ORGANIC LETTERS

2002
Vol. 4, No. 4
537−540

Spiroiminodihydantoin Is a Major Product in the Photooxidation of 2′-Deoxyguanosine by the Triplet States and Oxyl Radicals Generated from Hydroxyacetophenone Photolysis and Dioxetane Thermolysis

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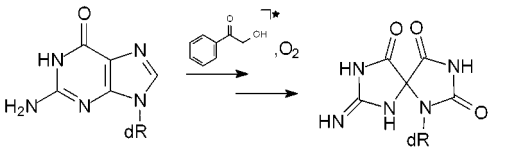
Received November 28, 2001

ABSTRACT



Photolysis of hydroxyacetophenone and thermolysis of the corresponding dioxetane afford spiroiminodihydantoin rather than 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (4-HO-8-oxodG) through the oxidation of 2′-deoxyguanosine (dG) by triplet-excited hydroxyacetophenone and the peroxyl radicals derived thereof by α cleavage and subsequent oxygen trapping. The structure of the spiroiminodihydantoin is assigned by the SELINQUATE NMR technique, which unequivocally establishes the spirocyclic connectivity.

The major photooxygenation product of 2′-deoxyguanosine (dG) has previously been assigned the 4-HO-8-oxodG structure,[1] which for almost the last two decades has served as a characteristic probe for singlet oxygen (type II photooxidation)[2] in photobiological studies.[1c] Recently, the 4-HO-8-oxodG structure has been questioned,[3] since it was shown that this compound is identical (HPLC analysis) to spiroiminodihydantoin, formed in the one-electron oxidation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and characterized on the basis of mass spectral analysis (MS/MS spectrum similar to that of the independently synthesized spiro compound but without the deoxyribose residue) and NMR spectroscopy.[3,4] These spectral data would allow a definitive differentiation between the spiroiminodihydantoin and 4-HO-8-oxodG structures if both were available as authentic materials. Since this is not the case, an unequivocal assignment requires the establishment of the connectivity of

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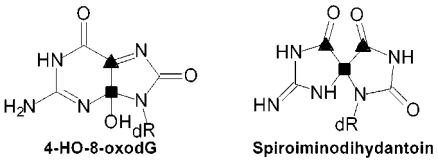
10.1021/ol017138m CCC: $22.00
Published on Web 01/25/2002

the marked carbon atoms in the 4-HO-8-oxodG and spiroiminodihydantoin structures given below.



This disputed oxidation product has also been observed in the photooxidation of the nucleosides dG and 8-oxodG by triplet-excited ketones and the radicals derived therefrom, generated either by the thermolysis of appropriate dioxetanes or by the photolysis of ketones.[5] The fact that this potentially photobiologically significant oxidation product[6] is also formed under the conditions of type I photooxidation (electron transfer, hydrogen abstraction) and oxyl-radical-mediated dG oxidation[7] urges a rigorous confirmation of the spiroiminodihydantoin structure.

For an unequivocal structural elucidation of the disputed dG oxidation product, we have chosen the SELINQUATE[8] NMR technique (*sel*ective *IN*ADE*QUATE*: *sel*ective *i*ncredible *n*atural abundance double *qua*ntum *t*ransfer *e*xperiment). This technique permits the definitive assignment of the atom connectivities through characteristic coupling patterns of nearest neighbors in the spirocyclic and annelated structures.

As a type I photooxidant of dG, we chose triplet-excited 2-hydroxyacetophenone (AP-OH). The AP-OH triplets were to be generated photochemically by direct excitation of the ketone and thermally by the decomposition of the hitherto unknown dioxetane **1** (for its preparation, see Supporting Information). The advantage of AP-OH triplets is that the involvement of singlet oxygen should be minimal and, thus, type II photooxidation of no concern. Moreover, it is known that AP-OH triplets cleave exclusively into benzoyl and hydroxymethyl radicals,[9] which on trapping with O_2 afford peroxyl radicals for the oxidation of dG. Photomechanistically relevant, the benzoyl chromophore in the AP-OH triplets, in contrast to the acetyl chromophore in previously used acetone derivatives,[5c] enables transient spectroscopy. This advantage should allow assessment of the relative importance of the direct photooxidation of the nucleoside dG by the triplet-excited AP-OH (electron transfer, hydrogen abstraction) versus oxidative damage by the radicals generated through α cleavage of the AP-OH triplets.

Our present results disclose the efficacious photooxidation of dG to spiroiminodihydantoin in the thermolysis of dioxetane **1** and in the photolysis of AP-OH; both modes of operation generate triplet-excited AP-OH and the latter fragments into carbon-centered radicals. For comparison, the photolysis of acetophenone (AP) was examined, a ketone substrate that is known to be photoresistant toward α cleavage into radicals.[10] We conclude that the direct photooxidation of the guanine in dG takes place by AP-OH triplets (presumably electron transfer) as well as radicals generated thereof; the latter dominate at low dG concentrations in the presence of molecular oxygen.

The SELINQUATE NMR technique distinguished unequivocally between the 4-HO-8-oxodG and spiroiminodihydantoin, since the two structures possess different carbon−carbon connectivities. The necessary amount of this oxidation product for NMR analysis was obtained by photooxygenation of dG with rose bengal.[11] The ^{13}C NMR spectrum showed five pairs of peaks (two diastereomers), which are attributed to the base part of the molecule, one at δ ca. 80 ppm and the other four between δ 155 and 182 ppm (see Figure 1). The structurally definitive resonance at

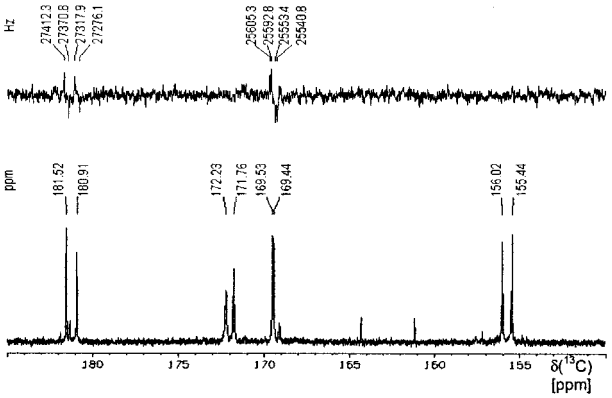


Figure 1. Top: 151 MHz ^{13}C-SELINQUATE spectrum of spiroiminodihydantoin 2′-deoxyribonucleosides in DMSO-d_6 obtained upon selective coherence transfer carried out by a selective 270° Gauss pulse (1 ms pulse length) at 80 ppm in the SELINQUATE[8] pulse sequence; only the region between 150 and 185 ppm of the base signals is shown. Bottom: ^{13}C NMR spectrum for comparison.

δ 80 ppm belongs either to the C-4 atom of 4-HO-8-oxodG[1d] or to the C-5 atom of spiroiminodihydantoin[4] (marked as squares; see above structures). Selective $^{13}C-^{13}C$ coherence transfer of this resonance resulted in distinct coupling patterns in the SELINQUATE spectra of the two structures: while the C-4 atom in 4-HO-8-oxodG possesses only one direct carbon neighbor (the imino functionality), the C-5 atom in spiroiminodihydantoin is flanked by two carbon atoms (the carbonyl groups, marked with triangles). The SELINQUATE spectrum (Figure 1) clearly shows two pairs of signals at δ 169.4 (52 Hz) and 169.5 ppm (52 Hz), as well as at δ 180.9 (42 Hz) and 181.5 ppm (42 Hz); the observed coupling constants (42 and 52 Hz) reveal that these carbon atoms

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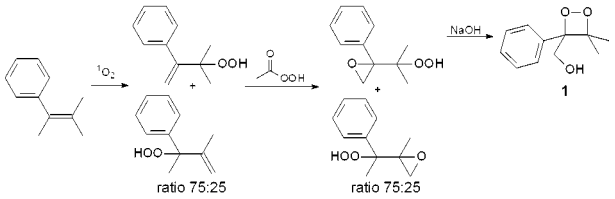
possess ^1J(C−C) couplings. Thus, this NMR spectral evidence speaks against the 4-HO-8-oxodG and supports the spiroiminodihydantoin structure. With the structure of the 2′-deoxyguanosine photooxidation product established as spiroiminodihydantoin, it was pertinent to confirm whether this photooxidation product is formed with triplet AP-OH, generated by photolysis of the ketone and thermolysis of the dioxetane **1**. For this purpose, the synthesis of the dioxetane **1** was achieved from the known 2-methyl-3-phenylbut-3-ene-2-yl hydroperoxide,[12] which was obtained as a mixture with the 2-methyl-3-phenylbut-1-ene-3-yl regioisomer by the ene reaction of 1,2-dimethyl-1-propylbenzene with singlet oxygen (Scheme 1). The corresponding epoxides were

Scheme 1. Synthesis of Dioxetane **1**



prepared from the crude mixture by epoxidation with peracetic acid, of which the 1-methyl-1-(2-phenyloxiranyl)-ethyl hydroperoxide was isolated and purified by silica gel chromatography. Subsequent base-catalyzed ring closure with dilute NaOH in methylene chloride gave the dioxetane **1** in 17% yield, which was fully characterized (see Supporting Information).

The rate of the dioxetane (1.00 mM) thermolysis was determined by monitoring the decay of the chemiluminescence intensity at various temperatures in 9:1 H_2O:CH_3CN (Table 1). The half-lives ($t_{1/2}$) were determined by fitting

Table 1. Half-Lives and Chemiluminescence Emission Intensities of the Thermal Decomposition of Dioxetane **1**

temp (°C)[a]	I_{max} (mV)	$t_{1/2}$ (h)	I_{max}·$t_{1/2}$ (mV h)
37	4.6 ± 0.2	42 ± 10	190
65	310 ± 10	4.1 ± 0.3	500
80	630 ± 30	0.82 ± 0.04	510
80[b]	550 ± 30	0.86 ± 0.09	480

[a] 1.00 mM in H_2O:CH_3CN (9:1). [b] In the presence of 0.50 mM dG and 5.00 mM phosphate buffer (pH 7.0).

the data to a monoexponential function (first-order kinetics). The maximum light intensity I_{max} that is reached within a few minutes of thermal decomposition was determined by monitoring the chemiluminescence emission with the Mitchell−Hastings photometer. From these values, the total light emission, which is proportional to the area under the chemiluminescence curve, was calculated by multiplying I_{max} and $t_{1/2}$. The results (Table 1) show that below 65 °C, the amount of emitted light decreases, which indicates that dark decomposition of the dioxetane dominates at low temperatures. To minimize such dark decomposition of the dioxetane **1**, decomposition temperatures >65 °C were employed.

The thermolysis of dioxetane **1**, as well as the photolysis of AP-OH, afforded the spiroiminodihydantoin as the major photooxidation product (Figure 2). Besides spiroiminodihydantoin, also 8-oxodG and guanidine-releasing products (GRP), e.g., oxazolone,[13] were formed in comparable amounts for both cases. Thus, irrespective of the mode of triplet AP-OH generation, a similar photooxidative reactivity was observed in both the dioxetane thermolysis and ketone photolysis. However, note in Figure 2 that in the photolysis of AP-OH, the relative yields of 8-oxodG and GRP are significantly smaller (ca. 63% for the dioxetane thermolysis versus ca. 34% for the AP-OH photolysis) compared to the conversion of dG. Evidently, due to the higher oxidative reactivity (dG conversion) in the AP-OH photolysis, these photoproducts (8-oxodG[14] and GRP) are subject to further oxidation. The similar product distributions in both cases imply common oxidizing species, presumably radicals formed by α cleavage of the triplet-excited state of AP-OH. The participation of singlet oxygen in this photooxidation is unlikely in view of the absence of a D_2O effect in the photolysis of AP-OH (data not shown).

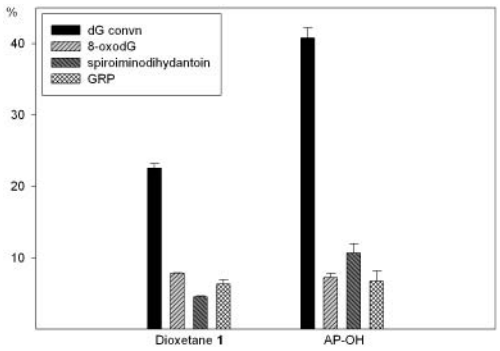


Figure 2. Oxidation of dG (0.200 mM) in the thermolysis of dioxetane **1** (5 equiv, 80 °C, 3 h) and the photolysis of AP-OH (2 equiv, 0 °C, 45 min) in 2.00 mM phosphate buffer:CH_3CN (9:1).

To assess the relative importance of the direct interaction of dG with the triplet-excited AP-OH in the dG photooxidation, half of the dG concentration was employed. No decrease of the photooxidative reactivity (dG conversion) at the lower dG concentration was observed for the ketone photolysis, as well as for the dioxetane thermolysis (data not shown). Since the direct oxidation of dG by triplet-excited AP-OH, a bimolecular process, should diminish due to its

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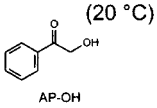
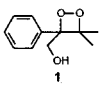
more efficient deactivation at lower dG concentrations, less dG conversion is expected. Such behavior was found for AP but not for AP-OH. Consequently, at the dG concentrations employed, the radical species formed from the AP-OH triplets by α cleavage must play the dominant role in the photooxidation process.

The kinetics of triplet-excited AP and AP-OH quenching by dG was determined through transient absorption spectroscopy. The triplet ketones were generated by excitation of degassed solutions at 308 nm and detected at 340 nm. The generation of AP-OH triplets by means of dioxetane **1** thermolysis for spectral monitoring is not feasible. Monoexponential fits of the decay curves gave the triplet lifetimes (in the absence of dG) of 6.6 ± 1 μs for AP and 1.1 ± 0.2 μs for AP-OH. The fact that the triplet lifetime of AP-OH is six times shorter than that of AP is attributed to the efficient Norrish-type I cleavage of AP-OH; the rate constant for α cleavage is estimated to be 5 (±2) × 10^5 s^{-1}. For the determination of the quenching rate constants of the triplet ketones by dG, the time dependence of the absorption decay curve obtained by time-resolved laser-flash spectroscopy was monitored at various dG concentrations. For both triplet-excited AP-OH and AP, quenching rate constants with dG of 3.3 (±0.2) × 10^9 M^{-1} s^{-1} were obtained. Thus, at the dG concentrations needed for the analysis of its photooxidation products (0.200 mM), direct photodeactivation of the AP-OH triplet by dG competes with α cleavage.

To confirm the formation of radicals through the α cleavage of AP-OH triplets, spin-trapping experiments for dioxetane **1** with DMPO have been conducted. When the dioxetane/DMPO solution was heated at 80 °C for 15 min, one radical product was observed, which was assigned to the hydroxymethyl radical[5b] adduct of DMPO. No benzoyl-radical product with DMPO was detected (Table 2), which is presumably due to the thermal instability of the benzoyl−DMPO radical adduct at 80 °C. Indeed, in a control experiment, the authentic radical adduct decomposed extensively already at 20 °C within 1 h. For the photolysis of AP-OH, a hydroxymethyl as well as a benzoyl radical adduct was observed (Table 2).

In the photolysis of AP, which does not undergo α cleavage,[10] no radical adducts were detected. These results confirm that during the dioxetane thermolysis and the ketone photolysis, a pair of benzoyl and hydroxymethyl radicals is formed from the triplet-excited AP-OH by α scission.[9]

Table 2. EPR Data of the DMPO Adducts Obtained in the Photolysis[a] of AP-OH and the Thermolysis[b] of Dioxetane **1**

substrate (T)	DMPO adduct of	α_N [G]	α_H [G]	g factor
(20 °C)  AP-OH	•COPh[c]	15.3 (15.3)	17.9 (18.7)	2.0056 (2.0055)
	$•CH_2OH$[d]	16.0 (15.7)	22.6 (22.7)	2.0055 (2.0056)
(80 °C)  1	$•CH_2OH$[d]	15.9 (15.7)	22.7 (22.7)	2.0057 (2.0056)

[a] 2.00 mM in a 9:1 H_2O:CH_3CN mixture at 300 nm for 30 min and 20 °C in a Rayonet photoreactor [sixteen 300-nm lamps (24 W)], [DMPO] = 50.0 mM. [b] 100 mM in a 9:1 H_2O:CH_3CN mixture, 15 min, 80 °C, [DMPO] = 50.0 mM. [c] In parentheses are given the data for an analogous acyl-radical adduct in H_2O:CH_3CN (9:1); see ref 5b. [d] In parentheses are given the literature data; see ref 5b.

We affirm that the major dG oxidation product in the dioxetane thermolysis and ketone photolysis (type I oxidation) is not 4-HO-8-oxodG but spiroiminodihydantoin, unequivocally characterized by means of the SELINQUATE NMR technique. Evidently, the spiroiminodihydantoin is the ubiquitous oxidation product of dG by metal-based one-electron oxidants, triplet ketones, singlet oxygen, and oxyl radicals. The advantage of the dioxetane **1** for photobiological studies is that oxidizing triplet ketones and radical species may be generated thermally without the direct exposure of biological materials to light.

Acknowledgment. Generous financial support by the Deutsche Forschungsgemeinschaft (SFB 172 "Molekulare Mechanismen kanzerogener Primärveränderungen") and by the Fonds der Chemischen Industrie is gratefully appreciated.

Supporting Information Available: Complete experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

OL017138M