Obtention of two anomers of imidazolone during the type I photosensitized oxidation of 2′-deoxyguanosine

Corine Vialas,[a] Geneviève Pratviel,[a] Albert Meyer,[b] Bernard Rayner[b] and Bernard Meunier *[a]

[a] *Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse cedex 04, France. E-mail: bmeunier@lcc-toulouse.fr*
[b] *Laboratoire de Chimie Bio-Organique, UMR-CNRS 5625, Université de Montpellier II, Place Eugène Bataillon, 34095 Montpellier cedex 5, France*

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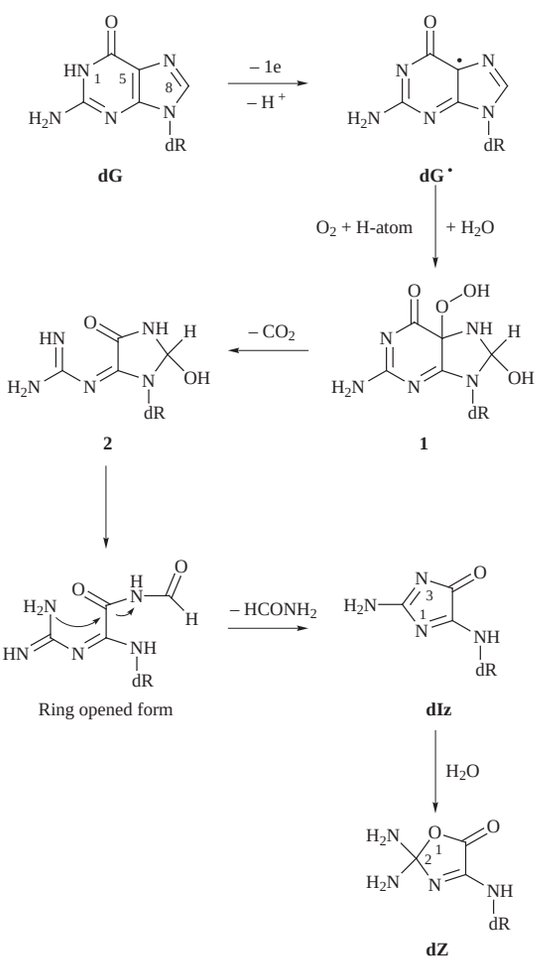
The β-furanose anomer of imidazolone (**β-dIz**) is the main product of the photosensitized oxidation of the natural 2′-deoxyguanosine in the presence of benzophenone, a type I photosensitizer, but the α-furanose anomer (**α-dIz**) is also formed through an imine intermediate during the photochemical reaction. This anomerization phenomenon has been confirmed by using the α-anomer of 2′-deoxyguanosine. In addition, the chemical oxidation of **α-** or **β-dG** by Mn-TMPyP–$KHSO_5$ provides the α- or β-furanose anomers, respectively, in an almost quantitative yield, and thus represents a method of choice for the synthesis of both **dIz** stereoisomers.

Introduction

Many studies are currently focused on the identification of oxidative damage to DNA in order to understand the biological role of these modifications.[1–3] Among the different oxidation products of 2′-deoxyguanosine (**dG**), two of them are now considered as key compounds: imidazolone, 2-amino-5-[(2′-deoxy-β-D-*erythro*-pentafuranosyl)amino]-4*H*-imidazol-4-one (**β-dIz**) together with its hydrolysis product oxazolone, 2,2-diamino-4-[(2′-deoxy-β-D-*erythro*-pentafuranosyl)amino]-2,5-dihydrooxazol-5-one (**β-dZ**).[1,4–6] These compounds were first described by Cadet *et al.*[7,8] as oxidation products of **dG** generated either by hydroxyl radicals or by type I photosensitizers (Scheme 1). Adam *et al.*[9] proposed later that **dIz** and **dZ** could be obtained by type I or II photooxidation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (**8-oxo-dG**). We recently reported that the chemical oxidation of **β-dG** with the artificial nuclease Mn-TMPyP–$KHSO_5$[10] gave within few minutes a nearly quantitative amount of **β-dIz** (90%).[11] This catalytic reaction is a convenient synthetic route for **β-dIz** since the photosensitized oxidation of **β-dG**, which may be considered as another method of **dIz** synthesis apart from not being quantitative, leads to a mixture of both anomers of **dIz**. The formation of the two anomers of **dIz** during the photosensitized oxidation of **dG** is reported here.



Scheme 1 Mechanism of **dIz** formation in type I photosensitized oxidation of **dG**.[7]

Results

Photosensitized compared to chemical oxidation of dG

The reactions were directly monitored by reverse-phase HPLC coupled to a UV-vis diode array detector or to an electrospray mass spectrometer allowing an easy recording of the UV-visible and the mass spectra of the oxidation products of **dG** as described previously.[11]

The photosensitized oxidation of **β-dG** in the presence of benzophenone was performed as described by Cadet and co-workers.[8] Half of the initial amount of **β-dG** was nearly quantitatively transformed into **β-dIz** after 2 h of irradiation. The HPLC peak corresponding to the **β-dIz** was observed at a retention time (t_R) of 10 min (compared to 25 min for **β-dG**). The UV-visible spectrum of **β-dIz** exhibits two λ_{max} values at 254 and 320 nm. The ESI-MS spectrum of **β-dIz** showed an $[M + H]^+$ signal at 229 and a fragmentation peak corresponding to the loss of the deoxyribose moiety $[M - dR + 2H]^+$ at 113. However, for longer irradiation times, as shown in Fig. 1A, the corresponding aglycone moiety of **dIz**, namely 2,5-diamino-4*H*-imidazol-4-one (**Iz**), was also generated

(t_R = 6 min; λ_{max} at 248 and 320 nm; ESI-MS: $[M + H]^+$at 113) as well as a product eluting after **β-dIz** (t_R = 11 min; λ_{max} at 254 and 320 nm; ESI-MS: $[M + H]^+$ at 229 and $[M - dR + 2H]^+$ at 113) with both UV-vis and MS spectra identical to those of **β-dIz**. This new photoproduct was tentatively attributed as being the α-anomer of **dIz** (**α-dIz**).

As illustrated in Fig. 1a (70% of **β-dG** being degraded), the reaction medium contained about 30% of **β-dG**, 20% of the major dIz isomer (**β-dIz**), 6% of the minor one (**α-dIz**) and 10% of **Iz** (these yields were calculated from the initial amount of **β-dG** by using calibration curves and by considering that **Iz** and **dIz** had nearly the same ε value).[11] Fluctuations were observed for these yields depending on slightly different experimental conditions (light intensity, temperature, *etc*.), the highest yield obtained for the minor **dIz** anomer reaching 10%.

The observed α/β ratio for **dIz** anomers increased with the advancement of the reaction. At low **dG** degradation level (less than 50%) the β-anomer is almost the only product observed during the two first hours of reaction (<10% of α-anomer). When **dG** was degraded up to 80%, after about 6 h of irradiation, the α/β ratio was about 1/9. Finally, at **dG** degradation of 90–95% and at longer irradiation times, the ratio could be as high as 1/3.

The reaction was further complicated by the degradation of **dIz** into **Iz**, **dZ** and into some other non-identified products. The transformation of **dIz** into **dZ** was reported to be relatively slow since the half-life of **dIz** was 10 h at pH 7 and ambient temperature.[4] The formation of **Iz** from **dIz**, under the present experimental conditions, was not negligible since for a **dG** degradation level of 70%, **Iz** represented 10% of the products of the reaction, while later, when **dG** is degraded at 95%, it represented 30% yield.

In order to confirm that the "second dIz product" was the α-anomer, we carried out the photosensitized oxidation of **α-dG** under the same experimental conditions as for **β-dG**. We verified that the reaction led to a mixture of two anomers of **dIz**, after 8 h of reaction, the major of which was the α-anomer (t_R = 11 min). The photosensitized oxidation of **α-dG** is illustrated in Fig. 1b where the ratio of the two anomers is high due to a large degradation of **α-dG** after 8 h of irradiation.

The formation of the two anomers of **dIz** was not due to anomerization of the starting **dG** since **α-** and **β-dG** were well separated under the chromatographic conditions used and no transformation of one into the other was observed during these photosensitized oxidations (t_Rs of **α-** and **β-dG** are 23 and 25 min respectively).

During the photosensitized oxidation of **α-** or **β-dG**, it was also possible to observe little amounts (<10%) of products probably corresponding to the α- and β-anomers of the pyranose derivatives of **dIz** at retention times of 8 and 7 min. These two compounds exhibited the same electrospray mass spectra as the furanose **dIz** derivatives ($[M + H]^+$ peak at 229 and the peak corresponding to the loss of the deoxyribose moiety, [M − dR + 2H], at 113) as well as the same UV-visible spectra.

On the other hand, the oxidation of **α-dG** by Mn-TMPyP–$KHSO_5$[11] led to quantitative transformation of **α-dG** to **α-dIz** within 1 min, as illustrated in Fig. 2. The chromatographic analysis of the two co-injected **α-dIz** products obtained by photosensitized or chemical oxidation confirmed that they were identical.

Both **α-** or **β-dIz** compounds were purified by collecting the corresponding HPLC peak from a large scale oxidation reaction (×15) of **α-** or **β-dG**, respectively, by Mn-TMPyP–$KHSO_5$, allowing the characterisation of these compounds by ^{1}H NMR (300 MHz). The ^{1}H NMR spectrum of **α-dIz** (D_2O) was consistent with the α-anomer structure. The signal at δ 5.77 (dd, J = 3.0 and 7.1 Hz, 1H) was assigned to the resonance of the proton H1′ of an α-anomer structure. The ^{1}H NMR spectrum of **α-dG** showed similar coupling pattern for H1′ proton.[17] In contrast, the ^{1}H NMR spectrum of **β-dIz** showed,

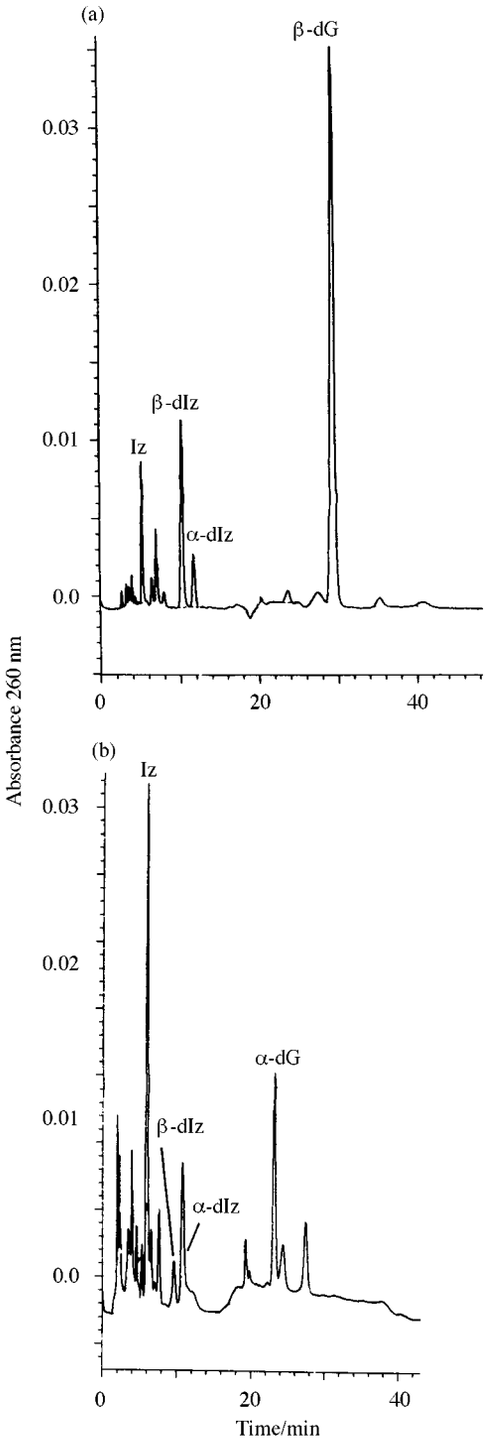


Fig. 1 Photosensitized oxidation of **β-dG** (a) or **α-dG** (b) in the presence of benzophenone after 8 h of irradiation (reverse-phase HPLC profile).

as previously published,[8] a pseudo triplet at δ 5.76 (J = 6.3 Hz) for the resonance of the H1′ proton. In both cases, the presence of impurities due to HPLC solvents did not allow a full description of the proton spectra.

Controls on the anomerization process

To understand the required conditions for the anomerization, the α- and β-anomers of **dIz** were purified from the metalloporphyrin-catalyzed oxidations **α-** and **β-dG** and subjected to different control experiments. No anomerization was observed when **β-dIz** was incubated at various pH (4.5 to 7.5) and temperatures (20 or 37 °C) for 8 h without irradiation. We observed only the already known transformation of **dIz** into **dZ**

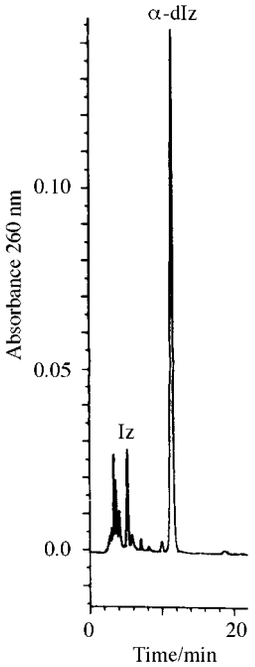


Fig. 2 Oxidation of **α-dG** in the presence of Mn-TMPyP–$KHSO_5$ after 1 min of reaction (reverse-phase HPLC profile).

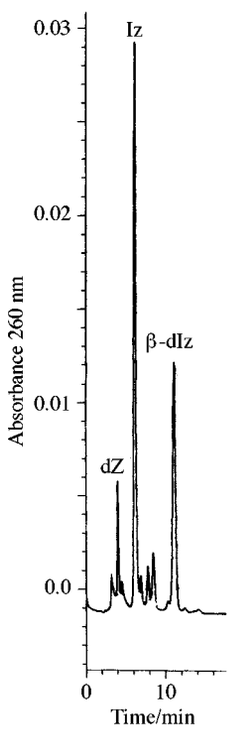


Fig. 3 Irradiation of purified **β-dIz** in the presence of benzophenone after 6 h (reverse-phase HPLC profile).

which was faster at higher pH and temperature.[4,7,8] The presence of benzophenone in these various incubation media did not promote the anomerization either. It is concluded that the ribosylamine structure of **dIz** is stable towards anomerization within the used temperature and pH range.

The purified **β-dIz** was then submitted to UV-irradiation in the presence of benzophenone under the same experimental conditions as those used for **dG** photooxidation. **β-dIz** was unstable and was degraded after 2 or 6 h of irradiation by 50 or 90% respectively. The formation of the two identified products, **Iz** and a minor amount of **dZ**, was not quantitative compared to the initial **dIz**. This was probably due to the formation of other non-identified oxidized derivatives. As shown in Fig. 3,

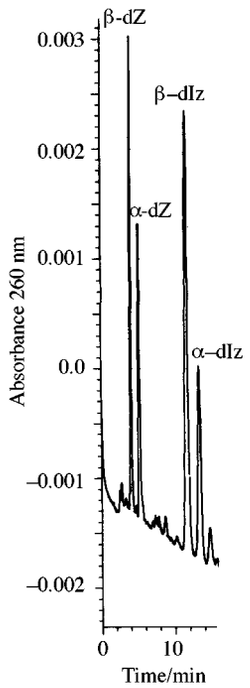


Fig. 4 Transformation of **β-** or **α-dIz** into **β-** or **α-dZ**. Incubation 6 h at 37 °C in water (reverse-phase HPLC profile).

18% of **Iz** (calculated from the initial amount of **dIz**) was formed after 6 h of irradiation (6% of **Iz** after 2 h) and no anomerization occurred. Thus the anomerization process must take place during the course of **dG** photooxidation but before the formation of **dIz**.

An additional control consisted of irradiating **β-dIz** in the absence of benzophenone. This compound was not stable in these reaction conditions and was degraded to the same extent as in the presence of benzophenone but without formation of **Iz**. The degradation of **β-dIz** was 60 and 90% respectively after 2 h and 6 h of irradiation. Only part of the degradation was accounted by the formation of **dZ**. Since **Iz** release was not observed here, the cleavage of the glycosidic bond, during the photosensitized oxidation, was thus probably due to sugar oxidation instead of to a simple hydrolysis of the ribosylamine structure of **dIz**. An H-abstraction on the C1′ (or C4′) position, followed by reaction of the carbon-centered radical with dioxygen, probably generates an oxidized abasic sugar.[2,12] The release of the free modified **Iz** base moiety of **dIz** was also reported to be due to sugar oxidation in the metalloporphyrin-mediated oxidation of **dG**.[11] Unfortunately, it was not possible to identify the sugar residue in these different experiments.

Identical chemical reactivity of both anomers of dIz

Apart from similar chemical reactivity during the photochemical or chemical oxidation reactions as described above it was possible to observe the same products derived from further chemical reaction of both anomers of **dIz**.

As expected, the stability of **α-dIz** was identical to that of **β-dIz**. A mixture of both purified anomers was incubated in water at 37 °C to monitor their respective hydrolysis into **α-** or **β-dZ**. The formation kinetics of both anomers of **dZ** were identical (under the HPLC conditions employed, retention times of **α-** and **β-dZ** were 5 and 4 min, respectively as shown in Fig. 4). The λ_{max} of **α-** or **β-dZ** was 234 nm. The ESI-MS spectra of the two anomers of **dZ** were identical and showed an $[M + H]^+$ signal at 247, fragmentation peaks corresponding to the loss of the deoxyribose moiety $[M - dR + 2H]^+$ at 131 or the loss of CO_2 at 203.[13]

The oxidation of both anomers of **dIz** by $KHSO_5$ (without metalloporphyrin) gave two products identified as being the

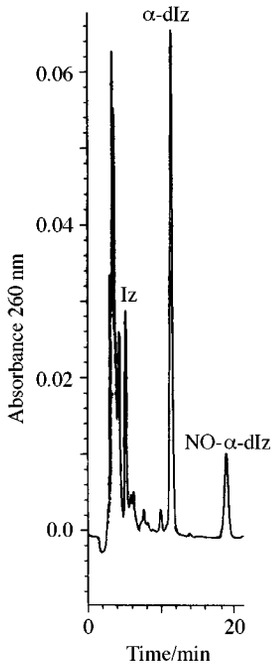


Fig. 5 Oxidation of **α-dG** in the presence of Mn-TMPyP–$KHSO_5$ after 5 min of reaction (reverse-phase HPLC profile). **NO-α-dIz** stands for the *N*-oxide of **α-dIz**.

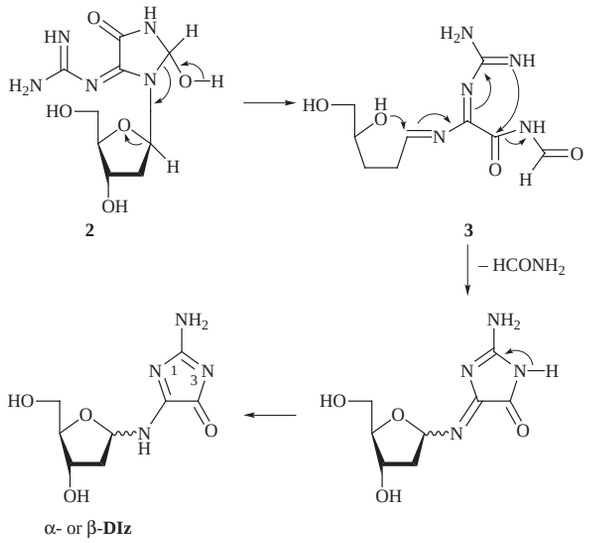
N-oxide derivatives of **dIz** (abbreviated **NO-α-dIz** in Fig. 5).[11] Under the HPLC conditions used, the retention time of the *N*-oxide derivatives of **dIz** were 14 and 19 min for the β- and α-anomer, respectively (λ_{max} value at 238 nm; ESI-MS: $[M + H]^+$ at 245 and $[M - dR + 2H]^+$ at 129).

Discussion

The photosensitized oxidation of **dG** in the presence of benzophenone led to the formation of two furanose anomers of **dIz**. The major one corresponds to the sugar configuration of the starting **dG**. The minor one, resulting from the anomerization process, represents 10% of the reaction products, depending on the reaction conditions. Due to long reaction times (several hours) the formation of degradation products decreases the yield of **dIz**. **dZ** and **Iz** have been identified among the degradation products.

The proposed mechanism of formation of **dIz** in a type I photosensitized oxidation (*i.e.*, *via* a one-electron oxidation of guanine base) is presented in Scheme 1.[7] After the addition of a water molecule at C8 and the loss of CO_2, the intermediate **2** is formed. This compound **2** would undergo either (as a major pathway) a ring-opening pathway by cleavage of the N9–C8 bond (Scheme 1) or (as a minor pathway) a sugar opening by cleavage of the C1′-O4′ bond (Scheme 2). The subsequent imine **3** (Scheme 2) would be the key step of the anomerization process since the sugar ring closure would give both stereoisomers of **dIz** by attack of the 4′-OH function on one or the other side of the carbon–nitrogen double bond. The presence of the two α- and β-anomers of the two pyranose derivatives further supports the importance of the intermediate imine **3** (a 5′-OH attack leading to ring expansion[12]).

This anomerization mechanism is dependent on the presence of the alcohol function at the former C8 position of **dG** on the 8-OH-7,8-dihydro intermediate **2**. Rearrangement inducing a delocalization of electrons from the heterocycle to the sugar oxygen atom leads to the intermediate imine **3** (the formation of an intermediate imine from **1** might also be considered as a possible alternative mechanism). When **α-dG** (this work) or **β-dG**[11] was oxidized into **dIz** by the Mn-TMPyP–$KHSO_5$ system, this hydroxylic function was quickly oxidized (within one minute) to give a 8-oxo-7,8-dihydro intermediate. We did not notice any isomerization of the produced **dIz** due to the absence of the hydroxylic function on C8 of the guanine in the intermediate compound leading to **dIz** (see ref. 11 for a detailed description of the mechanism of **dIz** formation from the 8-oxo-7,8-dihydro intermediate).



Scheme 2 Mechanism of formation of both anomers of **dIz** during the photooxidation of **β-dG**.

The same anomerization process was reported for purine nucleosides bearing an 8-OH-7,8-dihydro-modified base, precursors of formamidopyrimidine (**FAPy**) derivatives and well described for the 2′-deoxyadenosine (**dA**) species.[14] In the absence of molecular oxygen, γ-irradiation of β-dA led mainly to the β-furanose anomer of the **FAPy** derivative by the ring opening of an 8-OH-7,8-dihydro intermediate (a similar ring-opening is depicted in Scheme 1) as well as to about a 10% yield of the α-furanose anomer of the **FAPy** derivative. The β-isomer anomerized to a 50/50 equilibrium mixture of the α- and β-furanose anomers within 7 h. After 72 h the **FAPy-dA** compound was completely transformed to the more stable α- and β-pyranose anomers.[14] A mechanism involving the imine intermediate (Scheme 2) was proposed to account for these isomerizations. As can be noted from ref. 14, the equilibrium of α/β-furanose anomerization proved to be a slow process. During γ-irradiation, only 10% of the α-anomer of **FAPy** derivative was formed. So, the ring-opening of the 8-OH-7,8-dihydro-modified base is the kinetically favored pathway. It leads to the β-furanose **FAPy** derivative as the major product. The α-furanose derivative is formed by a minor sugar-opening process from the 8-OH-7,8-dihydro-modified base intermediate either directly or after ring closure of the opened major **FAPy** derivative (ring–chain tautomerism). Since the latter equilibrium of isomers is a slow process, a direct sugar-opening route may account for the low percentage of the minor anomer found after irradiation. In the reaction described here, the ring-opened form of the 8-OH-7,8-dihydro-modified intermediate is only a transient species with a low probability of ring closure, as in the case of the **FAPy** derivative, because the reaction proceeds further to the formation of imidazolone by the loss of formamide (Scheme 1). So we only observed the kinetic products of the 8-OH-7,8-dihydro-modified base intermediate; a less than 10% yield of the minor anomer is obtained. The higher degradation rate of the major β-anomer (into **Iz** by oxidation of the sugar and/or to other non-identified products) may explain the increase of the **α/β-dIz** ratio during the course of the reaction.

Anomerization was also reported during the oxidative degradation of β-thymidine that gave a mixture of α- and β-deoxyribosylurea and β-deoxyribosylformamide; the latter was stable in the β-configuration.[15] However, the anomerization

of the ribosylurea derivative seems to be due to the formation of the imine by loss of the anomeric amine proton that may be easier for the urea derivative than for the formamide one. This mechanism does not occur with **dIz**.

Isomerization, including also anomerization and ring expansion, are known a phenomenon when purine nucleosides are submitted to OH-radical attack.[12] It has been suggested that the sugar radical at C4′ was the precursor of these products because an epimerization at C4′ has also been observed. However, since the anomerization was only noticed for purine nucleosides, von Sonntag previously proposed[12] the involvement of base hydrates, instead of sugar radicals, for the mechanism of anomerization and ring expansion of purine nucleosides in oxidative photochemistry. The results reported here strongly support this hypothesis.

Conclusions

The photosensitized oxidation of **dG** leads to two anomers of imidazolone; the major one corresponds to the β-furanose conformation of the initial **dG**, while the second product corresponds to the α-furanose anomer. The yield of these products is dependent on the experimental conditions, due to long reaction times and to the instability of **dIz**. On the other hand, the chemical oxidation of **dG** by Mn-TMPyP–$KHSO_5$ offers the β-furanose anomer in an almost quantitative yield and thus represents a method of choice to the synthesis of **dIz**. These results were confirmed by the photosensitized or chemical oxidation of **α-dG**. A mechanism of anomerization of **dIz** has been proposed, which is not related to the ribosylamine structure of **dIz**, but rather to a C8-hydroxylated intermediate in the reaction pathway.

Experimental

Materials

Potassium monopersulfate, $KHSO_5$ (the triple salt $2KHSO_5 \cdot K_2SO_4 \cdot KHSO_4$, Curox®) was from Interox, 2′-deoxyguanosine was purchased from Sigma, benzophenone was from Aldrich. Mn-TMPyP was synthesized as described previously.[16] **α-dG** was obtained from the corresponding 2-*N*-isobutyryl derivative[17] upon treatment with methanolic ammonia and crystallization from water. Physico-chemical data were identical to those reported by Robins and Robins.[18]

Oxidation of dG anomers

The photooxidations were performed by irradiation, in a Pyrex flask containing 2 mL of a 1 mM aqueous solution of **dG**, in the presence of 4 mg of benzophenone, a predominant type I photosensitizer, in a Rayonet photochemical reactor equipped with black lamps emitting in the 350 nm range, under constant air bubbling and at constant temperature (~15 °C) for 8 h.

The chemical oxidation of **dG** was performed as described previously.[11] In a final volume of 250 μL of 20 mM triethylammonium acetate buffer pH 6.5, dG (1 mM) was incubated with Mn-TMPyP (8 μM) and $KHSO_5$ (10 mM). After 5 min of reaction at ambient temperature, the reaction was stopped by the addition of Hepes buffer pH 8 (100 mM) and directly injected for classical HPLC analysis.

The reactions were monitored by reverse-phase HPLC coupled to a UV-visible diode array detector or to an electrospray mass spectrometer allowing easy recording of the mass spectra of the oxidation products of **dG**. An analytical reverse-phase column (nucleosil C18, 10 μm, 250 × 4.6 mm) was eluted isocratically with MeOH–H_2O, 2 : 98 for 13 min then MeOH–H_2O, 10 : 90 at 1 mL min^{-1}. For details see ref. 11.

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