Singlet Oxygen ($O_2(^1\Delta_g)$) Quenching by Dihydropterins

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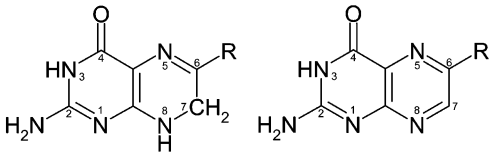
Pterins belong to a class of heterocyclic compounds present in a wide range of living systems. They participate in relevant biological functions and are involved in different photobiological processes. Dihydropterins are one of the biologically active forms of pterins. The photoinduced production and quenching of singlet oxygen ($^1O_2$) by a series of dihydropterins (7,8-dihydrobiopterin (DHBPT), 7,8-dihydroneopterin (DHNPT), 6-formyl-7,8-dihydropterin (FDHPT), sepiapterin (SPT), 7,8-dihydrofolic acid (DHFA), and 7,8-dihydroxanthopterin (DHXPT)) in aqueous solution at physiological pH (~7) were investigated, and the quantum yields of $^1O_2$ production (Φ_Δ) and rate constants of total quenching (k_t) of $^1O_2$ were determined. Studied compounds do not produce $^1O_2$ under UV-A irradiation and are very efficient $^1O_2$ quenchers. The chemical reactions between $^1O_2$ and dihydropterin derivatives were investigated, and the corresponding rate constants (k_r) were found to be particularly high. The oxidized pterin derivatives, biopterin (BPT), neopterin (NPT), 6-formylpterin (FPT), and folic acid (FA), were identified and quantified during the reaction of $^1O_2$ with DHBPT, DHNPT, FDHPT, and DHFA, respectively. Besides the oxidation of the dihydropyrazine ring to yield the corresponding oxidized pterins, a second oxidation pathway, leading to fragmentation of the dihydropterin and formation of non-pterinic products, was identified. Mechanisms and biological implications are discussed.

Introduction

Pterins, heterocyclic compounds widespread in biological systems, are derived from 2-aminopteridin-4(1*H*)-one or pterin (PT). The most common pterin derivatives are 6-substituted compounds. The molecular weight and functional groups of these substituents are quite different, e.g., pterins may have substituents with one carbon atom, with a short hydrocarbon chain or larger substituents containing a *p*-aminobenzoic acid (PABA) moiety (Figure 1). Derivatives with the latter type of substituents are frequently called conjugated pterins. Pterin derivatives can exist in different oxidation states and be divided into two classes according to this property: (a) oxidized or aromatic pterins containing the pyrazine[2,3-*d*]pyrimidine ring structure and (b) reduced pterins (Figure 1). Within the latter group, 7,8-dihydropterins and 5,6,7,8-tetrahydropterins are the most important derivatives due to their biological activity.

Oxidized and reduced pterins accumulate in the white skin patches of patients affected by vitiligo.[1] In this disease protection of the skin against UV radiation fails due to the lack of melanin. There are indications that the photochemistry and photophysics of pterins could play an important role in this disorder. Under UV-A radiation (320−400 nm) exposure oxidized pterins are able to generate reactive oxygen species (ROS),[2] very likely contributing to the oxidative stress in the affected tissues and deactivation of enzymes of the melanin metabolism. For instance, upon excitation at 350 nm, biopterin (BPT), 6-formylpterin (FPT), and 6-carboxyterin (CPT), compounds present in the white patches,[1] produce singlet molecular oxygen ($O_2(^1\Delta_g)$, denoted throughout as $^1O_2$).[3] In addition, photoinduced genera-



R	*dihydroderivative*	*Oxidized pterin*
-CHO	7,8-dihydro-6-formylpterin (FDHPT)	formylpterin (FPT)
$-(CHOH)_2-CH_3$	7,8-dihydrobiopterin (DHBPT)	biopterin (BPT)
$-(CHOH)_2-CH_2OH$	7,8-dihydroneopterin (DHNPT)	neopterin (NPT)
$-CO-CHOH-CH_3$	sepiapterin (SPT)	-
(*)	7,8-dihydrofolic acid (DHFA)	folic acid (FA)

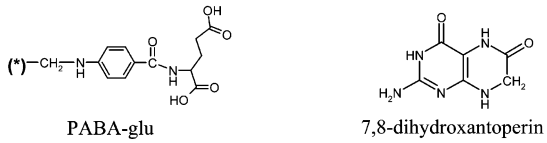


Figure 1. Molecular structures of the dihydropterins investigated at physiological pH (neutral forms); structures of corresponding oxidized pterins are also represented.

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tion of hydrogen peroxide (H_2O_2) by BPT and FPT[4,5] and of superoxide anion (O_2^-) by FPT[5] has recently been demonstrated or proposed.

Depending on their oxidation state, pterins have totally different reactivities toward oxidizing agents. Whereas oxidized pterins are quite stable in air, reduced pterins are able to react with molecular oxygen in its ground state. The stability of the reduced pterins in air-equilibrated solutions depends on the chemical structure of the pterin moiety as well as that of the substituent(s).[6,7] In a recent study we have shown that oxidized pterins, besides being good $^1O_2$ sensitizers,[3] are also $^1O_2$ quenchers and acceptors with varying reaction efficiencies depending on their structure.[8] Despite the biological and medical interest of the reactivity of dihydropterin derivatives with ROS, no systematic studies have been performed on reactions between these compounds and $^1O_2$. Photoinduced production of $^1O_2$ by reduced pterins has not been studied either.

Singlet oxygen ($O_2(^1\Delta_g)$), the lowest electronic excited state of molecular oxygen, is an important oxidizing intermediate in chemical processes and one of the main ROS responsible for the damaging effects of light on biological systems (photodynamic effects).[9] Photosensitization is primarily responsible for the production of $^1O_2$ in vivo.[10] In this process, $^1O_2$ is most often produced by energy transfer from the excited triplet state of a sensitizer (^{3}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 oxidize an acceptor molecule (reaction 5) and/or a physical quencher (reaction 6).

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

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

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

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

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

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

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 an important tool to analyze their antioxidant capability. Determination of the rate constants of $^1O_2$ physical quenching and chemical reaction with $^1O_2$ (k_q and k_r, reactions 6 and 5, respectively) allows evaluation of the efficiencies of these processes. Products identification provides information to elucidate oxidation mechanisms and discuss potential effects in vivo.

In the present article we describe the processes of deactivation of $^1O_2$ by a series of dihydropterins and analyze the effect of the 6 substituent on the quenching efficiency. We determined the k_t and k_r values and investigated the corresponding oxidation products. We also studied the capability of dihydropterins to generate $^1O_2$ upon excitation with UV-A radiation. Taking into account the biological importance of the results, the experiments were performed in aqueous solution at physiological pH conditions. Under these conditions, the studied compounds are in their neutral form ($pK_a \geq 9.5$).[6] We investigated the following dihydropterins: 7,8-dihydrobiopterin (DHBPT), 7,8-dihydroneopterin (DHNPT), 6-formyl-7,8-dihydropterin (FDHPT), sepiapterin (SPT), 7,8-dihydrofolic acid (DHFA), conjugated dihydropterin containing a *p*-aminobenzoylglutamic acid unit (PABA-Glu), and 7,8-dihydroxanthopterin (DHXPT). The corresponding molecular structures are shown in Figure 1. The results obtained are compared with those previously reported for oxidized pterins, and the biological implications are discussed.

Experimental Section

Chemicals and Preparation of Solutions. Pterins (Shircks Laboratories) and rose bengal (Aldrich) were of the highest purity available and 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 pH measurements were performed using a pH meter CG 843P (Schott, Mainz, Germany) with a pH-combination electrode BlueLine 14pH (Schott).

Solutions of dihydropterin derivatives were prepared in anaerobic conditions (argon atmosphere) using a glovebox. The O_2 concentration in the H_2O employed for preparing the pterin solutions within the air-free compartment was monitored using an O_2-selective electrode (Celiox 325) and kept below 0.02 ppm.

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.[11]

Determination of Quantum Yields of $^1O_2$ Production. Quantum yields of $^1O_2$ production (Φ_Δ) by the dihydropterins in aqueous solution were determined by direct analysis of the weak $^1O_2$ near-infrared (NIR) luminescence at 1270 nm.[12−14] Measurements were carried out under continuous irradiation of the dihydropterins. The main features of the method and equipment have already been described in detail.[15,16] Briefly, the sample solution in a quartz cell 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 cutoff filter (1000 nm), and an interference filter (1271 nm) detected at 90° with respect to the incident beam using a cooled NIR photomultiplier (Hamamatsu R5509-42).

Determination of the Rate Constants of $^1O_2$ Total Quenching by Dihydropterins (k_t). The values of k_t were determined by Stern−Volmer analysis of the $^1O_2$ luminescence in the near-IR (NIR)[17] with the same equipment described above. $^1O_2$ was generated by photosensitization using RB as a sensitizer. Groups of experiments were carried out at room temperature irradiating solutions of the different dihydropterins and RB at 547 nm, where the investigated compounds do not absorb. The RB concentration was kept constant, whereas the concentration of the dihydropterin derivative was varied within a series of experiments (up to 3×10^{-4} M). In addition, the concentration of RB ($A_{547\ nm} = 1$) was such that $^1O_2$ total quenching by the sensitizer itself was negligible compared with $^1O_2$ deactivation by the solvent.[18]

Under conditions for which Φ_Δ is not affected by the presence of the quencher (Q, e.g., the dihydropterin derivative) and assuming a dynamic quenching of $^1O_2$, a linear relationship between the ratio of the signals observed in the absence (S_e^0) and presence (S_e) of quencher and the quencher concentration should be obtained (eq 7)

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

where 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 3) in most solvents).[19] Values of τ_Δ 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 Dihydropterins (k_r). The rate of disappearance of a compound Q reacting with $^1O_2$ to yield an oxidized product (reaction 5) 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 (einstein 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 to deactivation by the solvent ($k_d \gg k_t^S[S]$), integration of eq 10 leads to eq 11[21]

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

In the cases where $k_t^Q[Q] \ll k_d$, 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 at room temperature irradiating aqueous solutions (H_2O, 3 cm^3, pH = 7.0−7.2) containing a dihydropterin derivative and RB as a $^1O_2$ sensitizer. RB was excited at 547 nm. Within each series of experiments, solutions of the pterin derivative (5×10^{-5} to 2×10^{-4} M depending on the compound) and RB (absorbance at 547 nm approximately 1.5) were irradiated during different periods of time in a 1 cm × 1 cm spectroscopic cell on the same optical bench as used for the determination of k_t and Φ_Δ (vide supra). The spectral changes were registered on a Cary 5 (Varian) spectrophotometer. The incident photon flux (P_o) at the wavelength of excitation of the sensitizer (547 nm) was determined by actinometry using Aberchrome 540 as an actinometer[22] ($P_o = 2.3 \times 10^{-6}$−4.5×10^{-6} Einstein L^{-1} s^{-1} at 547 nm). Aberchrome 540 is the anhydride form of the (*E*)-α-(2,5-dimethyl-3-furylethylidene)(isopropylidene)succinic acid which under irradiation in the spectral range 316−366 nm leads to a cyclized form. The reverse reaction to ring opening is induced by visible light. 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 (RB) at the excitation wavelength.

The experiments in D_2O were performed on an optical bench equipped with a halogen lamp. A cutoff filter at 515 nm was used to prevent irradiation of the dihydropterin derivatives. An experiment performed in H_2O for FDHPT under otherwise the same experimental conditions was used as an actinometer for calculating the photon flux absorbed by the sensitizer (P_a).

Consumption of the dihydropterin as a function of RB irradiation time and formation of photoproducts was analyzed by HPLC (vide infra). Absorption spectra were also registered at regular time intervals.

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

It should be noted that in the case of DHNPT, DHBPT, and DHFA the peak of the oxidized pterin could not be well separated from that of the corresponding dihydro compound. Therefore, calibration curves were established and integrations of the peaks at different wavelengths were performed. Assuming that the peak considered is only due to the reactant and its oxidized analogue, the concentration of both compounds can be calculated by resolving sets of equations as follows

$$\text{Area}_{\lambda 1} = f_{\lambda 1}^{\text{DHPT}}C^{\text{DHPT}} + f_{\lambda 2}^{\text{PT}}C^{\text{PT}} \quad (13)$$

$$\text{Area}_{\lambda 2} = f_{\lambda 2}^{\text{DHPT}}C^{\text{DHPT}} + f_{\lambda 2}^{\text{PT}}C^{\text{PT}} \quad (14)$$

where $\text{Area}_{\lambda 1}$ and $\text{Area}_{\lambda 2}$ are the values resulting from integration of the chromatograms at analysis wavelengths $\lambda 1$ and $\lambda 2$, C^{DHPT} and C^{PT} are the concentrations of the reactant (DHPT) and the corresponding oxidized derivative (PT), $f_{\lambda 1}^{\text{DHPT}}$, $f_{\lambda 1}^{\text{PT}}$, $f_{\lambda 2}^{\text{DHPT}}$, and $f_{\lambda 2}^{\text{PT}}$ are the factors obtained from the calibration curves for the reduced and oxidized pterins at $\lambda 1$ and $\lambda 2$. Although only two equations are required for calculating C^{DHPT} and C^{PT}, more equations were used in order to check the results obtained.

H_2O_2 Determination. For determination of H_2O_2, the cholesterol CHOD-PAP kit from Roche was used. H_2O_2 was quantified by its color reaction with 4-aminophenazone and phenol.[23,24] Irradiated solutions (0.5 mL) containing a given dihydropterin and RB as a sensitizer were added to 1.0 mL of reagent. The absorbance at 505 nm of the solution containing

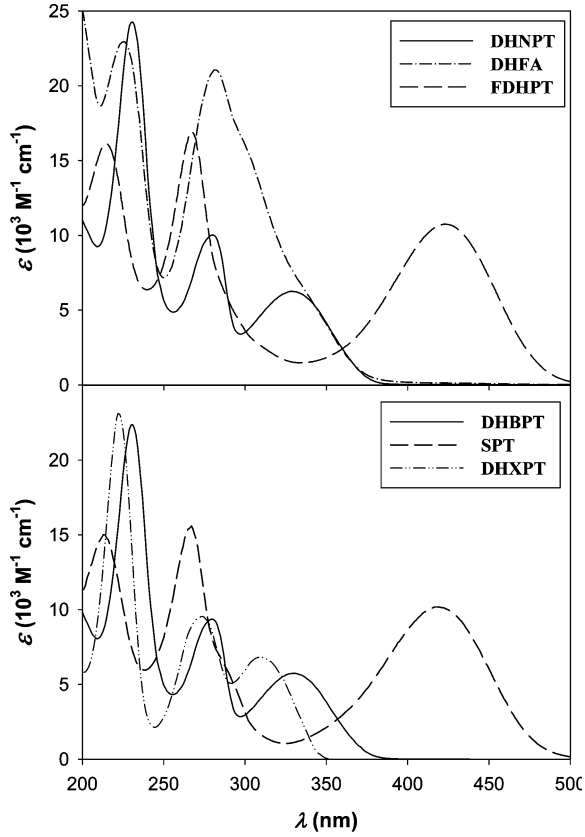


Figure 2. Absorption spectra of DHNPT, DHFA, FDHPT, DHBPT, DHXPT, and SPT in H_2O at pH 7.0.

the mixture of irradiated sample and reagent was measured after 30 min of incubation at room temperature using the reagent as a blank. Aqueous solutions of H_2O_2, prepared from commercial standards (Sigma), were employed for obtaining the corresponding calibration curves.

Results and Discussion

Absorption Spectra. The absorption spectra of the dihydropterins investigated are considerably affected by the chemical nature of the substituent (Figure 2). FDHPT and SPT, two derivatives bearing a carbonyl group in the α position of the 6 substituent (Figure 1), absorb at longer wavelength (maximum of absorption, λ_{max}, in the visible at ca. 420 nm) than the other dihydropterins investigated (λ_{max} lower than 350 nm). This bathochromic shift of the absorption band may be explained by a charge transfer, across the conjugated system of the pterin moiety, between the amino group at position 2 and the carbonyl group of the substituent at position 6 (Figures 1 and 2).[6]

The molar absorption coefficient ($\epsilon_{\lambda max}$) at λ_{max} of the low-energy band of each compound was determined (Table 1) by means of spectrophotometric analysis of standard solutions. The values obtained for BPT and NPT are in good agreement with previous results reported by Pfleiderer.[6] For DHFA the low-energy band is superimposed with a band of higher energy. As a result, the corresponding λ_{max} and ϵ cannot be determined independently.

Quantum Yields of $^1O_2$ Production (Φ_Δ) by Dihydropterin Derivatives. The compounds were excited into their corresponding low-energy bands and according to the emission maxima of the Xe−Hg lamp employed for irradiation. Thus, the excitation wavelength was 310 nm for DHBPT, DHNPT, DHXPT, and DHFA, whereas FDHPT and SPT were excited at 436 nm (see spectra in Figure 2). Although these dihydropterin derivatives are poorly fluorescent compounds (fluorescence quantum yields lower than 0.02),[25] control experiments in argon-saturated solutions were carried out in order to check possible tailing of the fluorescence emission of the dihydropterins in the NIR spectral region. No luminescence at 1270 nm could be detected under those conditions. Within experimental error, no $^1O_2$ emission was detected in air-equilibrated solutions, indicating that dihydropterins are not $^1O_2$ sensitizers. The values of the quantum yields of $^1O_2$ production (Φ_Δ) were lower than 10^{-3}.

In contrast, oxidized unconjugated pterins are good $^1O_2$ sensitizers with Φ_Δ values ranging from 0.2 to 0.5.[3] Oxidized pterins contain an aromatic pyrazine ring, whereas the aromaticity is lost in the reduced derivatives (Figure 1). Apparently this structural change is responsible for a considerable decrease of the quantum yield of formation of the triplet excited state (eq 1). This result is consistent from a biological point of view. Dihydropterins are biologically active, present in the skin of human beings, and cofactors of many enzymes. Therefore, if these compounds would generate $^1O_2$ upon irradiation, very harmful effects would result for skin cells.

Folic acid (FA), a conjugated oxidized pterin and the most important pterin derivative in mammalians, is a very poor $^1O_2$ sensitizer.[3] In this case the substituent at position 6 (PABA-Glu) enhances the deactivation of the singlet excited state, and as a result, intersystem crossing becomes very inefficient. From this point of view, the negligible Φ_Δ found for DHFA was not surprising.

Rate Constants of $^1O_2$ Total Quenching by Dihydropterin Derivatives (k_t). The Stern−Volmer plots of the quenching of the near-infrared $^1O_2$ luminescence (eq 7) were linear within the range of concentrations used (Figure 3). The values of k_t obtained from the slopes of these plots are in the range from 1.9 to 6.8×10^8 M^{-1} s^{-1} (Table 1), showing that dihydropterins are among the most efficient $^1O_2$ quenchers.[26] These k_t values are 1−2 orders of magnitude higher than those obtained for oxidized pterin derivatives.[3,8] The k_t values reported for a series of 11 of the latter compounds were strongly dependent on the substituent at position 6 and in the range between 1.4×10^6 and 6.7×10^7 M^{-1} s^{-1}. Moreover, it was shown that 6-substituted conjugated pterins bearing a PABA-Glu or PABA group (FA, 10-methylfolic acid, pteroic acid) were the most efficient $^1O_2$ quenchers in this series with k_t values (2.9×10^7 to 6.7×10^7 M^{-1} s^{-1}) an order of magnitude larger than those of 6-substituted unconjugated pterins (1.4×10^6 to 8.0×10^6 M^{-1} s^{-1}). Since amines are known to deactivate efficiently $^1O_2$ by charge-transfer-induced physical quenching,[27,28] the larger k_t values for conjugated pterins could be attributed to the quenching of $^1O_2$ by the aromatic amino group contained in the PABA substituent of these compounds.[8] However, the contribution of the PABA moiety to $^1O_2$ quenching remains an order of magnitude lower than the total quenching by dihydropterins. For these compounds (Table 1) the PABA-Glu substituent in DHFA does not play a particular role and the variation of k_t does not exceed a factor of 3. It should be noted that the two dihydropterins bearing a carbonyl group in the α position of the 6 substituent (FDHPT and SPT) are the least efficient $^1O_2$ quenchers in the series ($k_t = 2.0 \times 10^8$ M^{-1} s^{-1}, Table 1). Focusing on the comparison between pterins and corresponding dihydropterins bearing identical substituents at position 6 (Table 1), it is clear that the dihydropyrazine ring is considerably more efficient in quenching $^1O_2$ than the pyrazine one. Therefore, the rate constants of the chemical reaction between $^1O_2$ and dihydropterin derivatives (k_r) had to be

TABLE 1: Absorption Maxima of the Low-Energy Band (λ_{max}), Molar Absorption Coefficient at λ_{max} ($\epsilon_{\lambda max}$), Rate Constant of $^1O_2$ Total Quenching (k_t) by Dihydropterin Derivatives, and Rate Constant of their Chemical Reaction with $^1O_2$ (k_r)

compound	λ_{max} (nm)	$\epsilon_{\lambda max}$ (10^3 M^{-1} cm^{-1})	k_t (10^8 M^{-1} s^{-1}) (D_2O)	k_r (10^8 M^{-1} s^{-1}) (D_2O)	k_r (10^8 M^{-1} s^{-1}) (H_2O)	oxidized derivative s	k_t[a] (10^8 M^{-1} s^{-1}) (D_2O)
DHFA			5.5 ± 0.9		5.3 ± 0.6	FA	0.30
DHBPT	330	6.2	3.7 ± 0.3		3.1 ± 0.4	BPT	0.024
DHNPT	330	6.3	4.6 ± 0.4		4.2 ± 0.5	NPT	0.023
FDHPT	423	11.3	2.1 ± 0.2	1.4 ± 0.3	3.2 ± 0.4	FPT	0.014
SPT	418	10.3	1.9 ± 0.2	1.6 ± 0.3	3.5 ± 0.4		
DHXPT	309	6.8	6.8 ± 0.4		7.6 ± 0.8		

[a] Given for comparison (data from ref 3).

determined for evaluating the contribution of this reaction to the total $^1O_2$ quenching by these compounds.

Rate Constants of the Chemical Reaction between $^1O_2$ and Dihydropterin Derivatives. The rate constants of the chemical reaction between $^1O_2$ and several dihydropterin derivatives (k_r) in aqueous solution were determined from HPLC analyses of the disappearance of the dihydropterin (Q) during the photosensitized oxidation using RB as a $^1O_2$ sensitizer. In the case of FDHPT and SPT, the disappearance of the dihydropterin could also be followed directly by UV−vis spectrophotometry as the products formed did not absorb significantly at the λ_{max} of the dihydropterin. Comparing the two methods, the same results were obtained within experimental error. In a first series of experiments the values of k_r were evaluated in H_2O at pH $\approx$ 7 (Table 1). Under the experimental conditions used, the disappearance of the dihydropterin followed in all cases first-order kinetics as $^1O_2$ deactivation by the dihydropterin itself was negligible compared to $^1O_2$ quenching by the solvent (k_t^Q[Q] $\ll$ $k_d(H_2O)$ (approximately 2.5×10^5 s^{-1}), eq 11′, Experimental Section). The corresponding first-order plots are shown in Figure 4. As observed for k_t, the values of k_r determined in these experiments vary in a narrow range ($3.0-7.6 \times 10^8$ M^{-1} s^{-1}) (Table 1) and are particularly high, showing that dihydropterins are very efficient $^1O_2$ acceptors. The k_r values determined in H_2O are very similar to the corresponding k_t values obtained in D_2O (Table 1). Moreover, for most of the compounds k_r and k_t are equal within experimental error, indicating that deactivation of $^1O_2$ by dihydropterins is mainly a chemical process (reaction 5). However, for FDHPT and SPT, k_r values in H_2O appear to be higher than the corresponding k_t values in D_2O, the difference exceeding experimental error (Table 1). Therefore, a deuterium isotope effect on these reactions had to be taken into account, and the values of k_r for FDHPT and SPT were also evaluated in D_2O. When using D_2O as solvent, $^1O_2$ deactivation by the dihydropterin itself was not negligible anymore compared to $^1O_2$ quenching by the solvent ($k_d(D_2O)$ approximately 1.6×10^4 s^{-1}), and the values of k_r were obtained from the linear plots of f([Q]) as a function of the irradiation time (eq 11, Experimental Section). These plots are shown in Figure 5. Values of k_r in D_2O for FDHPT and SPT (1.4 and 1.6×10^8 M^{-1} s^{-1}, respectively) are about one-half of the values of k_r in H_2O, showing an isotopic effect of about 2 (Table 1). They are only slightly lower than the corresponding k_t values in D_2O ($1.9-2.1 \times 10^8$ M^{-1} s^{-1}), confirming that deactivation of $^1O_2$ by dihydropterins is mainly a chemical process (reaction 5). Physical quenching (reaction 6) appears to be a minor $^1O_2$ deactivation pathway by dihydropterins; nevertheless, the rate constants of physical quenching

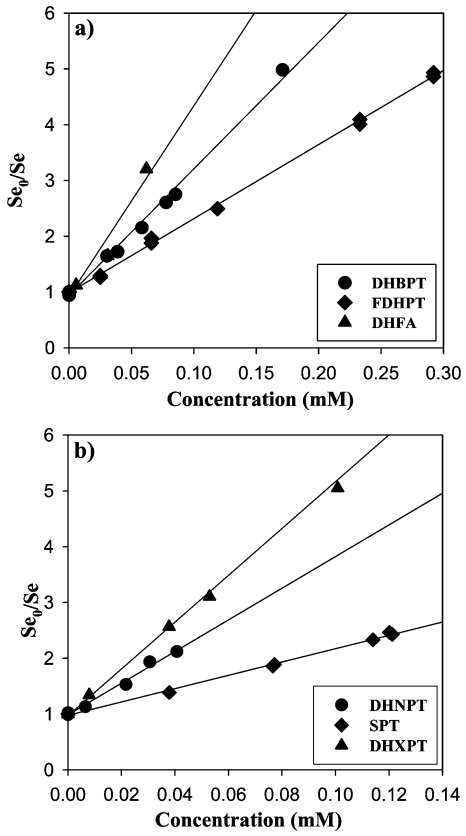


Figure 3. Stern−Volmer plots for quenching of the $^1O_2$ near-infrared luminescence by (a) DHBPT, DHFA, and FDHPT and (b) DHNPT, SPT, and DHXPT in D_2O (pD = 7.0−7.2, RB was used as a sensitizer, λ_{ex} = 547 nm).

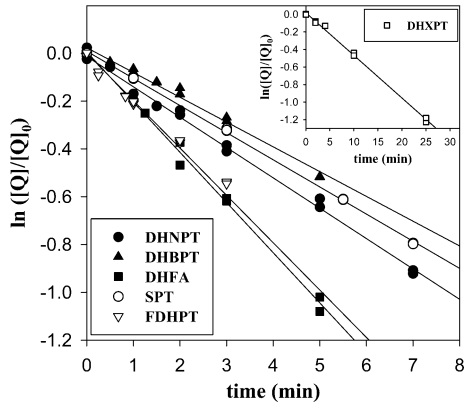


Figure 4. First-order plots of the photosensitized oxidation of the dihydropterin derivatives in H_2O solutions (eq 11′, Experimental Section). Sensitizer: rose bengal (λ_{ex} = 547 nm), pH = 7.0−7.2, concentrations of dihydropterins determined by HPLC analysis and UV−vis spectrophotometry (FDHPT and SPT). The ranking of the plots does not correspond to that of k_r values as P_a varied between 2.3 and 4.5×10^{-6} einstein L^{-1} s^{-1} depending on the compound. The DHXPT experiment is shown in a separate plot (inset) as the incident photon flux applied was approximately an order of magnitude lower than that used for the other compounds ($P_a = 4 \times 10^{-7}$ einstein L^{-1} s^{-1}).

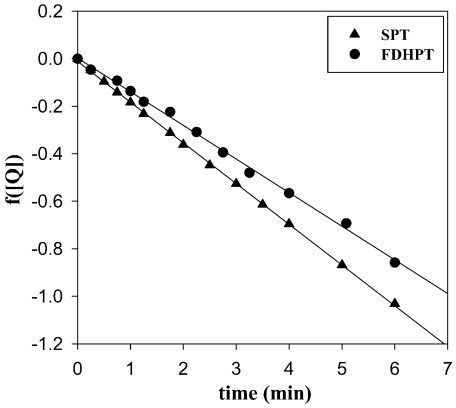
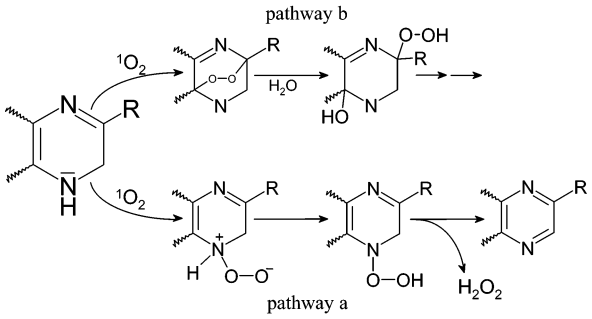


Figure 5. Photosensitized oxidation of FDHPT and SPT in air-equilibrated D_2O solutions: plot of $f([Q]) = \ln([Q]/[Q]_o) - [(k_t^Q/k_d)-([Q]_0 - [Q])]$ as a function of the irradiation time (eq 11, Experimental Section). Sensitizer: rose bengal (irradiation wavelength: 547 nm), pD = 7.0−7.2, concentrations of FDHPT and SPT determined by UV−vis spectrophotometric analysis.

SCHEME 1: Mechanisms Proposed for the Reaction of $^1O_2$ with the Dihydropterin Moiety of Dihydropterins[a]



[a] Pathway a: aromatization via charge-transfer reaction between the N(8) atom and $^1O_2$ and subsequent H_2O_2 release. Pathway b: reaction of $^1O_2$ with the aza-dienic system (−C(9)═C(10)−N(5)═C(6)−).

($k_q = k_t - k_r$) may be on the order of 10^7 M^{-1} s^{-1}. In contrast, oxidized pterins are primarily $^1O_2$ physical quenchers, although k_q values do not exceed 3×10^6 M^{-1} s^{-1} for unconjugated pterins (dominant quenching by the pterin moiety) and 5.5×10^7 M^{-1} s^{-1} for conjugated pterin (dominant quenching by the PABA or PABA-Glu substituents). Values of k_r for oxidized pterins are comparatively very low, being only 2.5×10^5 M^{-1} s^{-1} for pterin itself and reaching 10^7 M^{-1} s^{-1} only for pterins with a sufficiently activated C(6)−C(7) double bond (6,7-dimethylpterin) or with an unsubstituted PABA unit (pteroic acid).[8] When comparing pterins and the corresponding dihydropterins bearing identical substituents at position 6 (Table 1, Figure 1), it is evident that the dihydropyrazine ring is much more reactive toward $^1O_2$ than the pyrazine one. For conjugated oxidized pterins the attack of $^1O_2$ on the PABA substituent, in addition to the attack on the pyrazine ring, has been demonstrated.[8] This pathway, resulting in formation of 6-formylpterin (FPT) due to oxidation and cleavage of the PABA moiety between the methylene group and the amino group, should also be operating for DHFA. However, the k_r value reported for this process with folic acid (FA) is negligible in comparison to k_r determined for DHFA (Table 1).

Two sites in the dihydropyrazine ring may contribute to $^1O_2$ deactivation: the −N(8)H−C(7)H_2− group and the aza-dienic system (−C(9)═C(10)−N(5)═C(6)−) (Scheme 1). Although $^1O_2$ deactivation by the former site is probably dominated by physical quenching as observed for other amines,[27,28] the [4 + 2] cycloaddition of $^1O_2$ to the 1,3-aza-diene moiety might occur

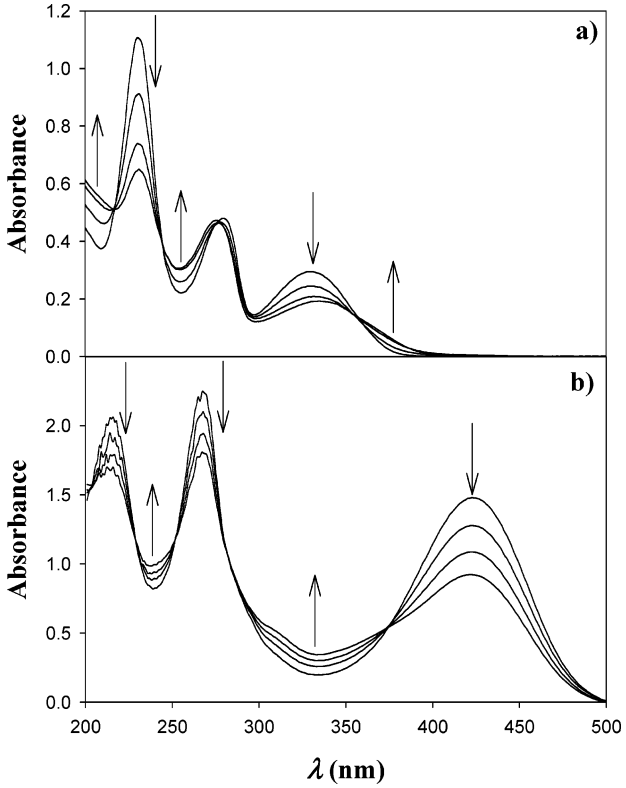


Figure 6. Photosensitized oxidation of dihydropterins in air-equilibrated aqueous solution: Evolution of the absorption spectra as a function of irradiation time (the spectra of the sensitizer were subtracted at each time). Arrows indicate the changes observed at different wavelengths. Sensitizer: rose bengal (irradiation wavelength: 547 nm), pH ≈ 7.0. (a) DHBPT, spectra were recorded every 5 min, optical path length = 1 mm. (b) FDHPT, spectra were recorded every 1 min, optical path length = 1 cm.

with high efficiency, leading to endoperoxide formation as in the case of 1,3-dienes.[29,30] The corresponding rate constants in aqueous solutions may exceed 10^8 M^{-1} s^{-1} for some heterocyclic 1,3-dienes, such as furfuryl alcohol.[31,32] In order to gain more insight into the mechanistic pathways involved, the products formed during the sensitized photooxidation of dihydropterins were investigated.

Products of the Reactions between $^1O_2$ and Dihydropterin Derivatives. Products of the chemical reactions between $^1O_2$ and dihydropterins in aqueous solution were investigated by UV−vis spectrophotometry and HPLC analysis. Considerable spectral changes were registered as a function of irradiation time (Figure 6), concerning primarily the absorption range of the dihydropterins. In contrast, no modification of the sensitizer (RB) absorbance was detected, indicating that RB was stable during irradiation under the experimental conditions applied.

In most cases, reaction of $^1O_2$ with dihydropterins yielded the analogous oxidized pterins. Indeed, biopterin (BPT), neopterin (NPT), FPT, and FA were detected in the experiments performed with DHBPT, DHNPT, FDHPT, and DHFA, respectively. Identification of the oxidized pterins was carried out comparing retention times of samples and references using different elution conditions. Spectra of irradiated and standard solutions were also compared using the DAD detector of the HPLC equipment. Such analyses were carried out especially for long irradiation times, where conversion of the reactants could be expected to be relatively high. In the case of DHXPT, the corresponding oxidized derivative, xanthopterin (XPT), was not detected. Finally, for SPT, the corresponding oxidized pterin

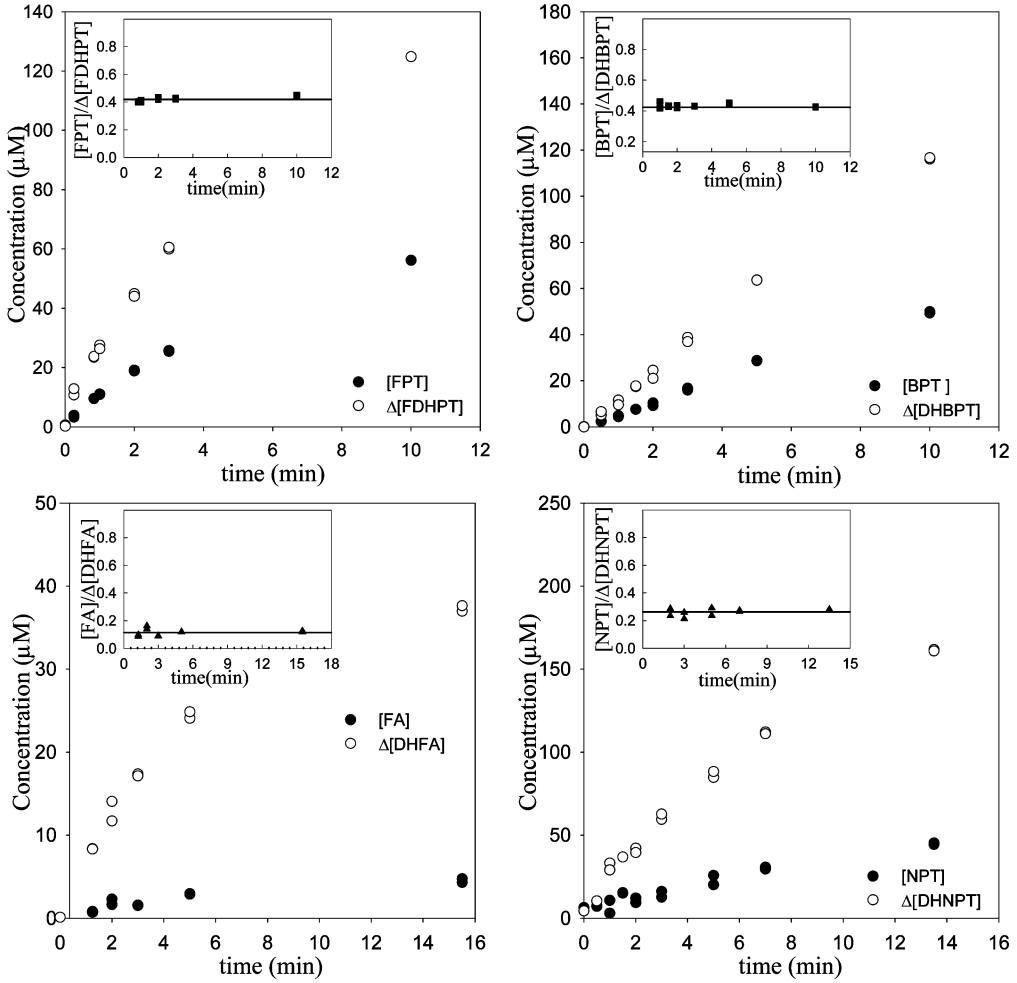


Figure 7. Photosensitized oxidation of dihydropterins (DHPT) in air-equilibrated aqueous solution. Evolution of the consumption of DHPT (Δ-[DHPT] = $[DHPT]_o - [DHPT]_t$) and production of the corresponding oxidized pterin ([PT]) as a function of irradiation time. Sensitizer: rose bengal (irradiation wavelength: 547 nm), pH = 7.0−7.2, concentrations determined by HPLC analysis. (Insets) Plot of the fraction of DHPT converted into PT ([PT]/Δ[DHPT]) as a function of irradiation time.

derivative could not be investigated because the standard is not commercially available.

In order to check if the reaction between $^1O_2$ and the 6 substituent on DHFA (PABA-Glu) takes place to a significant extent, FDHPT and FPT, two possible products of such a reaction, were searched. As expected, i.e., in accord with the evaluation of k_r (vide supra), neither product could be detected. Therefore, the investigated pathway did not contribute significantly to the oxidation of DHFA.

Oxidized pterins were quantified as a function of irradiation time. In all cases investigated, the rate of oxidized pterin production was lower than the corresponding rate of dihydropterin consumption (Figure 7). The extent of the transformation of the dihydropterin into the corresponding oxidized pterin was evaluated by calculating the fraction of dihydropterin converted to oxidized pterin (Δ[PT]/Δ[DHPT]) during irradiation. Data at very short times (<2 min) were discarded due to the high relative error. Within experimental error, values of Δ[PT]/Δ-[DHPT] remained constant (Figure 7, insets) and depending on the dihydropterin varied between 0 and 0.42 (Table 2). Consequently, although the percentage of substrate transformed into oxidized pterin was significant in most cases, a large fraction of dihydropterin was converted into other products.

Production of oxidized pterins by these processes has important biomedical implications. Indeed, unconjugated oxidized pterins are $^1O_2$ sensitizers with quantum yields ranging from 0.1 to 0.45 depending on their structure.[2] If in a pathological situation oxidative stress is induced by $^1O_2$, generated by photosensitization, reaction of $^1O_2$ with dihydropterin derivatives will produce more sensitizer (oxidized pterin), thus enhancing the oxidative stress.

TABLE 2: Generation of Oxidized Pterins and H_2O_2 in the Reaction of $^1O_2$ with Dihydropterins in H_2O[a]

compound	Δ[PT]/Δ[DHPT]	$\Delta[H_2O_2]/\Delta$[DHPT]
DHFA	0.12 ± 0.02	
DHBPT	0.42 ± 0.03	0.17 ± 0.03
DHNPT	0.26 ± 0.03	0.07 ± 0.01
FDHPT	0.40 ± 0.05	0.14 ± 0.02
DHXPT	~0	

[a] Sensitizer: rose bengal (irradiation wavelength: 547 nm), pH = 7.0−7.2. Δ[PT]/Δ[DHPT]: average fraction of dihydropterin converted to oxidized pterin. $\Delta[H_2O_2]/\Delta$[DHPT]: ratio calculated from experimentally determined initial rate of H_2O_2 production $(d[H_2O_2]/dt)_o$ (Figure 8) and calculated initial rate of each dihydropterin consumption $(d[DHPT]/dt)_o$.

Besides oxidized pterins, several additional products were detected by HPLC analysis, all of them having retention times lower than those corresponding to both reduced and oxidized pterins. They must therefore be very polar substances, most probably because of incorporation of oxygen into their structures. Moreover, analysis of the absorption spectra suggests that the pterin moiety was oxidized and cleaved, yielding a group of non-pterinic products, i.e., the characteristic absorption bands

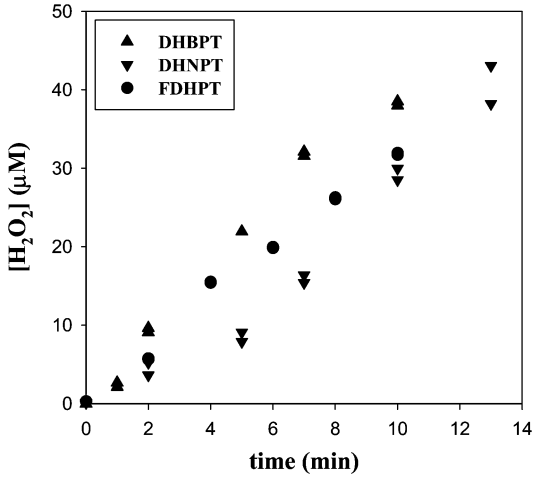


Figure 8. Photosensitized oxidation of dihydropterins in air-equilibrated aqueous solution. Evolution of the H_2O_2 concentration as a function of irradiation time. Sensitizer: rose bengal (irradiation wavelength: 547 nm), pH = 7.0−7.2. Initial concentrations: $[DHBPT]_o$ = 358 μM, $[DHNPT]_o$ = 281 μM, $[FDHPT]_o$ = 227 μM.

of dihydropterins in the UV-A and visible regions were lost. These non-pterinic substances cannot originate from the further oxidation of oxidized pterins because of their relatively low reactivity. Simple calculations, taking the k_r values of oxidized pterins[8] and the steady-state concentration of $^1O_2$, allow discarding a significant oxidation of oxidized pterins due to their reaction with $^1O_2$. Therefore, at least two reaction pathways have to be considered for the reaction of $^1O_2$ with dihydropterins: oxidation of the dihydropyrazine ring yielding the aromatic pyrazine moiety and oxidation and cleavage of the dihydropterin to yield non-pterinic substances.

The former reaction may be explained by 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:[33] the charge-transfer reaction between the N(8) atom of the −HN(8)−C(7)H_2− group and $^1O_2$ leads to oxidation of the amine and subsequent aromatization with concomitant production of H_2O_2 (Scheme 1, pathway a). The oxidation and cleavage of the dihydropterin to yield non-pterinic polar products may result from reaction of $^1O_2$ with the aza-dienic system (−C(9)═C(10)−N(5)═C(6)−), giving the endoperoxide as an initial intermediate (Scheme 1, pathway b). Hydrolysis and further reactions would lead to fragmentation of the dihydropterin.[34] Depending on the relative efficiencies of pathways a and b (Scheme 1), a higher or lower proportion of oxidized pterin should be observed. In the case of DHXPT, there is no detectable amount of XPT in the system, indicating that the reaction on the 1,3-aza-diene could be so fast that the reaction with the amino group of the dihydropyrazine ring cannot compete and no detectable amount of XPT could be found.

With DHBPT, DHNPT, and DHFPT the H_2O_2 concentration increased with irradiation time. Although the photoinduced production of $^1O_2$ by pterins in vivo has not been proven so far, it should occur in tissues where these compounds accumulate and are exposed to UV-A radiation. Therefore, the reaction between $^1O_2$ and dihydropterin derivatives might be an additional source of H_2O_2 in vitiligo.

The concentration profiles of H_2O_2 formation (Figure 8) cannot be directly compared with consumption of the substrate or formation of other products in experiments performed for k_r determination because some experimental conditions, such as the RB concentration or the initial concentration of the dihydropterin derivatives, were different. However, in the present set of experiments the initial rates of reactant consumption $(d[DHPT]/dt)_o$ can be calculated from the corresponding k_r values (Table 1) and the steady-state $^1O_2$ concentration estimated with eq 9 for each experiment. These rates were compared with the initial rates of H_2O_2 production, $(d[H_2O_2]/dt)_o$ (Figure 8). The ratios obtained $((d[H_2O_2]/dt)_o/(d[DHPT]/dt)_o)$ are listed in Table 2 and show that the apparent rate of H_2O_2 production is much smaller than the corresponding rate of substrate consumption. Moreover, for the three cases analyzed, the amount of H_2O_2 released is lower than the amount of oxidized pterins produced. However, there seems to be a relation between the rates of oxidized pterin production and H_2O_2 release. H_2O_2 might therefore be produced by the same sequence of reactions that yield oxidized pterins. This hypothesis is in good agreement with the mechanism proposed for aromatization of the dihydropterin moiety upon attack of $^1O_2$ (Scheme 1, pathway a).

The lower proportion of H_2O_2 released in comparison to the proportion of oxidized pterin produced ($\Delta[H_2O_2]/\Delta[DHPT] < \Delta[PT]/\Delta[DHPT]$, Table 2) might be due to the reaction of H_2O_2 with some compounds present in the irradiated solutions. In order to check a possible reaction of H_2O_2 with dihydropterins, experiments were performed in aqueous solutions (pH 7.0) containing these compounds and H_2O_2, the latter at a concentration at least 1 order of magnitude higher than the substrate concentration. In all cases, the rates of the reaction were not significant and, as expected, the same results were obtained in control experiments using oxidized pterins. However, reactions with intermediates of proposed pathway b may be responsible for the relatively low proportion of H_2O_2 determined.

Conclusions

The photoinduced production and quenching of $^1O_2$ by a series of 7,8-dihydropterins, one of the biologically active forms of pterins, have been investigated in aqueous solution at physiological pH (∼7). The series includes 7,8-dihydrobiopterin (DHBPT), 7,8-dihydroneopterin (DHNPT), 6-formyl-7,8-dihydropterin (FDHPT), sepiapterin (SPT), 7,8-dihydrofolic acid (DHFA), and 7,8-dihydroxanthopterin (DHXPT) (Figure 1). Evidence is given that all studied compounds, in contrast to oxidized unconjugated pterins[3] (Figure 1), do not produce $^1O_2$ under UV-A irradiation ($\Phi_\Delta < 10^{-3}$). The values of the rate constants of total quenching of $^1O_2$ (k_t) by the dihydropterin derivatives investigated are in the range from 1.9 to 6.8 × 10^8 M^{-1} s^{-1} (Table 1). These k_t values are strikingly larger than those reported for oxidized pterin derivatives,[3,8] indicating that the dihydropyrazine ring is much more efficient in quenching $^1O_2$ than the pyrazine one.

The rate constants of the chemical reaction between $^1O_2$ and dihydropterin derivatives (k_r) investigated in aqueous solution are also in a narrow range and particularly high (3.0−7.6 × 10^8 M^{-1} s^{-1}) (Table 1), showing that dihydropterins are very efficient $^1O_2$ acceptors. These k_r values determined in H_2O are very similar to the corresponding k_t values obtained in D_2O, indicating that deactivation of $^1O_2$ by dihydropterins is mainly a chemical process. In contrast, oxidized pterins are predominantly $^1O_2$ physical quenchers.

Biopterin (BPT), neopterin (NPT), 6-formylpterin (FPT), and folic acid (FA) were detected during the reaction of $^1O_2$ with DHBPT, DHNPT, FDHPT, and DHFA, respectively. In the case of DHXPT, the corresponding oxidized derivative xanthopterin (XPT) was not detected. In all cases, a high percentage of substrate consumed was converted into other products. These additional products could be formed as a consequence of the

oxidation and cleavage of the pterin moiety. Therefore, at least two chemical pathways have to be considered: oxidation of the dihydropyrazine ring to yield the aromatic pyrazine moiety and oxidation and cleavage of the dihydropterin moiety to yield non-pterinic substances. The former reaction may be explained considering the charge-transfer reaction between N(8) atom and $^1O_2$ that leads to oxidation of the amine and subsequent aromatization with concomitant H_2O_2 elimination (Scheme 1, pathway a). In agreement with the proposed pathway, H_2O_2 was detected and its concentration increased with irradiation time. The latter reaction may result from the attack of $^1O_2$ to the aza-dienic system, yielding the endoperoxide as initial intermediate (Scheme 1, pathway b). Hydrolysis and further reactions would lead to fragmentation of the dihydropterin.

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