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Reactivity of Conjugated and Unconjugated Pterins with Singlet Oxygen ($O_2(^1\Delta_g)$): Physical Quenching and Chemical Reaction†

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ABSTRACT

Pterins (PTs) belong to a class of heterocyclic compounds present in a wide range of living systems. They participate in relevant biological functions and are involved in different photobiological processes. We have investigated the reactivity of conjugated PTs (folic acid [FA], 10-methylfolic acid [MFA], pteroic acid [PA]) and unconjugated PTs (PT, 6-hydroxymethylpterin [HPT], 6-methylpterin [MPT], 6,7-dimethylpterin [DPT], rhamnopterin [RPT]) with singlet oxygen ($^1O_2$) in aqueous solutions, and compared the efficiencies of chemical reaction and physical quenching. The chemical reactions between $^1O_2$, produced by photosensitization, and PT derivatives were followed by UV-visible spectrophotometry and high-performance liquid chromatography, and corresponding rate constants (k_r) were evaluated. Whenever possible, products were identified and quantified. Rate constants of $^1O_2$ total quenching by the PT derivatives investigated were obtained from steady-state $^1O_2$ luminescence measurements. Results show that the behavior of conjugated PTs differs considerably from that of unconjugated derivatives, and the mechanisms of $^1O_2$ physical quenching by these compounds and of their chemical reaction with $^1O_2$ are discussed in relation to their structural features.

INTRODUCTION

Singlet molecular oxygen ($O_2(^1\Delta_g)$, denoted throughout as $^1O_2$), the lowest electronic excited state of molecular oxygen, is an important oxidizing intermediate in chemical processes and one of the main reactive oxygen species responsible for the damaging effects of light on biological systems (photodynamic effects) (1). This activated metastable state is much more reactive than the triplet ground state ($O_2(^3\Sigma_g^-)$, denoted throughout as $^3O_2$) and both applied and fundamental aspects of its reactivity have been attracting considerable interest (1–4).

Photosensitization is primarily responsible for the production of $^1O_2$ *in vivo* (3). In this process, $^1O_2$ is most often produced by energy transfer from the excited triplet state of a sensitizer (Sens) to dissolved molecular oxygen (Reactions (1) and (2)). Subsequently, $^1O_2$ relaxes to its ground state $^3O_2$ through solvent induced radiationless and radiative pathways (Reactions (3) and (4)). It may also be deactivated by a physical quencher (Reaction (5)) and/or oxidize an acceptor molecule (Reaction (6)). The physical mechanisms proposed for these reactions have been recently reviewed (4).

$$^1\text{Sens} \xrightarrow{h\nu} {}^1\text{Sens}^* \xrightarrow{k_{ISC}} {}^3\text{Sens}^* \quad (1)$$

$$^3\text{Sens}^* + {}^3O_2 \xrightarrow{k_{et}} {}^1\text{Sens} + {}^1O_2 \quad (2)$$

$$^1O_2 \xrightarrow{k_d} {}^3O_2 \quad (3)$$

$$^1O_2 \xrightarrow{k_e} {}^3O_2 + h\nu'' \quad (4)$$

$$Q + {}^1O_2 \xrightarrow{k_r} QO_2 \quad (5)$$

$$Q + {}^1O_2 \xrightarrow{k_q} Q + {}^3O_2 \quad (6)$$

If a biological compound is able to deactivate $^1O_2$ efficiently by means of physical quenching, such a compound may have a protective role against $^1O_2$ *in vivo* and, very likely, against other reactive oxygen species. On the other hand, an efficient chemical reaction with $^1O_2$ may be beneficial or harmful to biological systems, depending on the nature of the oxidized products. Therefore the study of the reactivity of $^1O_2$ with biomolecules (physical quenching and chemical reaction) is a powerful tool to analyze their antioxidant capability. The determination of the rate constants of $^1O_2$ physical quenching and of the chemical reaction with $^1O_2$ (k_q and k_r, Reactions (5) and (6), respectively) allows the evaluation of the efficiencies of these processes. Product identification provides information to elucidate oxidation mechanisms and to discuss potential effects *in vivo*.

We have recently been interested in the production and deactivation of $^1O_2$ by PTs, a family of heterocyclic compounds derived from 2-aminopteridin-4(1*H*)-one (PT) and containing the pyrazine[2,3-d]pyrimidine ring structure (Scheme 1) (5–8). These compounds, widespread in nature, are involved in relevant biological functions (9) and participate in many photochemical and photobiological processes (10). PTs present, in aqueous solution, an acid–base equilibrium that involves an amide group (acid form) and a phenolate group (basic form) (Scheme 1), with a pK_a close to 8 (11–13). Other functional groups of the PT moiety (*e.g.* 2-amino group or ring nitrogen atoms) have pK_a values lower than 2 (11).

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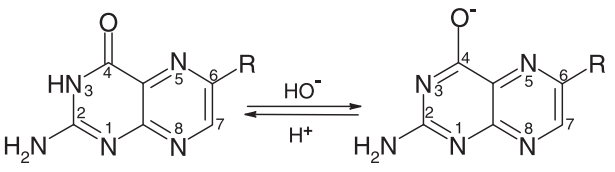
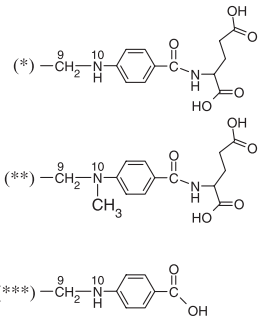


-R	Compound
-H	pterin (PT)
$-CH_2OH$	6-hydroxymethylpterin (HPT)
$-CH_3$	6- methylpterin (MPT)
$-CH_3$	6,7- dimethylpterin (DPT)[a]
$-(CHOH)_3-CH_3$	rhamnopterin (RPT)
(*)	folic acid (FA)[b,c]
(**)	10-methylfolic acid (MFA)[b,c]
(***)	pteroic acid (PA)[b]



Scheme 1. Molecular structures of common pterin derivatives and the acid–base equilibrium in aqueous solution. (a) DPT has an additional methyl group at position 7 of the pterin moiety; (b) these compounds contain a *p*-aminobenzoic acid (PABA) unit in the substituent at position 6; (c) in these compounds, a glutamic acid unit (Glu) has been attached to the PABA moiety.

The most common PT derivatives are 6-substituted compounds. The molecular weight and the functional groups of these substituents are quite different (Scheme 1); *e.g.* PTs may have substituents with one carbon atom, with a short hydrocarbon chain or larger substituents containing a *p*-aminobenzoic acid (PABA) moiety. Derivatives with the latter type of substituents are frequently called conjugated PTs.

We have reported previously the rate constants of $^1O_2$ total quenching ($k_t = k_q + k_r$, Reactions (5) and (6)) by a few PT derivatives (5–7). The rate constant k_t appears to be very dependent on the chemical nature of the 6-substituent. However, more investigations are needed because, up to now, k_t has only been determined for one conjugated PT, folic acid (FA) (5). Moreover, to the best of our knowledge, the rate constants of the chemical reaction between $^1O_2$ and PT derivatives (k_r) have been only reported for PT (8) and 6-methylpterin (MPT) (7) and the corresponding reaction products have not been studied.

In the present article we describe the processes of deactivation of $^1O_2$ by a series of conjugated and unconjugated PT derivatives in aqueous solutions and analyze the effect of the 6-substituent on the quenching efficiency. To avoid a mixture of acid and basic forms of the PT derivatives in the solution (*vide supra*), we have chosen to work at alkaline pH. We have expanded our previous studies on $^1O_2$ total quenching (5–7) by determining k_t values for new compounds. We have determined the k_r values for a series of conjugated and unconjugated PTs and investigated the corresponding oxidation products. Knowing k_t and k_r, rate constants of $^1O_2$ physical quenching (k_q) could be calculated. Taking into account this ensemble of results, we compare the efficiencies of chemical reaction and physical quenching, and discuss the effects of the structural features of the PT derivatives on their reactivity with $^1O_2$ within the general context of the mechanisms of $^1O_2$ deactivation proposed in the literature.

MATERIALS AND METHODS

Chemicals and preparation of solutions. Pterins (Shircks Laboratories) and rose bengal (RB; Aldrich) were used without further purification. The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 to 2 *M*. The ionic strength was approximately 10^{-3} *M* in all the experiments. The pH measurements were performed using a pH-meter CG 843P (Schott, Mainz, Germany) with a pH-combination electrode Blue-Line 14pH (Schott).

D_2O (Euriso-top; Groupe CEA, Saclay, France, minimum isotopic purity of 99.9%), a solution of DCl (Aldrich, 99.5% D) in D_2O and a solution of NaOD (CEA) in D_2O were employed for preparing solutions in D_2O. The pD values were calculated by adding 0.4 to the apparent pH values measured with the pH meter (14).

H_2O_2 determination. For determination of H_2O_2, the cholesterol CHOD-PAP kit from Roche Diagnostics (Germany) was used. H_2O_2 was quantified by its color reaction with 4-aminophenazone and phenol catalyzed by peroxidase. Five hundred microliters of irradiated solution of FA was added to 1 mL of reagent. The absorbance at 505 nm of the solution containing a mixture of irradiated solution and the reagent was measured after 20 min of incubation at room temperature, using the nonirradiated sample solution as a blank. Aqueous solutions of H_2O_2, prepared from commercial standards, were employed for obtaining the corresponding calibration curve.

Determination of the rate constants of $^1O_2$ total quenching by PTs. The rate constants of $^1O_2$ total quenching (k_t) by PTs were determined by Stern–Volmer analysis of the $^1O_2$ luminescence in the near-infrared (NIR) (15). Our equipment used to monitor the $^1O_2$ luminescence at 1270 nm upon continuous monochromatic excitation of a sensitizer has already been described (16). For the experiments reported in this article, a cooled (−80°C) NIR photomultiplier (Hamamatsu) was used as a $^1O_2$ detector, instead of a Ge photodiode. Briefly, the sample solution in a quartz cell (1 cm × 1 cm) was irradiated with a xenon/mercury arc through a water filter, focusing optics and a monochromator. The $^1O_2$ luminescence was collected with a mirror, chopped and, after passing through a focusing lens, a cut-off filter (1000 nm) and an interference filter (1271 nm), was measured at 90° with respect to the incident beam.

Singlet oxygen was generated by photosensitization, using RB as a sensitizer. Groups of experiments were carried out irradiating solutions of PTs and RB at 547 nm, where PT derivatives do not absorb. The RB concentration was kept constant, whereas the PT derivative concentration was varied within a series of experiments (Stern–Volmer analysis). Besides, the concentration of RB was such that $^1O_2$ total quenching by the sensitizer itself was negligible compared with $^1O_2$ deactivation by the solvent (17). Under conditions where the quantum yield of $^1O_2$ production by the sensitizer (Φ_Δ) is not affected by the presence of the quencher (a PT derivative) and assuming a dynamic quenching of $^1O_2$, a linear relationship between the ratio of the signals observed in the absence (Se^0) and in the presence (Se) of quencher and the quencher concentration should be obtained (Eq. 7).

$$S_e^0/S_e = 1 + k_t\tau_\Delta[Q] \quad (7)$$

τ_Δ is the $^1O_2$ lifetime in the solvent used in the absence of Q ($\tau_\Delta = 1/k_d$, as the radiative rate constant [k_e, Reaction (4)] is negligible compared to the radiationless deactivation rate constant [k_d, Reaction (5)] in most solvents) (18).

Therefore, knowing τ_Δ, k_t can be calculated from the slope of the Stern–Volmer plot (Eq. 7). Because of the short τ_Δ in H_2O (3.8 μs), D_2O (where τ_Δ is much longer: 62 ± 3 μs [19]) was used as a solvent in all experiments. Singlet oxygen lifetimes were determined by time-resolved phosphorescence detection. The laser system and the custom-built detectors employed (a Ge photodiode, Judson, or an InGaAs photodiode, IR Components) have already been described (20).

Determination of the rate constants of the chemical reaction between $^1O_2$ and PTs (k_r). The rate of disappearance of a compound Q reacting with $^1O_2$ is given by Eq. (8).

$$-d[Q]/dt = k_r[^1O_2][Q] \quad (8)$$

If $^1O_2$ is produced by sensitization and applying the quasi-stationary hypothesis to the concentrations of excited states (Reactions (1)–(6)), Eq. (9) gives the steady-state concentration of $^1O_2$,

$$[^1O_2] = P_a\Phi_\Delta/(k_d + k_t^S[S] + k_t^Q[Q]) \quad (9)$$

where P_a (E L^{-1} s^{-1}) is the photon flux absorbed by the sensitizer, Φ_Δ the quantum yield of $^1O_2$ production by the sensitizer, [S] the concentration of the sensitizer, k_t^S the rate constant of $^1O_2$ total quenching by the sensitizer and k_t^Q the rate constant of $^1O_2$ total quenching by Q.

Combining Eqs. (8) and (9), and assuming that there is no interference by the oxidation product(s), Eq. (10) is obtained for the rate of disappearance of a compound Q reacting with $^1O_2$.

$$-\frac{d[Q]}{dt} = P_a\Phi_\Delta\frac{k_r[Q]}{k_d + k_t^S[S] + k_t^Q[Q]} \quad (10)$$

If the rate of $^1O_2$ total quenching by the sensitizer is negligible compared with deactivation by the solvent ($k_d >> k_t^S[S]$), integration of Eq. (10) leads to Eq. (11).

$$f([Q]) = \ln([Q]/[Q]_O) - \left[\left(k_t^Q/k_d\right)([Q]_0 - [Q])\right] = -P_a\Phi_\Delta(k_r/k_d)t \quad (11)$$

and the plot of $f([Q])$ as a function of time should be linear (21). In the cases where $k_t^Q[Q] << k_d$, Eq. (11) simplifies to:

$$\ln([Q]/[Q]_O) = -P_a\Phi_\Delta(k_r/k_d)t \quad (11')$$

and first-order kinetics should be observed for the disappearance of Q.

For determining k_r, groups of experiments were carried out irradiating alkaline solutions (3 cm^3, pH = 10.5) containing a PT derivative and RB as a $^1O_2$ sensitizer. RB was excited at 547 nm (Φ_Δ = 0.75 [22,23]). The experiments were performed in D_2O. Within each series of experiments, the initial concentrations of the PT derivative and RB were kept constant, but the solutions were irradiated during different periods of time.

A high-performance liquid chromatography (HPLC) equipment (Hewlett Packard Series 1100) and an RP 18 LiChro CART 125-4 column were used for the determination of the evolution of the concentration of the PT derivatives as a function of irradiation time and for the analysis of the products of the reaction. Solutions containing 0–10% of acetronitrile and 90–100% of potassium phosphate aqueous solution (20 m*M*, pH 5.5) were used as eluents. The HPLC was equipped with a diode array detector (HP 1100 DAD) and a software (HP ChemStation for LC) for registering and analyzing spectra of the separated substances.

The solutions were irradiated in a 1 cm × 1 cm spectroscopic cell, on the same optical bench as used for the determination of k_t values (*vide supra*). The incident photon flux (P_o) at the wavelength of excitation of the sensitizer (547 nm) were determined by actinometry, using Aberchrome 540 as an actinometer (24) ($P_o = 2.8 \times 10^{-6}$–3.6×10^{-6} E L^{-1} s^{-1} at 547 nm). Values of P_a (photon flux absorbed by the sensitizer) were calculated from P_o using the Beer–Lambert law:

$$P_a = P_o(1 - 10^{-A}) \quad (12)$$

where A is the absorbance of the sensitizer at the excitation wavelength.

The spectral changes were registered on a Cary 5 (Varian) spectrophotometer. The irradiation experiments were performed using quartz cells of 1 cm of optical path length, whereas the absorbance measurements were carried out with quartz cells of 0.1 cm or 1 cm of optical path length.

RESULTS AND DISCUSSION

Rate constants of $^1O_2$ total quenching (k_t) by PT derivatives

The values of the rate constants of $^1O_2$ total quenching (k_t) by the series of PT derivatives investigated in this work (Scheme 1)—three conjugated PTs (FA, 10-methylfolic acid [MFA], pteroic acid [PA]) and five unconjugated PTs (PT, 6-hydroxymethylpterin [HPT], MPT, 6,7-dimethylpterin [DPT], rhamnopterin [RPT])—are listed in Table 1. These values were determined as indicated in Materials and Methods. The Stern–Volmer plots of the quenching of the NIR $^1O_2$ luminescence (Eq. (7)) were linear within the range of concentrations used and are shown in Fig. 1 for MFA, PA, RPT and DPT. The values of k_t were calculated from the slopes of these plots. Values of k_t obtained in this work and in former studies (5–7) lie in the range 3×10^6–7×10^7 M^{-1} s^{-1}.

The k_t value obtained for RPT ($3.6 \pm 0.4 \times 10^6$ M^{-1} s^{-1}) is of the same order of magnitude as k_t values found for PT and HPT (approx. 3×10^6 M^{-1} s^{-1}, Table 1). Among unconjugated PTs, MPT and particularly DPT have larger k_t values ($8.0 \pm 0.6 \times 10^6$ and $3.1 \pm 3 \times 10^7$ M^{-1} s^{-1}, respectively) than PT, HPT and RPT.

The k_t values obtained for FA, MFA and PA are about 1 order of magnitude larger than the k_t values obtained for most unconjugated PTs (Table 1). It should be noted that the

Table 1. Rate constants of $^1O_2$ total (k_t) and physical quenching (k_q) by pterin derivatives, and rate constants of their chemical reaction with $^1O_2$ (k_r) in D_2O (pD = 10.5).

Compound	k_t (10^6 M^{-1} s^{-1})	k_r (10^6 M^{-1} s^{-1})	k_q (10^6 M^{-1} s^{-1})
Unconjugated pterins			
PT	2.9 ± 0.3 (5)	0.25 ± 0.03 (8)	2.6 ± 0.3
HPT	3.1 ± 0.4 (6)	1.2 ± 0.1	1.9 ± 0.5
RPT	3.6 ± 0.4	2.4 ± 0.2	1.2 ± 0.6
MPT	8.0 ± 0.6 (7)	4.9 ± 0.7 (7)	3 ± 1
DPT	31 ± 3	10 ± 2	21 ± 5
Conjugated pterins			
FA	30 ± 3 (5)	2.8 ± 0.3	27 ± 3
MFA	44 ± 4	1.9 ± 0.2	42 ± 4
PA	67 ± 7	12 ± 2	55 ± 9

PT = pterin; HPT = 6-hydroxymethylpterin; RPT = rhamnopterin; MPT = 6-methylpterin; DPT = 6,7-dimethylpterin; FA = folic acid; MFA = 10-methylfolic acid; PA = pteroic acid.

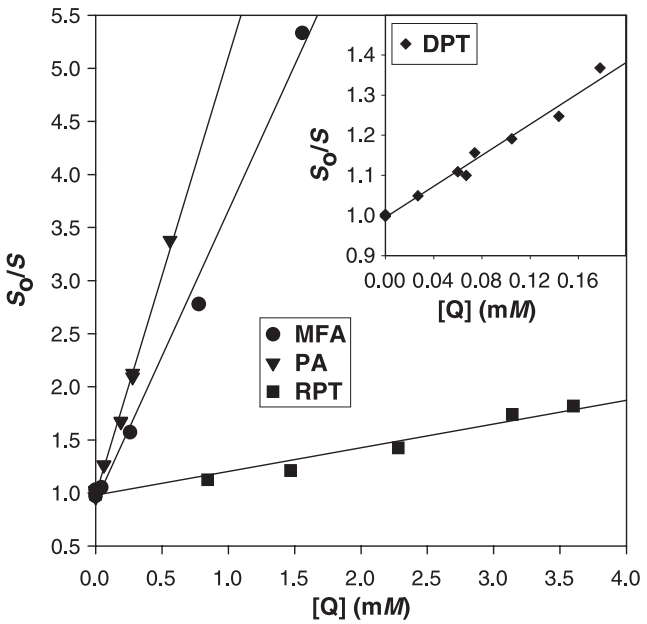


Figure 1. Stern–Volmer plots of the quenching of the $^1O_2$ near-infrared luminescence by MFA, PA, RPT and DPT (inset) in D_2O (pD = 10.5, rose bengal was used as a sensitizer, λ_{ex} = 547 nm); due to solubility problems the concentration range for DPT was limited.

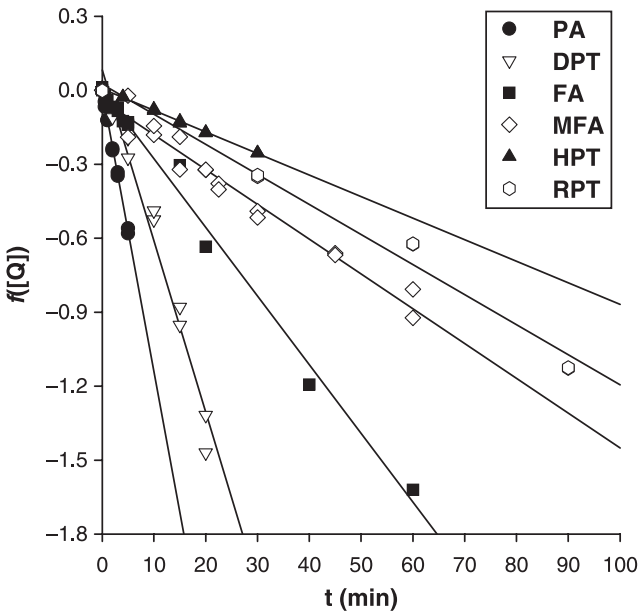


Figure 2. Photosensitized oxidation of pterin derivatives in air-equilibrated alkaline D_2O solution: plot of $f([Q]) = \ln([Q]/[Q]_o) - [(k_t^Q/k_d)([Q]_0 - [Q])]$ as a function of the irradiation time (Eq. 11, Materials and Methods). Sensitizer: rose bengal (irradiation wavelength: 547 nm), pD = 10.5, concentrations of pterin derivatives determined by HPLC analysis. Note that the slopes of these plots ($-P_a\,\Phi_\Delta\,[k_r/k_d]$) do not reflect directly the values of k_r, as experiments were carried out using different incident photon flux.

conjugated PTs (FA, MFA, PA) contain an additional amino group in the PABA unit (N-10, Scheme 1) and that amines are known to deactivate efficiently $^1O_2$ by charge-transfer-induced physical quenching (25–27). Therefore, the presence of the PABA amino group in FA, MFA and PA could explain the larger k_t values observed for these compounds. The comparison of k_t for FA and MFA with that of PA reveals that the amide group introduced in the former compounds by the attachment of the glutamic acid unit (Glu) to the PABA moiety (Scheme 1) has a negative effect on the total $^1O_2$ quenching efficiency (Table 1). However, detailed reactivity comparisons based on k_t values only are difficult to make because k_t represents the sum of k_r and k_q (rate constants corresponding to chemical reaction and physical quenching, respectively) and these two kinetics constants may be governed by different factors.

Chemical reaction between $^1O_2$ and unconjugated PTs

The rate constants of the chemical reaction between $^1O_2$ and the basic forms of several unconjugated PT derivatives (HPT, DPT and RPT) (k_r) in D_2O were determined from HPLC analysis of the disappearance of the PT derivative (Q) during the photosensitized oxidation, using RB as a $^1O_2$ sensitizer. The values of k_r listed in Table 1 were obtained from the linear plot of $f([Q])$ or $\ln([Q]/[Q]_0)$ as a function of the irradiation time (Eqs. 11 or 11′, Materials and Methods, and Fig. 2). For comparison purposes, we have included in Table 1, the k_r values previously reported for PT (8) and MPT (7). The values of the rate constants of $^1O_2$ physical quenching (k_q) listed in Table 1 were obtained by subtracting the k_r values from the corresponding k_t values.

The k_r value for PT ($2.5 \pm 0.3 \times 10^5\ M^{-1}\ s^{-1}$) is very low in comparison with the corresponding k_t value, indicating that the deactivation of $^1O_2$ by PT is mainly a physical quenching process ($k_q = 2.6 \pm 0.3 \times 10^6\ M^{-1}\ s^{-1}$ is an order of magnitude larger than k_r). The UV-visible spectrophotometric analysis of the irradiated solutions and the HPLC analysis of the products of the reaction between $^1O_2$ and PT showed that the PT moiety was oxidized and cleaved yielding a group of nonpterinic products; *i.e.* the characteristic absorption band of the PT in the UV-A region was lost (see Supplemental Materials). The absorption also decreased at shorter wavelength (in the UV-B and UV-C) indicating that products formed lack aromatic character.

The same analysis performed for MPT and DPT yielded similar results—the oxidation of these compounds by $^1O_2$ leads to the cleavage of the PT moiety and the formation of several nonpterinic products. As an example, the HPLC analysis and the disappearance of the two typical absorption bands of the PT moiety during the sensitized oxidation of MPT, using RB as a sensitizer (irradiation at 547 nm) can be observed in Fig. 3. Similar spectrophotometric behavior was observed for all unconjugated PTs (see Supplemental Materials).

However, significant differences can be appreciated within this series of compounds in the chemical reactivity toward $^1O_2$. Values of k_r increase in the order $k_r(PT) << k_r(MPT) < k_r(DPT)$, which suggests that the higher electronic density on the pyrazine ring induced by the methyl group(s) favors the electrophilic attack of $^1O_2$. Introduction of a methyl substituent at position 6 (MPT) leads to an increase in k_r by a factor of 20 relative to PT ($k_r(MPT) = 4.9 \pm 0.7 \times 10^6\ M^{-1}\ s^{-1}$), and introduction of a second methyl substituent at position 7 (DPT) leads to an increase in k_r by a factor of 40 ($k_r(DPT) = 10 \pm 2 \times 10^6\ M^{-1}\ s^{-1}$). Therefore, taking into

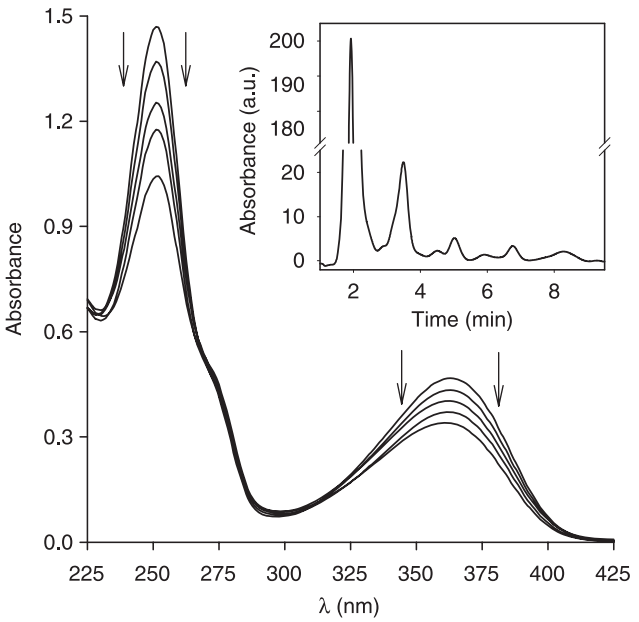
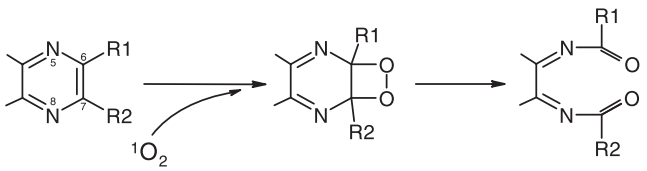


Figure 3. Photosensitized oxidation of 6-methylpterin ($[MPT]_o$ = 634 μ*M*) in air-equilibrated alkaline D_2O solution: Evolution of the absorption spectra as a function of irradiation time. Sensitizer: rose bengal (irradiation wavelength: 547 nm), pD = 10.5. Spectra were recorded every 30 min; arrows indicate the changes observed at different wavelengths. Optical path length: 1 cm for irradiation and 1 mm for absorbance measurements. Inset: Chromatogram obtained in HPLC analysis at 30 min of irradiation. All the picks shown correspond to products of the reaction. λ = 230 nm.

account the known reactivity of $^1O_2$ with C=C (28,29), the most probable reaction of this species with the PT moiety is a [2 + 2] cycloaddition to the C6–C7 double bond. Such a reaction would yield a dioxetane which may subsequently decompose, resulting in the cleavage of the pyrazine ring and the formation of two carbonyl groups in the first step (Scheme 2). As expected in the context of such an hypothesis, k_r(HPT) ($1.2 \pm 0.1 \times 10^6$ M^{-1} s^{-1}) and k_r(RPT) ($2.4 \pm 0.1 \times 10^6$ M^{-1} s^{-1}) are higher than k_r(PT), but smaller than k_r(MPT), the electron donor effect of the C6-substituent of HPT and RPT being smaller than that of a methyl group. Moreover, the reactivity of HPT in comparison with that of PT and MPT follows a pattern already observed in previous studies performed on other series of compounds; *e.g.* the k_r value obtained for 5-hydroxymethyl-2-furaldehyde is in between the k_r values of 2-furaldehyde and 5-methyl-2-furaldehyde (30).

Physical quenching of $^1O_2$ by unconjugated PTs

It is noteworthy that the rate constants of $^1O_2$ physical quenching (k_q) obtained for PT, HPT, RPT and MPT are similar ($1.2–3 \times 10^6$ M^{-1} s^{-1}), whereas the corresponding values of k_r are quite different ($2.5 \times 10^5–5 \times 10^6$ M^{-1} s^{-1}) (Table 1). Only for DPT, which has the largest k_r value of all unconjugated PTs investigated, k_q (2.1×10^7 M^{-1} s^{-1}) is about an order of magnitude larger than k_q for other PTs in this series. Although such a large effect on k_q of the 6,7-dimethyl substitution on the PT moiety was rather surprising, it confirms that this substitution affects considerably the reactivity of PT derivatives with $^1O_2$. However, if the chemical reaction is clearly dependent on the electronic activation of the C6–C7 double bond of the pyrazine ring, $^1O_2$ physical quenching does not show such a dependence and involves most probably different sites on the PT derivative. Thus, charge-transfer deactivation by the amino group at position 2 may contribute to $^1O_2$ physical quenching by PT, HPT, RPT and MPT, although primary amines may exhibit rather low k_q values ($< 5 \times 10^5$ M^{-1} s^{-1}) (26).



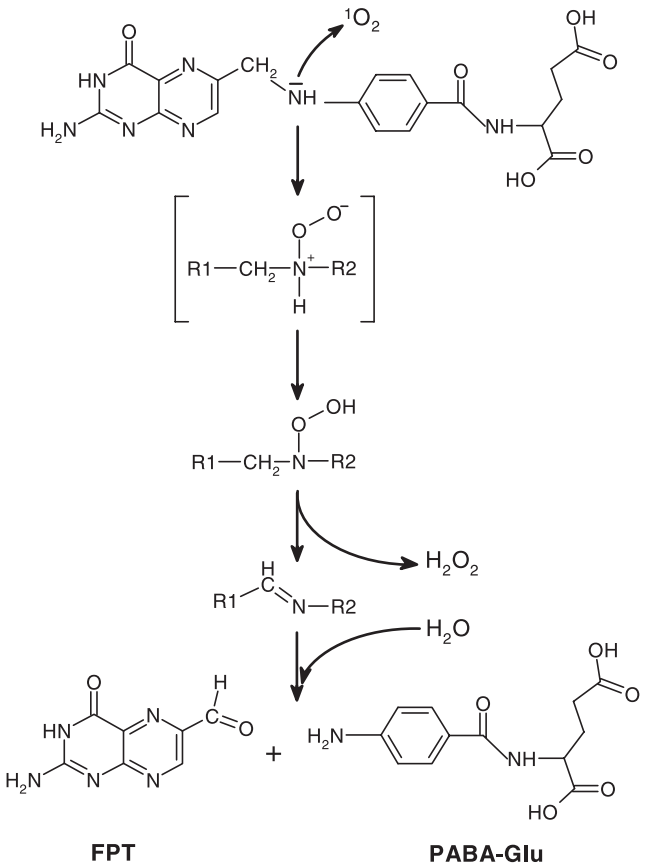
Scheme 2. Mechanism proposed for the attack of $^1O_2$ onto the pyrazine ring of the pterin moiety.

Chemical reaction between $^1O_2$ and conjugated PTs

The k_r value for FA, the most important PT derivative present in mammalians, and two related compounds, MFA and PA (Scheme 1), have been determined (Materials and Methods and Table 1). These values are much lower than the corresponding k_t values, thus indicating that the quenching of $^1O_2$ by conjugated PTs is mainly a physical process. Nevertheless, k_r values remain in the range similar to that obtained for C6-substituted unconjugated PTs (Table 1).

The k_r value for FA is about 1 order of magnitude larger than that found for PT. The HPLC analysis of the products of the reaction between $^1O_2$ and FA revealed the formation of 6-formylpterin (FPT). Therefore, the 6-substituent on the FA molecule is oxidized and cleaved between the methylene group linked to the PT moiety and the amino group of the PABA unit (Scheme 1). The mechanism proposed by Gollnick and Lindner for the attack of $^1O_2$ on primary and secondary amines bearing a nitrogen atom substituted by a CH group (31) can explain the observed reaction (Scheme 3). Accordingly, we propose that the charge-transfer reaction occurring between the N-10 atom and $^1O_2$ leads to the oxidation of the amine (–CH_2–NH–) to an imine (–CH = N–) with concomitant H_2O_2 elimination. Hydrolysis of the imine functional group in aqueous solution induces cleavage of the 6-substituent (–CHO + –NH_2), yielding FPT and *p*-aminobenzoylglutamic acid (PABA-Glu) as products. Formation of H_2O_2 was confirmed using a colorimetric method based on the reaction of H_2O_2 with 4-aminophenazone and phenol in the presence of peroxidase (Materials and Methods).

The HPLC analysis of the reaction mixture also shows that other products were formed, in addition to FPT, and PABA-Glu. The spectral features of these latter products suggest that the pterinic moiety is also oxidized to yield non pterinic compounds. In order to evaluate the extent of the oxidation that yields FPT as product (oxidation of the 6-substituent), the FPT concentration was determined as a function of irradiation time and compared with the corresponding evolution of the FA concentration. In the experiment shown in Fig. 4a, the initial rate of FA consumption was 1.9 ± 0.2 μ*M* min^{-1}, whereas the initial rate of FPT production was 0.51 ± 0.04 μ*M* min^{-1}. These results show that 27% of the FA molecules consumed are transformed into FPT. Therefore



Scheme 3. Mechanism proposed for the photosensitized oxidation of FA in aqueous solution to yield FPT and PABA-Glu as main products.

we can propose two different pathways on two different sites of the molecule, C6–C7 double bond of the PT moiety and N-10 of the PABA substituent (Reactions (13) and (14), respectively).

$$FA + {}^1O_2 \xrightarrow{k_{r1}(FA)} \text{Non pterinic compounds} \quad (13)$$

$$FA + {}^1O_2 \xrightarrow{k_{r2}(FA)} FPT + PABA - Glu \quad (14)$$

The rate constants of these reactions, k_{r1}(FA) and k_{r2}(FA), were calculated from the corresponding initial rates of FA consumption and FPT formation. Values of 2.0×10^6 and 8×10^5 M^{-1} s^{-1} were obtained, respectively (Table 2). It is interesting to compare the value of k_{r1}(FA) with k_r values obtained for unconjugated PTs: k_{r1}(FA) is almost 10 times larger than k_r(PT), but lower than k_r(MPT). This could be expected, as Reaction (13) corresponds to chemical modifications similar to those undergone by other unconjugated PTs. The methylene group of the 6-substituent should enhance the reaction of the PT moiety with $^1O_2$ with respect to PT, but such an effect should be weaker than that of the methyl group in MPT.

The k_r and k_t values found for PA are significantly larger than corresponding values obtained for FA (Table 1). The only structural difference between PA and FA is the lack of the glutamic (Glu) unit attached to the PABA substituent of PA. Therefore steric effects due to the Glu unit present in FA might

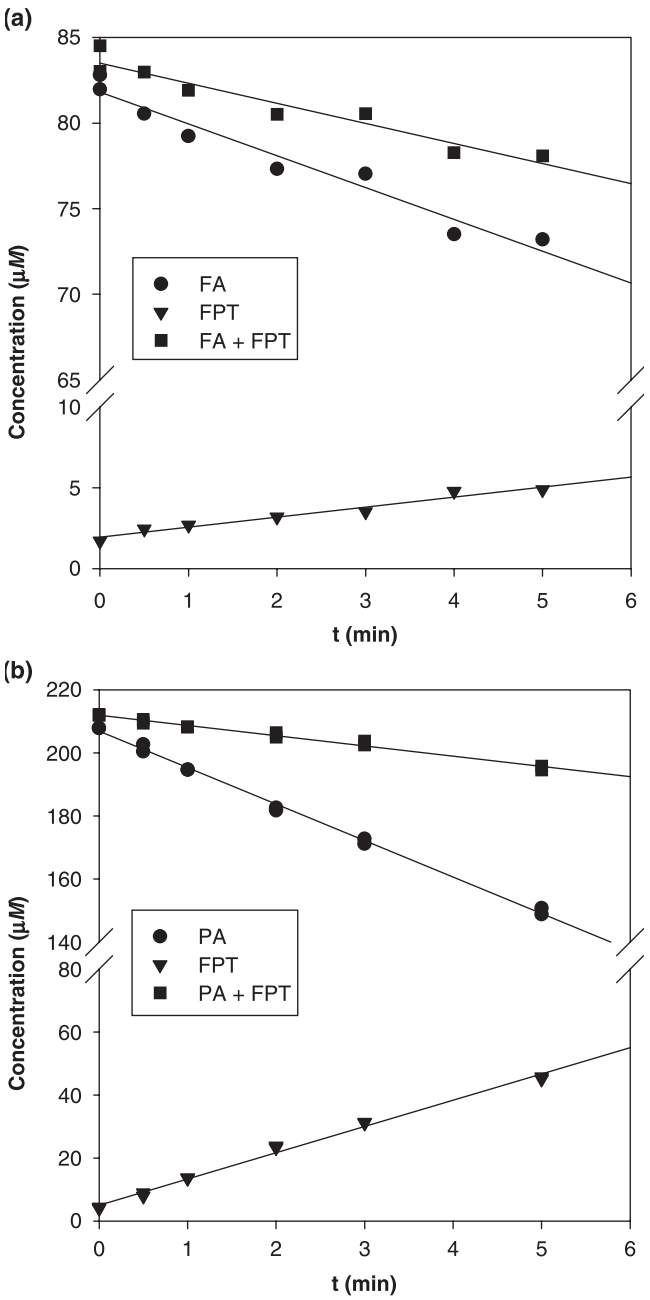


Figure 4. Evolution of pterin derivative and product concentrations as a function of irradiation time during sensitized photooxidation. Chemical reaction between $^1O_2$ and: (a) FA, (b) PA. Conditions: sensitizer, RB; irradiation wavelength, 547 nm; solvent, D_2O; pD, 10.5; concentrations of FA, PA and FPT determined by HPLC analysis.

hinder the quenching of $^1O_2$ by the PABA moiety (N-10 atom). Otherwise, PA is expected to undergo similar reactions with $^1O_2$ (Reactions (15) and (16)) as already proposed for FA (Reactions (13) and (14)).

$$PA + {}^1O_2 \xrightarrow{k_{r1}(PA)} \text{Non pterinic compounds} \quad (15)$$

$$PA + {}^1O_2 \xrightarrow{k_{r2}(PA)} FPT + PABA \quad (16)$$

Indeed, FPT is a product of the reaction between $^1O_2$ and PA. Therefore, similar to FA (Scheme 3), the amino group of the

Table 2. Rate constants of the chemical reaction of conjugated pterins with $^1O_2$ in D_2O (pD = 10.5). k_{r1} and k_{r2}: reaction of $^1O_2$ with the pterin moiety and the PABA substituent, respectively.

Compound	k_{r1} (10^6 M^{-1} s^{-1})	k_{r2} (10^6 M^{-1} s^{-1})
FA	2.0 ± 0.4	0.8 ± 0.1
MFA	1.9 ± 0.2	–
PA	~2.4	9.5 ± 1

PABA = *p*-aminobenzoic acid; FA = folic acid; MFA = 10-methylfolic acid; PA = pteroic acid.

PABA unit of PA reacts with $^1O_2$, leading to the cleavage of the PABA substituent and the formation of FPT and PABA (Reaction (16) and Scheme 3). The evolution of FPT and PA concentrations as a function of irradiation time is shown in Fig. 4b. The initial rate of PA consumption was 11.6 ± 0.5 μM min^{-1}, whereas that of FPT production was 9.2 ± 0.4 μM min^{-1}. These results show that, in this case, approximately 80% of the PA molecules consumed were transformed into FPT. The value of k_{r2}(PA) was calculated from the latter rate to be 9.5×10^6 M^{-1} s^{-1} (Table 2). This value is 1 order of magnitude higher than that of k_{r2}(FA), indicating that the chemical reaction of $^1O_2$ with the N-10 atom of the PABA substituent is much more efficient for PA than for FA. As mentioned above, steric effects of the Glu unit in FA may explain this result. Indeed, in a study on a series of 22 amines, Monroe has shown that $^1O_2$ quenching is highly sensitive to steric effects, and is inhibited in sterically hindered amines (26). As expected, the rate constants for the chemical reaction of $^1O_2$ with the PT moiety (k_{r1}(PA) and k_{r1}(FA), Table 2) are close (2.4×10^6 and 2.0×10^6 M^{-1} s^{-1}, respectively) and similar to those observed for C-6 substituted unconjugated PTs (Table 1).

6-Formylpterin was not detected as a product of the reaction between $^1O_2$ and MFA. In contrast to the case of FA and PA, only non pterinic compounds were found as products by HPLC analysis. These results strongly support the mechanism proposed in Scheme 3 for FA. Indeed such a sequence of reactions as proposed in this Scheme is not possible if the N-10 atom bears a methyl group, as it is the case for MFA. Moreover, the k_r value obtained for MFA (Table 1) is similar to k_{r1}(FA) and k_{r1}(PA) (reaction with the PT moiety).

Physical quenching of $^1O_2$ by conjugated PTs

The results presented above on the reactivity of $^1O_2$ with FA, MFA and PA confirm the important contribution of the amine group of the PABA unit to the deactivation of $^1O_2$ through charge-transfer-induced quenching. Several studies have shown that the singlet exciplex ($^1(Q\cdots O_2)$) formed by interaction of $^1O_2$ with the N-lone electron pair of aliphatic and aromatic amines is stabilized by the transfer of charge from the amine to O_2 (4,25,27,32), and may follow three different pathways: (1) decay by intersystem-crossing to a triplet ground-state complex ($^3(Q\cdots O_2)$), which finally dissociates to the quencher and $^3O_2$, without charge separation; (2) chemical reaction due to the formation of a chemical bond between a nitrogen atom of the quencher and the O_2 molecule (oxygenation); (3) dissociation with charge separation, *i.e.* formation of free radical ions. In most cases, the first pathway (physical quenching) is quantitatively more important than the latter ones (chemical reactions) (4). In agreement, the singlet exciplex formed by interaction between the N-10 atom of FA and PA with $^1O_2$ leads mainly to $^1O_2$ physical quenching (k_q(FA) = 2.7×10^7 and k_q(PA) = 5.5×10^7 M^{-1} s^{-1}), the corresponding chemical reaction (Scheme 3) contributing to a much lesser extent to the deactivation (k_{r2}(FA) = 8×10^5 and k_{r2}(PA) = 9.5×10^6 M^{-1} s^{-1}). k_q(PA) is larger than k_q(FA) by a factor of 2, showing that $^1O_2$ physical quenching is less sensitive than the chemical reaction to steric effects of the Glu unit present in FA. The case of MFA emphasizes the important contribution of the amine group of the PABA unit to the physical deactivation of $^1O_2$: although the chemical reaction can only take place on the PT moiety as for unconjugated PTs, k_q(MFA) (4.2×10^7 M^{-1} s^{-1}) is much higher than k_q of the latter compounds, and is comparable to k_q values for PA and FA. As expected for a charge-transfer-induced $^1O_2$ quenching, k_q for MFA (N-10 tertiary amine) is larger than k_q for FA (N-10 secondary amine).

CONCLUSIONS

The reactivity of a series of conjugated (FA, MFA, PA) and unconjugated PTs (PT, HPT, DPT, RPT) (Scheme 1) with singlet oxygen ($^1O_2$) in alkaline aqueous solutions has been investigated.

The rate constant of the chemical reaction between $^1O_2$ and the basic forms of unconjugated PT derivatives (k_r) is strongly affected by the chemical nature of the 6-substituent; and the values lie in a wide range (2.5×10^5–10^7 M^{-1} s^{-1}, Table 1). These values increase with the electronic activation of the C6–C7 double bond of the pyrazinic ring of the PT moiety, suggesting that the electrophilic attack of $^1O_2$ takes place preferentially on this bond. The PT moiety was oxidized and broken yielding a group of non pterinic products; *e.g.* the characteristic absorption band of PTs in the UV-A region was lost. In contrast to k_r, the rate constants of $^1O_2$ physical quenching (k_q in the range 1.2–3×10^6 M^{-1} s^{-1} for PT, HPT, RPT and MPT) do not depend on the electronic activation of the C6–C7 double bond of the pyrazine ring. The exception in the series of unconjugated PTs investigated is the 6,7-dimethyl derivative (DPT) with a k_q value an order of magnitude larger (2.1×10^7 M^{-1} s^{-1}).

In the chemical reaction of $^1O_2$ with conjugated PTs, two different processes have been observed: (1) the attack of $^1O_2$ on the PT moiety is responsible for the formation of nonpterinic products as in the case of unconjugated derivatives (corresponding rate constants are comparable); (2) the attack of $^1O_2$ on the secondary amine group (N-10 atom) of the PABA unit leads to the cleavage of the 6-substituent and formation of 6-formyl pterin (FTP, Scheme 3). The k_r value for this latter reaction (k_{r2}) is 1 order of magnitude smaller for FA (8×10^5 M^{-1} s^{-1}) than for PA (9.5×10^6 M^{-1} s^{-1}), due to steric effects of the Glu unit attached to the PABA substituent of FA. In the case of MFA, the N-10 substitution by a methyl group prevents this reaction, confirming the mechanism proposed in Scheme 3.

In contrast to unconjugated PTs, rate constants of $^1O_2$ physical quenching by conjugated derivatives are larger than corresponding rate constants for the chemical reaction. Values of k_q for conjugated PTs are also at least 1 order of magnitude larger than those for unconjugated PTs. These results show

that the amine group of the PABA unit of the former compounds plays a dominant role in the physical deactivation of $^1O_2$ through charge-transfer-induced quenching, in agreement with published data for different types of amines (25,26).

Finally the biological relevance of the results obtained can also be evaluated. At physiological pH (~7.4) both acid and basic forms of PTs are present. Several unconjugated PTs accumulate in white skin patches of patients affected by vitiligo, zones of the skin where the protection against UV radiation fails due to the lack of melanin (33). In addition, unconjugated PTs are good singlet oxygen photosensitizers. Therefore the reactions studied in this work could also take place *in vivo*.

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SUPPLEMENTAL MATERIALS

Additional figures are available for this article:

Evolution of the absorption spectra as a function of irradiation time.

Sensitizer: rose bengal (irradiation wavelength: 547 nm), pD = 10.5.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/full/10.1111/j.10.1562/2006-09-15-RA-1041

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