the presence of diasteromers and is observed to be two peaks at −9.2 ppm. Furthermore, the ^{31}P NMR associated with the β phosphorus was complicated by the splitting by both α ^{31}P and γ ^{31}P and appeared as a broad multiplet at −17.8 ppm.

The heteronuclear multiple bond correlation (HMBC) NMR experiment of 2′,3′,5′-tri-*O*-acetylspiroiminodihydantoin (SpOAc) demonstrated the correlation of H1′ proton with the C2 carbonyl on the hydantoin ring, which was reported by Adam and co-workers for the spiroiminodihydantoin 2′-deoxynucleoside.[14] The resonance for the H1′ proton was assigned based on the fact that it is the only doublet in the sugar region. The same correlation of the H1′ proton and C2 carbonyl of the hydantoin ring was also found for the 2′,3′,5′-tri-*O*-acetylguanidinohydantoin (GhOAc, see the Supporting Information). The HMBC of the dinucleoside monophosphate (d(GhpT)) containing Gh has been reported previously,[15] but the correlation between the H1′ proton and the C2 carbonyl carbon of the hydantoin ring was not observed. The results of the current NMR study support the structures of Sp and Gh as assigned based upon mass spectroscopy plus ^{13}C and ^{15}N NMR experiments.[4,12]

In conclusion, we have described effective methods to prepare highly purified dSpTP. Our protocol involves oxidation of either G by singlet oxygen or OG by potassium persulfate, desalting by size exclusion chromatography, and purification by ion pair chromatography. The purified dSpTP will be utilized in subsequent enzymatic studies. The same methods are applicable to dGhTP by lowering the pH of the solution, although the yield is lower than that of dSpTP. The cellular relevance of Sp has recently been reported for repair-deficient *E. coli* cells under oxidative stress,[36] and this finding spurs further interest in determining the molecular basis for the high mutagenicity of these lesions.

Experimental Section

Synthesis of Spiroiminodihydantoin-2′-deoxynucleoside-5′-triphosphate from 2′-Deoxyguanosine-5′-triphosphate and 7,8-Dihydro-8-oxo-2′-deoxyguanosine-5′-triphosphate. A solution consisting of dOGTP (0.04 mg, 0.08 μmol), 100 μL of 40 mM $K_2S_2O_8$ (1.1 mg, 4 μmol) dissolved in 75 mM potassium phosphate (pH 8.0, adjusted with NaOH), was irradiated at a distance of 7−8 cm with a short wavelength UV lamp for 30 min at room temperature (yield 75%). The resulting solution was passed through a Sephadex G-25 column to remove salts. The resultant fractions were further purified by ion pair HPLC with a 4.6 mm × 250 mm C-18 reversed-phase column and a gradient solvent system consisting of 5% solvent B to 20% solvent B over 40 min. Solvent A consisted of 5 mM tetrabutylammonium sulfate and 10 mM sodium phosphate (pH 7), while solvent B was acetonitrile. The flow rate was 1 mL min^{-1}. The UV spectra were recorded at 220 nm. Overall yield ≈ 50%.

Alternatively, a solution consisting of dGTP (2 mg, 4 μmol), 1 mL of 0.3 mM Rose Bengal (0.3 mg, 300 μmol) dissolved in 75 mM potassium phosphate (pH 8.0), was incubated at 4 °C in a water bath for 5 min followed by irradiation at a distance of 7−8 cm with a high-intensity sunlight lamp (wavelength > 500 nm) for 3 h. The resulting solution was passed through a Sephadex G-25 column to remove the Rose Bengal. The resultant fractions were further purified by ion pair HPLC with a 4.6 mm × 250 mm C-18 reversed-phase column and a gradient solvent system consisting of 5% solvent B to 20% solvent B over 40 min. Solvent A consisted of 5 mM tetrabutylammonium sulfate and 10 mM sodium phosphate (pH 7), while solvent B was acetonitrile. The flow rate was 1 mL min^{-1}. The UV spectra were recorded at 220 nm. Overall yield ≈ 35%. ^{31}P NMR (H_2O) δ −17.8 (br), −9.3 (s), −9.2 (s), −4.6 (d, J = 36 Hz), −4.5 (d, J = 36 Hz). Negative ion ESI-MS, m/z 538 [M − H]$^-$. λ_{max} (H_2O) = 230 nm.

Synthesis of Guanidinohydantoin-2′-deoxynucleoside-5′-triphosphate from 2′-Deoxyguanosine-5′-triphosphate and 7,8-Dihydro-8-oxo-2′-deoxyguanosine-5′-triphosphate. A solution consisting of dOGTP (0.04 mg, 0.08 μmol), 100 μL of 40 mM $K_2S_2O_8$ (1.1 mg, 4 μmol) dissolved in 75 mM potassium phosphate (pH 4.4), was irradiated at a distance of 7−8 cm with a short wavelength UV lamp for 30 min at room temperature (yield 50%). The resulting solution was passed through a Sephadex G-25 column to remove salts. The resultant fractions were further purified by ion pair HPLC with a 4.6 mm × 250 mm C-18 reversed-phase column and a gradient solvent system consisting of 5% solvent B to 20% solvent B over 40 min. Solvent A consisted of 5 mM tetrabutylammonium sulfate and 10 mM sodium phosphate (pH 7), while solvent B was acetonitrile. The flow rate was 1 mL min^{-1}. The UV spectra were recorded at 220 nm.

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Further characterization of dSpTP and dGhTP are based on HMBC and ESI-MS/MS experiments compared to nucleosides. See the Supporting Information.

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Supporting Information Available: ^{1}H and HMBC NMR spectra of SpOAc and GhOAc; ^{31}P NMR spectra of dSpTP; LC-ESI/MS spectra of dSpTP and dGhTP; ESI-MS parent ion scan spectra of [$dSpDH_2$]$^-$; and ESI-MS/MS spectra of dSpTP. This material is available free of charge via the Internet at http://pubs.acs.org.

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