Type I and Type II Photosensitized Oxidative Modification of 2′-Deoxyguanosine (dGuo) by Triplet-Excited Ketones Generated Thermally from the 1,2-Dioxetane HTMD

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Abstract: The nucleoside 2′-deoxyguanosine (dGuo) was treated with 3-(hydroxymethyl)-3,4,4-trimethyl-1,2-dioxetane (HTMD), the latter generates efficiently triplet-excited carbonyl products on thermal decomposition *in the dark*. The type I photooxidation products, 2,2-diamino-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-amino]-5(2*H*)-oxazolone (oxazolone) and the cyclic nucleoside 2-(*S*)-2,5′-anhydro-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-guanidinylidene-2-hydroxy-4-oxoimidazolidine (oxoimidazolidine), as well as the type II photooxidation products 4-(*R*)*- and 4-(*S*)*-4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine (4-HO-8-oxodGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), were quantitatively determined by appropriate selective and sensitive HPLC assays. The concentration and time profiles revealed that about 40% of the triplet ketones derived from the thermal decomposition of HTMD led to photooxidation of dGuo. Essentially equal amounts of type I and type II photooxidation products were found, as could be established by comparison with predominant type I (benzophenone, riboflavin) and type II (Rose Bengal, methylene blue) photosensitizers. The participation of singlet oxygen (type II activity) was confirmed by the substantial D_2O effect in the formation of 8-oxodGuo. The results demonstrate that dioxetanes, particularly HTMD, are efficient photooxidants of dGuo on *thermal activation in the dark* and constitute excellent chemical tools to study *photobiological processes without the use of light*, in the present case, photogenotoxicity.

Introduction

Due to the importance of the oxidative degradation of nucleic acids in mutagenesis, carcinogenesis, and aging,[1−4] numerous chemical and biological investigations have been made on this subject in the past decade.[5−7] These intensive studies have contributed significantly in understanding the reaction mechanism of nucleic acid oxidations mediated by photosensitizers,[8−11] hydroxyl radicals,[12,13] singlet oxygen,[14,15] and other reactive oxygen species which are involved in oxidative stress.[16,17]

The purine base guanine is the predominant target in the photosensitized oxidation of DNA.[18,19] The highly mutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)[20−23] and the 2,2-diamino-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-amino]-5(2*H*)-oxazolone (oxazolone), which results from hydrolysis of the corresponding imidazolone precursor,[24] constitute the major photooxidation products of DNA. While 8-oxodGuo can be formed either directly through type I (hydrogen atom abstraction or electron transfer)[24c] or indirectly through type II (singlet oxygen, energy transfer)[25,26] photooxidation processes, the oxazolone is derived from a type I photooxidation.[5,27,28] Recently, it was reported[29] that oxazolone is also produced by singlet oxygen oxidation of 8-oxodGuo, which is a primary photooxidation product of dGuo.

The photosensitized oxidation of the monomeric nucleoside 2′-deoxyguanosine (dGuo) affords (Scheme 1), in addition to 8-oxodGuo and the oxazolone, the two 4*R** and 4*S** diastereomers of 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine

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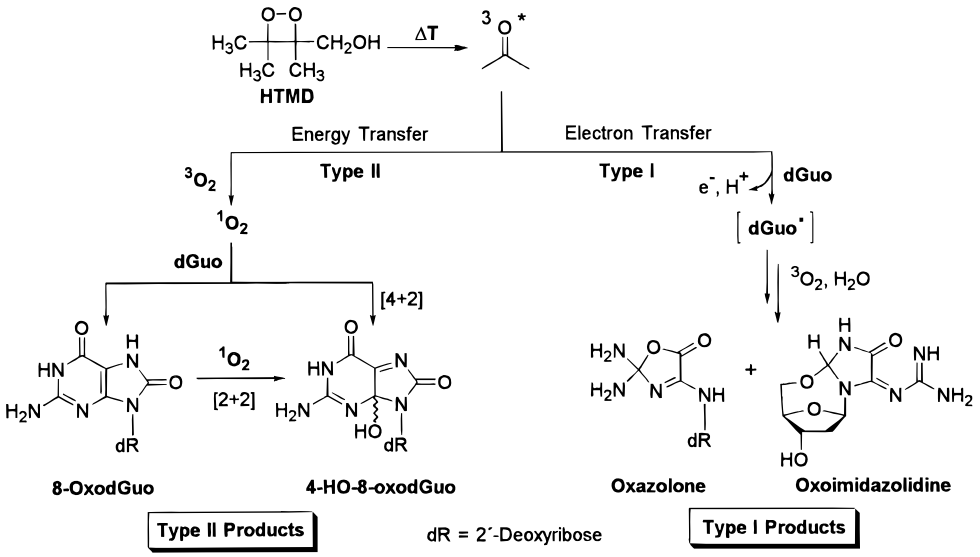
Scheme 1. Photochemical Mechanisms of the Oxidation of 2′-Deoxyguanosine (dGuo) in the Thermal Decomposition of the 1,2-Dioxetane HTMD



(4-HO-8-oxodGuo) as characteristic singlet oxygen products either by [4 + 2] cycloaddition with dGuo[28,30] or by [2 + 2] cycloaddition with 8-oxodGuo.[30] Furthermore, 2-(*S*)-2,5′-anhydro-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-guanidinylidene-2-hydroxy-4-oxoimidazolidine (oxoimidazolidine) was characterized as a type I photooxidation product of dGuo,[31] which results from intramolecular attack of the 5′-hydroxyl functionality on the C-8 position of the intermediary guanine radical. In contrast to the photooxidation of guanine in DNA, the formation of 8-oxodGuo through type I photooxidation is essentially negligible for the nucleoside dGuo as substrate. Presumably, in this case the fast deprotonation of the intermediary, highly acidic guanosine radical cation[32] prevents the addition of water to afford 8-oxodGuo. Therefore, significant amounts (>0.1%) of 8-oxodGuo are exclusively produced by the type II process in the photosensitized oxidation of dGuo.[5]

An important class of photooxidative sensitizers constitutes the triplet-excited ketones,[16,33] which are of biological interest since they may be generated in cellular systems upon exposure of endogeneous chromophores to UV irradiation or by dark reactions [e.g., lipid peroxidation (Russell mechanism) and enzymatic oxidation].[34−36] Alternative to their conventional photochemical generation, triplet-excited ketones may be conveniently produced by the thermal decomposition of 1,2-dioxetanes,[37,38] which are high-energy, four-membered ring cyclic peroxides. The thermally generated triplet-excited species, analogous to those produced photochemically, also operate as type I or type II photooxidants. The advantageous feature that their thermal generation circumvents the exposure of biological systems, particularly cells, directly to UV radiation.

This attractive opportunity of employing 1,2-dioxetanes as chemical sources for electronically excited ketones paved the way for extensive studies on the new bioorganic topic "*photobiology without light*" to explore the biological function, in particular genotoxicity, of such peroxides.[39−41] These studies revealed that, on thermal decomposition, 1,2-dioxetanes efficiently damage DNA in cell-free and cellular systems.[39] The dioxetane-mediated DNA lesions are mostly sensitive to formamidopyrimidine DNA−glycosylase (FPG protein),[40] which strongly indicates that 1,2-dioxetanes preferentially generate guanine lesions (e.g., 8-oxodGuo) besides AP sites and formamidopyrimidine (Fapy) residues.[42,43]

To evaluate the chemical nature of these dioxetane-induced DNA modifications, we have recently investigated the oxidation of *calf thymus* DNA by 1,2-dioxetanes *in the dark*.[44] It has been established that 1,2-dioxetanes, in particular those that are alkyl-substituted, oxidize efficiently and almost exclusively the guanine bases in DNA to form 8-oxodGuo in high yields. Triplet-excited states generated by thermal decomposition of dioxetanes were found to be responsible for the observed guanine oxidation in DNA.

The mechanistic origin of guanosine photooxidation products is less clear-cut. For example, although in DNA 8-oxodGuo can be formed through type I and type II photooxidations, this particular photooxidation product of the dGuo nucleoside is derived exclusively through a type II process ($^1O_2$). Therefore, the relative contribution of type I versus type II photooxidation of dGuo is a more definitive monitor of the photooxidation mechanism. Consequently, in order to define the predominant chemical oxidation mode of the triplet-excited species generated thermally from 3-(hydroxymethyl)-3,4,4-trimethyl-1,2-dioxetane (HTMD), we have investigated its reaction with the nucleoside dGuo. The results are compared with those of the photosen-

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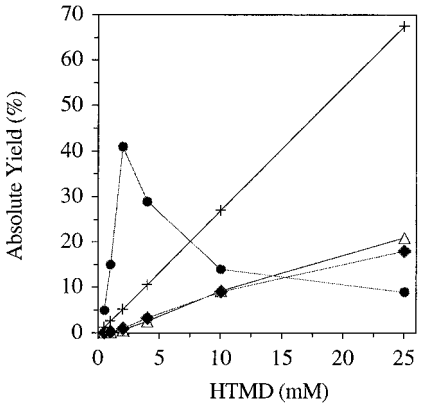

Figure 1. Concentration profile for the thermally HTMD-induced photooxidation of dGuo. In the dark with 0.5 mM dGuo at 50 °C for 15 h in 10 mM sodium cacodylate buffer (pH 7.0) and acetonitrile as cosolvent (10 vol %), 70% consumption of dioxetane (chemiluminescence measurements). Absolute yield derived from the mean values of three independent runs, error ±10% of the stated value, conversion dGuo (crosses), yields of type II products 8-oxodGuo × 100 (filled circles) and 4-HO-8-oxodGuo (filled squares) and type I products oxazolone and oxoimidazolidine (open triangles).

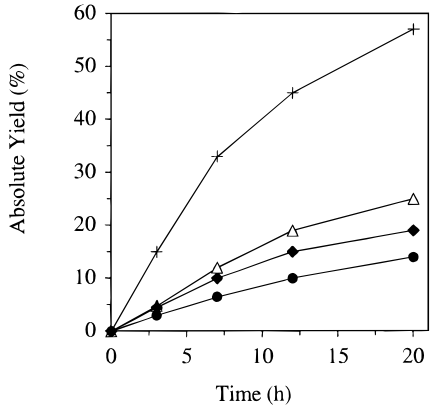

Figure 2. Time profile for the thermally HTMD-induced photooxidation of dGuo. In the dark at 50 °C with 0.5 mM dGuo in 10 mM sodium cacodylate buffer (pH 7.0) and 25 mM HTMD dissolved in acetonitrile (10 vol %). Absolute yield derived from the mean values of at least three independent runs, error ±10% of the stated value, conversion dGuo (crosses), yields of type II products 8-oxodGuo × 100 (filled circles) and 4-HO-8-oxodGuo (filled squares) and type I products oxazolone and oxoimidazolidine (open triangles).

sitized oxidation of dGuo by characteristic type I[45] (benzophenone and riboflavin) and predominant type II (Rose Bengal and methylene blue) photosensitizers.[46,47]

Results

On thermal treatment of dGuo with HTMD at 50 °C, five major oxidation products of the guanine base were detected by means of appropriate HPLC assays. These products are the type II photooxidation products 8-oxodGuo and the two 4*R** and 4*S** diastereomers of 4-HO-8-oxodGuo, in addition to the type I photooxidation products oxazolone (also produced by singlet oxygen oxidation of 8-oxodGuo,[29] the primary type II photooxidation product of dGuo) and oxoimidazolidine.[5] The latter two products were assessed together by the indirect fluorescence-labeling HPLC assay of the alkaline-released guanidine, introduced by Ravanat *et al*.;[28] whereas, 8-oxodGuo and 4-HO-8-oxodGuo were directly detected, either by electrochemical[48,49] or by spectrophotometric HPLC analyses.[50] Furthermore, the amount of decomposed HTMD was monitored by chemiluminescence measurements ($t_{1/2}$ of 6 ± 1 h at 50 °C).

The concentration and time profiles (Figures 1 and 2) for the HTMD-induced oxidation of dGuo were determined by using sodium cacodylate buffer as the reaction medium with 10 vol % acetonitrile as cosolvent. As shown in Figure 1, when dGuo was thermally treated with HTMD at 50 °C for 15 h, a linearly dependent degradation of dGuo was observed with increasing HTMD concentration (about 70% dGuo conversion at 25 mM, 50 equiv of HTMD). The absolute yield (based on initial amount of dGuo) of 8-oxodGuo increased continuously up to ca. 0.4% (in Figures 1 and 2 yields of 100[8-oxodGuo] are given), but at HTMD concentrations above 2 mM, it dropped significantly to *ca*. 0.1% 8-oxodGuo at 25 mM HTMD (i.e., the 8-oxodGuo yield went through a maximum). The other characteristic type II photooxidation product, 4-HO-8-oxodGuo, was formed in up to 20% absolute yield at 25 mM HTMD, while the type I photooxidation products oxazolone and oxoimidazolidine amounted to ca. 18% at the same HTMD concentration. In these reactions, the product balance (i.e., the sum of quantified products relative to consumed dGuo) amounted to ca. 55% and was essentially independent of the HTMD concentration. The time profile (Figure 2), determined with 25 mM HTMD, revealed a gradual increase of all dGuo oxidation product yields with reaction time. Also in this case, the product balance amounted to 55 ± 3% and was independent of reaction time.

To define the predominant photooxidation mechanism (type I versus type II) for the oxidation of dGuo by electronically excited species generated thermally from HTMD, the results of the HTMD reaction were compared with those of the dGuo photooxidation by the predominant type I (benzophenone and riboflavin) and type II (Rose Bengal and methylene blue) photosensitizers.[46,47] For this purpose, the product distributions of dGuo photooxidation induced by these sensitizers were investigated over a wide range of sensitizer concentrations and irradiation times at 20−80% conversion of dGuo (Table 1).

In the type II sensitized photooxidation of dGuo by Rose Bengal and methylene blue, as well as in the HTMD-induced oxidation (cf. entries 1, 4, and 5, Table 1), the minor product 8-oxodGuo (>1%) was detected in significant yields (relative to dGuo conversion), whereas the type I photosensitizers riboflavin and benzophenone (cf. entries 2 and 3) gave only negligible amounts of 8-oxodGuo (less than 0.1%). Since the relative yields (based on converted dGuo) of the major type II (4-HO-8-oxodGuo) and type I (oxazolone and oxoimidazolidine) photooxidation products remained within the experimental error independent on sensitizer concentration and reaction time for all employed photosensitizers, the type II/type I product ratio may serve as a mechanistic probe to assess the predominant photooxidation mode. For example, for predominant type I photooxidation, this product ratio lies *below 1* (entries 2 and 3, Table 1) while for predominant type II photooxidation it is *above 4* (entries 4 and 5). For HTMD the type II/type I product ratio is 1.3 ± 0.2 (entry 1) and, thus, entails both photooxidation modes. Also for the product balance, the HTMD oxidation (56 ± 2%, entry 1) lies between the type I (44−47%, entries 2 and 3) and type II (73−78%, entries 4 and 5) processes. Further-

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Table 1. Product Studies of the dGuo Oxidation by the Dioxetane HTMD and by Predominantly Type I (Benzophenone, Riboflavin) and Type II (Rose Bengal, Methylene Blue) Photosensitizers[a]

			product yields (%)[b]				
			type II		type I		
entry	oxidant	concn (μM)	8-oxodGuo	4-HO-8-oxodGuo	products[c]	product balance	type II/type I
1	HTMD/50 °C[d]	10000	<1.2	31 ± 2	24 ± 2	56 ± 2	1.3 ± 0.2
2	benzophenone/350 nm[e]	500−2500	<0.1	17 ± 3	27 ± 4	44 ± 5	0.7 ± 0.2
3	riboflavin/564 nm[f]	1−10	<0.1	15 ± 2	32 ± 2	47 ± 1	0.5 ± 0.1
4	Rose Bengal/564 nm[f]	2−20	<2.1	65 ± 2	13 ± 1	78 ± 2	5.0 ± 0.9
5	methylene blue/564 nm[f]	2−20	<3.0	58 ± 3	15 ± 2	73 ± 3	4.0 ± 0.9

[a] dGuo (0.5 mM) in 10 mM sodium cacodylate buffer (pH 7.0). [b] Relative yields based on consumed dGuo, mean values of at least 10 independent runs at 20−80% conversions of dGuo. [c] Oxazolone and oxoimidazolidine. [d] In the dark, 15 h, acetonitrile (10 vol %) as cosolvent. [e] Blacklight lamp (125 W), irradiation distance (10 cm), 30−180 min, 4 °C, acetonitrile (1 vol %) as cosolvent. [f] Sodium lamp (150 W), irradiation distance (20 cm), 30−180 min, 4 °C.

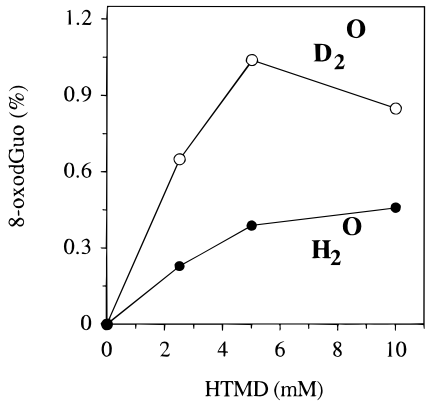

Figure 3. Effect of D_2O on the yield of 8-oxodGuo in the thermally HTMD-induced photooxidation of dGuo. In the dark at 50 °C for 12 h with 0.5 mM dGuo in 10 mM sodium cacodylate buffer [pH(D) 7.0] and acetonitrile as cosolvent (10 vol %). Absolute yield derived from the mean values of at least three independent runs, error ±10% of the stated value.

more, the relative yield of the type II product 4-HO-8-oxodGuo in the HTMD-induced oxidation (ca. 31%) is significantly higher (ca. 16%) than that with type I photooxidants and smaller than that in the type II case (ca. 60%). In contrast, the yields of the type I products in the HTMD-induced oxidation (ca. 24%) are comparable with those of type I sensitizers (ca. 30%) and significantly higher than those in the type II photooxidation (ca. 14%).

To confirm the participation of singlet oxygen, the HTMD-mediated oxidation of dGuo was examined in deuterium oxide, which is known to prolong the lifetime of singlet oxygen about 10-fold.[51,52] The results for the HTMD-induced formation of 8-oxodGuo in H_2O and D_2O are shown in Figure 3. At HTMD concentrations up to 5 mM (10 equiv), the yield of 8-oxodGuo is almost 3-fold enhanced in D_2O (1.04%) compared to the yield of the reaction in H_2O (0.38%). However, at higher HTMD concentration (10 mM), the yield of 8-oxodGuo in D_2O decreases slightly to 0.85%, while in H_2O it increases insignificantly to 0.45%.

Discussion

Our present investigation reveals that triplet-excited ketones generated in the thermal decomposition of the dioxetane HTMD oxidize dGuo efficiently to the established guanine photooxidation products 8-oxodGuo, 4-HO-8-oxodGuo, oxazolone, and oxoimidazolidine.[5] Control experiments at 0 °C (HTMD is not significantly decomposed at this temperature during 15 h) showed that HTMD does not oxidize dGuo directly. Approximately 60% of the consumed dGuo is accounted for in terms of these guanine oxidation products. The remaining products were not pursued, since some of them most likely result from multiple oxidative degradation. Indeed, 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid and its precursor 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)tetrahydro-2,4,6-trioxo-1,3,5-triazine-1(2*H*)-carboximidamide have been recently identified[29] as major singlet oxygen products in the subsequent photooxidation of 8-oxodGuo, the primary oxidation product of dGuo.

Generally, photosensitizers with ketone chromophores operate through low-lying triplet n,π* states and are highly efficient for electron transfer with substrate or hydrogen abstraction from the substrate.[53] Consequently, they react preferentially through the type I photooxidation mechanism. However, the dioxetane HTMD as an efficient thermal source of triplet-excited ketones, yields large amounts of the characteristic type II products 8-oxodGuo and 4-HO-8-oxodGuo, indicative of singlet oxygen.[5,54] It was, therefore, important to compare the product composition of the dGuo photooxidation by predominant type I (benzophenone and riboflavin) and type II (methylene blue, Rose Bengal) photosensitizers[8,46] with those observed in the thermally-promoted dGuo oxidation by the dioxetane HTMD. In this comparison it should be kept in mind that the photosensitized oxidations operate catalytically [i.e., the photosensitizers are not consumed (except for photobleaching) and are recycled to afford a steady-state concentration of the triplet-excited species]. In contrast, the thermal dioxetane oxidations are stoichiometric in type since on fragmentation of the dioxetane, concentration- and time-dependent quantities of triplet-excited ketones are produced irreversibly. Moreover, another significant difference is the necessity that, in the thermal cleavage of the dioxetane to triplet-excited ketone products, *elevated temperatures* must be employed.

The type II/type I product ratio and product balance were shown to differ characteristically with the photosensitizer employed (Table 1) and may, therefore, serve as probe for the predominant photooxidation mode. The type II/type I product ratio (Table 1, entries 2 and 3) for the characteristic type I photosensitizers benzophenone and riboflavin (poor singlet oxygen generators) is quite high (0.7 and 0.5). However, the product balance (i.e., the sum of the quantified products relative to consumed dGuo) in the benzophenone- and riboflavin-sensitized photooxidation of dGuo (Table 1, entries 2 and 3) is only ca. 45%. Presumably, a significant fraction of the unidentified photooxidation products may derive from the type

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I process, and since these unidentified products are not accounted for, a high type II/type I product ratio is observed. The comparison of the thermally HTMD-induced oxidation of dGuo with that of authentic type I and type II photosensitized processes establishes, however, that HTMD is neither a typical type I (riboflavin, benzophenone) nor a characteristic type II (Rose Bengal, methylene blue) photooxidant; in particular, both photooxidation modes occur quite efficiently.

Proof of the involvement of singlet oxygen in the HTMD-induced oxidation of dGuo comes from the substantial effect of D_2O on the formation of the primary type II oxidation product 8-oxodGuo. Unfortunately, singlet oxygen quenchers such as DABCO, β-carotene, and sodium azide could not be employed to corroborate the involvement of singlet oxygen in the HTMD photooxidation because they react with the dioxetane.[55−57] However, the up to three times higher yields of 8-oxodGuo in D_2O compared to those in H_2O implicate that singlet oxygen is involved in the dioxetane-mediated photooxidation of dGuo. Singlet oxygen persists 10-fold longer in D_2O,[51,52] and photooxidation is more effective in this medium. Moreover, the observed decrease of the 8-oxodGuo yield in D_2O at dioxetane concentrations higher than 2.5 mM may be rationalized by the high reactivity of 8-oxodGuo toward further oxidation by singlet oxygen.[58,59]

The linear increase of dGuo conversion (Figure 1) with increasing HTMD concentration (up to 25 mM) suggests that the HTMD oxidation of dGuo is directly proportional to the amount of triplet-excited ketones formed by the thermal decomposition of the dioxetane. At a triplet yield of ca. 5% for HTMD in aqueous solution[40] and a half-life of $t_{1/2} = 6$ h at 50 °C (measured by chemiluminescence decay), it may be estimated that ca. 40% of the thermally produced triplet-excited ketones cause the oxidation of dGuo, either through direct (type I) or indirect (type II) photoreaction. Indeed, this efficiency is remarkable in view of the numerous competitive physical quenching processes that deactivate triplet-excited ketones. In addition to this physical deactivation of the triplet-excited ketones, it should be noted that successive oxidation reactions (e.g., the oxidation of 8-oxodGuo to 4-HO-8-oxodGuo)[59] also require triplet states without consuming dGuo. For example, for singlet oxygen, generated by quenching of triplet-excited species through energy transfer, the reactivity toward 8-oxodGuo was estimated to be more than 100 times faster than with dGuo.[58] Thus, high amounts of singlet oxygen will be consumed by its reaction with 8-oxodGuo without further consumption of dGuo. In fact, a maximum in the yield of 8-oxodGuo is expected at low HTMD concentration because with a larger excess of dioxetane the 8-oxodGuo is consumed by further oxidation, as is confirmed experimentally (Figure 1).

In contrast, the relative yields of the type II and type I photooxidation products of guanine remain approximately constant with reaction time. The time profile of the thermally HTMD-induced photooxidation of dGuo exhibited a nearly exponential decrease of the dGuo concentration with reaction time (Figure 2). This reflects that the dGuo substrate is consumed in parallel with the generation of triplet-excited ketones, which are formed thermally from the HTMD by first-order kinetics. As in the concentration profile, the time profile revealed that about 40% of the thermally generated triplet-excited ketones react with dGuo.

Conclusion

Our results on the oxidation of dGuo by the thermal treatment with HTMD reveal that through the action of triplet-excited ketones derived from the dioxetane, photooxidation products of the guanine base are formed in relatively high yields. Comparison with well-established type I and type II photosensitizers clearly points out that HTMD acts through both photooxidation types on thermal activation. This was convincingly demonstrated by the type II/type I product ratio, which serves as a mechanistic probe to assess the predominant photooxidation mode. The pronounced D_2O effect (a 3-fold increase in 8-oxodGuo product) confirms the participation of singlet oxygen. The concentration and time profiles for the HTMD-mediated photooxidation of dGuo establish the direct proportionality between product yield and availability of triplet ketones. The approximate 40% quantum yield of triplet-ketone-promoted dGuo oxidation demonstrates the high efficiency of the dioxetane HTMD.

We emphasize that dioxetanes, particularly HTMD, constitute unique chemical sources for the efficient photooxidation of biological substrates through the action of their thermally generated triplet-excited ketones. In aqueous media, HTMD serves as convenient chemical photooxidant for photobiological studies *in the dark*, which circumvents the disadvantages of direct exposure of the biological material, in particular isolated or cellular DNA, to ultraviolet radiation.

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Supporting Information Available: A listing of the Experimental Section (4 pages). See any current masthead page for ordering and Internet access instructions.

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