Photochemistry of dihydrobiopterin in aqueous solution†

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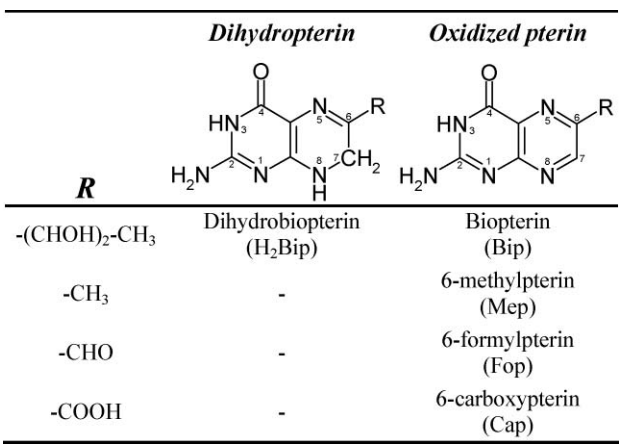
Dihydrobiopterin (H_2Bip) and its oxidized analogue, biopterin (Bip), accumulate in the skin of patients suffering from vitiligo, a chronic depigmentation disorder in which the protection against UV radiation fails. The photochemistry of H_2Bip was studied in neutral aqueous solutions upon UV-A irradiation (320–400 nm) at room temperature. The photochemical reactions were followed by UV/vis spectrophotometry, HPLC and enzymatic methods for hydrogen peroxide (H_2O_2) determination. Photoproducts were analyzed by means of electrospray ionization mass spectrometry. Under anaerobic conditions, excitation of H_2Bip leads to the formation of at least two isomeric dimers with molecular masses equal to exactly twice the molecular mass of the reactant. This reaction takes place from the singlet excited state of the reactant. To the best of our knowledge, this is the first time that the photodimerization of a dihydropterin is reported. In the presence of air, the dimers are again the main photoproducts at the beginning of the reaction, but a small proportion of the reactant is converted into Bip. As the reaction proceeds and enough Bip accumulates in the solution, a photosensitized process starts, where Bip photoinduces the oxidation of H_2Bip to Bip, and H_2O_2 is formed. As a consequence, the rates of H_2Bip consumption and Bip formation increase as a function of irradiation time, resulting in an autocatalytic photochemical process. In this process, Bip in its triplet excited state reacts with the ground state of H_2Bip. The mechanisms involved are analyzed and the biological implications of the results are discussed.

Introduction

Pterins, heterocyclic compounds derived from 2-aminopteridin-4-(3*H*)-one or pterin,[1] are present in biological systems in multiple forms, and play different roles ranging from pigments to enzymatic cofactors for numerous redox and one-carbon transfer reactions.[2,3] Pterins can exist in different oxidation states and can be divided into two classes: (a) oxidized or aromatic pterins and (b) reduced pterins. Within the latter group, 7,8-dihydropterins and 5,6,7,8-tetrahydropterins (denoted throughout as dihydropterins and tetrahydropterins, respectively) are the most important derivatives due to their biological activity. In particular, tetrahydrobiopterin (H_4Bip) is involved in the metabolism of amino acids.[4] This compound is present in the skin of human beings acting as a cofactor of phenylalanine hydroxylase, an enzyme that catalyzes the oxidation of phenylalanine to tyrosine. This latter amino acid is the precursor of melanin, the pigment of human skin and the main natural protection against the harmful effects of UV radiation.

Dihydrobiopterin (H_2Bip), biopterin (Bip) (Scheme 1) and other pterin derivatives accumulate in the skin of patients suffering from vitiligo,[5] a chronic depigmentation disorder. These patients express a characteristic fluorescence in their white skin patches upon Wood's light examination. This fluorescence is due to the accumulation of oxidized unconjugated pterins,[6] compounds with high fluorescence quantum yields.[7,8] In the tissues affected by this disease, hydrogen peroxide (H_2O_2) is present in high concentrations and the cells undergo oxidative stress, deactivation of enzymes of the melanin biosynthesis takes place and the protection of the skin against UV radiation fails because of



Scheme 1 Chemical structures of some pterin derivatives mentioned in this work.

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the lack of melanin. Therefore, the photochemistry of pterins is of particular interest for the study of this disease. Moreover, 6-carboxypterin (Cap), a product of Bip photolysis (*vide infra*) that is not synthesized in the skin cells, has been isolated from the affected skin,[9] thus proving that photooxidation of pterins occurs *in vivo* under pathological conditions.

The photochemistry of Bip and other oxidized pterins has been previously studied in detail.[10–13] Briefly, UV-A irradiation (320–400 nm) of Bip in aqueous media leads to oxidation of the 6-substituent to yield 6-formylpterin (Fop), which in turn is photooxidized to Cap. The formation of reactive oxygen species has been recently demonstrated. In particular, H_2O_2 and a superoxide anion ($O_2^{\bullet-}$) are formed during the photooxidation of Bip[11] and Fop[14] in air-equilibrated aqueous solution. Moreover, unconjugated oxidized pterins, such as those mentioned above, are good singlet oxygen ($^1O_2$) photosensitizers.[15] In addition, oxidized pterins are able to photoinduce oxidation of DNA and its components.[16–18] Therefore, accumulation of oxidized pterins in the skin enhances the harmful effects of UV radiation and very likely contributes to the oxidative stress in vitiligo.

Bip is formed *in vivo* from H_2Bip by a non-enzymatic oxidation. However, the specific chemical process that leads to this conversion in human skin under pathological conditions is still unknown. The formation of Bip might result from the oxidation of H_2Bip by H_2O_2,[19] but it was recently demonstrated that this reaction yields dihydroxanthopterin (H_2Xap) as a product.[20] The same main product was obtained for the "autooxidation" of H_2Bip (reaction with O_2 in an air-equilibrated solution).[21] Moreover, H_2Xap is recognized as the typical oxidation product of 7,8-dihydropterin derivatives bearing a –CHOH group linked to C6[22] (Scheme 1). However, it has been reported that oxidation of H_2Bip by $^1O_2$ yields Bip.[23]

On the other hand, non-enzymatic oxidation of H_4Bip by O_2 or other oxidants leads to the formation of H_2Bip *in vivo*.[24] The absorption spectrum of this compound, as well as that of Bip, has intense bands in the UV-A and UV-B (280–320 nm) spectral regions (Fig. 1). Despite this fact and the presence of H_2Bip in human skin, especially in pathological situations of UV protection failure, to the best of our knowledge, the photochemical behavior of H_2Bip has not been studied in detail, even though photochemical oxidation of H_2Bip might be a source of Bip production.

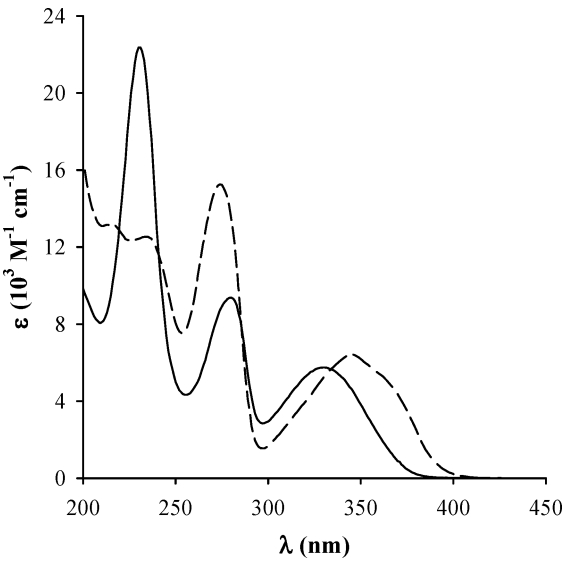


Fig. 1 Absorption spectra of H_2Bip and Bip in aqueous solutions (pH 6.8–7.0); solid line: H_2Bip; dashed line: Bip.

In the context of our investigations on the photochemistry of pterins, we herein report a systematic study of the photochemistry of H_2Bip under UV-A irradiation in neutral aqueous solutions, in the presence and in the absence of O_2. In particular, we have determined the quantum yields, identified the photoproducts, and analyzed the kinetics under various conditions. The results are evaluated in connection with its biological implications and compared with those reported for the photochemistry of related pterins.

Experimental

General

H_2Bip, Bip and other pterins were purchased from Schircks Laboratories (Switzerland) and used without further purification. Other chemicals were from Sigma Chemical Co. The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH solutions 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.

Steady-state irradiation

Irradiation set-up. The continuous photolysis of H_2Bip aqueous solutions was carried out in quartz cells (1 cm optical path length) at room temperature. Two radiation sources were employed: (I) Rayonet RPR lamps emitting at 350 nm (Southern N.E. Ultraviolet Co., Branford, CT) and (II) Xe/Hg Osram 1000 W with a monochromator ISA Jobin-Yvon B204. Irradiation experiments were performed in the presence and in the absence of air. Deaerated or O_2-saturated solutions were obtained by bubbling with Ar gas and O_2 for 20 min, respectively.

Actinometry. Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. The method for the determination of P_0 has been described in detail elsewhere.[25,26] Values of the photon flux absorbed (P_a) were calculated from P_0 according to the Lambert–Beer law:

$$P_a = P_0 (1 - 10^{-A}) \quad (1)$$

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

Analysis of irradiated solutions

UV/vis analysis. Electronic absorption spectra were recorded on diode array spectrophotometers 8452A and 8453 (Hewlett Packard). Measurements were made using quartz cells of 1 cm optical path length. The absorption spectra of the solutions were recorded at regular time intervals during irradiation.

High-performance liquid chromatography. Two chromatographic systems were employed for monitoring the reaction: (I) a Prominence equipment from Shimadzu with a Pinnacle-II C18 column (250 × 4.6 mm, 5 μm; Restek); (II) a Waters Alliance equipment with an XTerra RP18 column (250 × 4.6 mm, 5 μm, Waters). The mobile phase consisted of 4–5% of methanol and 95–96% of an aqueous solution containing 15 mM TRIS-HCl and 1 mM NaCl (pH = 6.8) or 10 mM ammonium acetate (pH = 7). Aqueous solutions of commercial standards were employed for obtaining calibration curves of reactants and products.

In the case of H_2Bip, the peak of the reactant could not be well separated from that of the corresponding oxidized product (Bip). Therefore, integrations of the peaks at different wavelengths were performed. Assuming that the peak considered is only due to the reactant and one known product, the concentration of both compounds can be calculated by resolving sets of equations as follows:

$$\text{Area}_{\lambda 1} = f_{\lambda 1}{}^{R}\, C^{R} + f_{\lambda 1}{}^{P}\, C^{P} \quad (2)$$

$$\text{Area}_{\lambda 2} = f_{\lambda 2}{}^{R}\, C^{R} + f_{\lambda 2}{}^{P}\, C^{P} \quad (3)$$

where $\text{Area}_{\lambda 1}$ and $\text{Area}_{\lambda 2}$ are the values resulting from integration of the chromatogram peaks at analysis wavelengths $\lambda 1$ and $\lambda 2$, C^R and C^P are the concentrations of the reactant and the product, $f_{\lambda 1}{}^{R}$, $f_{\lambda 1}{}^{P}$, $f_{\lambda 2}{}^{R}$ and $f_{\lambda 2}{}^{P}$ are the factors obtained from the calibration curves for the reactant and the product at $\lambda 1$ and $\lambda 2$. Although only two equations are required for calculating C^R and C^P, more equations were used in order to check the results obtained.

Detection and quantification of H_2O_2. For the determination of H_2O_2, a Cholesterol Kit (Wiener Laboratorios S.A.I.C.) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol.[27,28] Briefly, 400 μL of irradiated solution was added to 1.8 mL of reagent. The absorbance at 505 nm of the resulting mixture was measured after 30 min at room temperature, using the reagent as a blank. Aqueous H_2O_2 solutions prepared from commercial standards were employed for calibration.

Mass spectrometry analysis. An LC/MS system, equipped with an HPLC chromatograph (Agilent 1100) and a triple quadrupole mass spectrometer (Q TRAP Applied Biosystems), was employed. HPLC analyses were performed by using an XTerra RP18 column (*vide supra*), and isocratic elution with 96% ammonium acetate (10 mM) and 4% methanol at a flow rate of 0.6 mL min^{-1}. The mass spectrometer was equipped with an electrospray ion (ESI) source (Turbo Ion Spray (TIS)) and was operated in both positive and negative ion modes. Nitrogen served as auxiliary, collision gas and nebulizer gas. The nitrogen temperature of the TIS source was 450 °C and the declustering potential (DP) 60 V. The detection was scan mode with a step size of 0.1 atomic mass units (amu) and a scan range of 150–500 amu. Mass chromatograms, representations of mass spectrometry data as chromatograms (the *x*-axis represents time and the *y*-axis represents signal intensity) were registered using different scan ranges.

Quantum yield determinations

The quantum yields of reactant disappearance (Φ_R) and product formation (Φ_P) were determined in experiments performed under different conditions. Values were obtained using the following equations:

$$\Phi_R = -\frac{(d[R]/dt)_0}{P_a} \quad (4)$$

$$\Phi_P = \frac{(d[P]/dt)_0}{P_a} \quad (5)$$

where $(d[R]/dt)_0$ and $(d[P]/dt)_0$ are the initial rates of reactant consumption and product formation, respectively, and P_a is the photon flux absorbed by the reactant (eqn (1)). For the determination of the initial rates, the experiments were carried out using solutions of relatively high reactant concentration (H_2Bip). Under these conditions, the time evolution of the concentrations of H_2Bip and products followed a pseudo-zero-order rate law as long as P_a did not change significantly. The initial rates were obtained from the slope of the corresponding plots of concentration *vs.* irradiation time within such time windows. The evolution of the concentrations during irradiation was followed by HPLC (*vide supra*).

Fluorescence measurements

Steady-state fluorescence measurements were performed in quartz cells (1 cm path length) using a Perkin-Elmer LS 50B spectrofluorometer. Fluorescence spectra of aqueous solutions of Bip and H_2Bip were obtained by excitation at different wavelengths (λ_{exc} = 330–390 nm) and were recorded between (λ_{exc} + 10) nm and 650 nm. The spectra were corrected for differences in instrumental response and light scattering. For determining the quenching of fluorescence of Bip by iodide (I^-), emission spectra of Bip and H_2Bip solutions (25 μM) were recorded in the absence and in the presence of I^- (0–10 mM). The total fluorescence intensities (I_F) were calculated by integration of the corresponding fluorescence bands.

Results

Photolysis of H_2Bip in aqueous solution in the absence of O_2

Aqueous solutions containing H_2Bip (50–200 μM, pH = 7.0), previously saturated with Ar, were irradiated at 335 and 350 nm using both radiation sources described in the Experimental section. The irradiation-induced spectral changes (Fig. 2a) revealed that the characteristic low energy band of H_2Bip, centered at 330 nm, decreased in intensity, whereas a product (or products) absorbing at wavelengths lower than 300 nm was (were) formed. In addition, the same isosbestic points could be observed for more than 10 min of irradiation, suggesting that the same photochemical transformation occurred during that time window.

HPLC analysis of the irradiated H_2Bip solutions showed that the H_2Bip concentration decreased as a function of irradiation time (Fig. S1, ESI†). A value of $(5.3 \pm 0.3) \times 10^{-2}$ was obtained for the quantum yield of H_2Bip consumption (Φ_{H_2Bip}). This value is lower than the quantum yields associated with the disappearance of Bip ($\Phi_{Bip} = 0.10 \pm 0.01$) and the related derivative neopterin,[11] under similar conditions.

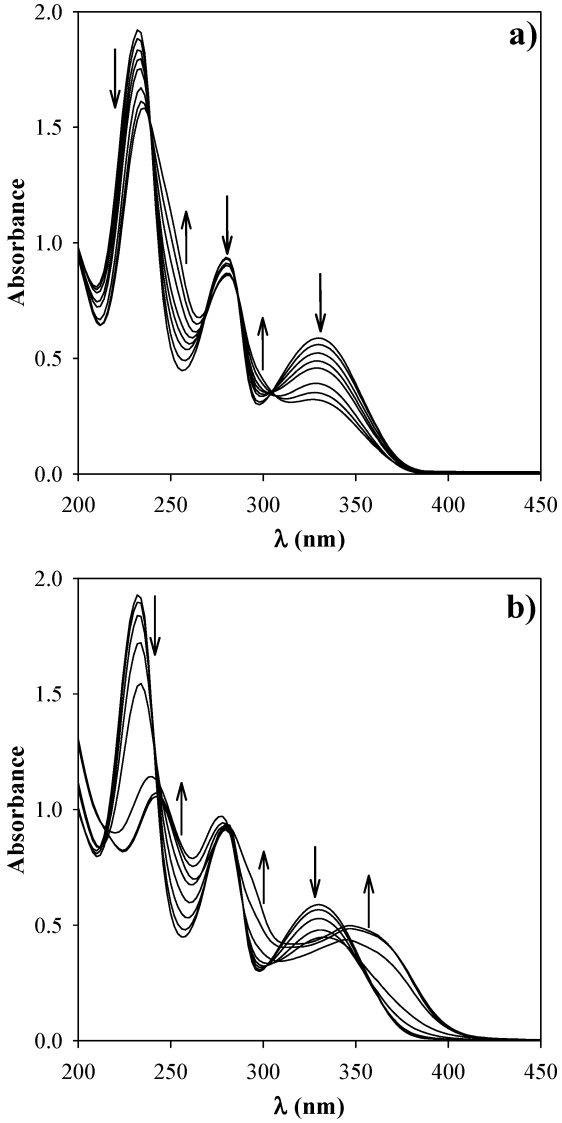


Fig. 2 Evolution of the absorption spectra of irradiated solutions of H_2Bip ($[H_2Bip]_0 = 100$ μM, pH = 7.0) as a function of irradiation time. Spectra were recorded at 0, 1, 2, 3, 4, 6, 8 and 10 min. Optical path length: 10 mm, irradiation: source I (350 nm). Arrows indicate the changes observed at different wavelengths. (a) Ar-saturated aqueous solutions; (b) air-equilibrated aqueous solutions.

As H_2Bip was consumed, at least 3 photoproducts (P1, P2, P3, Fig. 3), with retention times lower than that of the reactant, were formed. Analysis of the chromatograms obtained at different wavelengths showed that the areas of the P2 and P3 peaks increased simultaneously, and were much larger than that corresponding to P1, suggesting that this latter compound was probably a minor photoproduct. The changes in the peak area of P1 were difficult to quantify due to relatively large errors in integrating very small peaks. The spectra of the photoproducts P2 and P3 were also quite similar, with no absorption above 320 nm and a band centered at 246 nm (Fig. 3). These results are consistent with the absorption spectra shown in Fig. 2. As expected, neither Bip nor H_2O_2 were detected by HPLC and the enzymatic method, respectively.

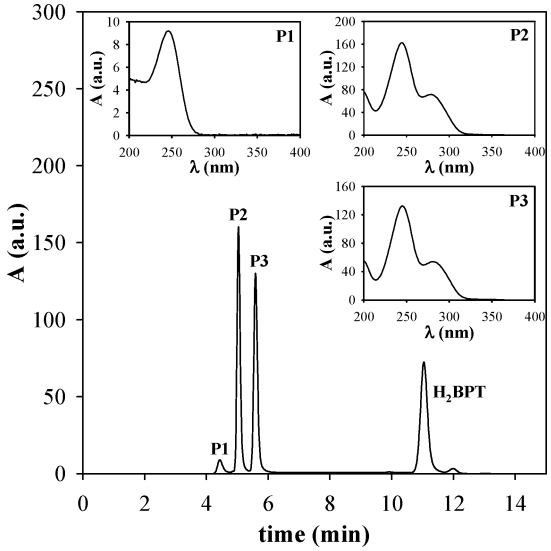


Fig. 3 Chromatogram ($\lambda_{an} = 245$ nm) and absorption spectra obtained by HPLC analysis of Ar-saturated solutions of H_2Bip after 10 min of irradiation. $[H_2Bip]_0 = 100$ μM, pH = 7.0. Experiments performed using irradiation source I (350 nm) and chromatographic system I (Experimental).

Photolysis of H_2Bip in aqueous solution in the presence of O_2

Air-equilibrated aqueous solutions of H_2Bip (50–200 μM, pH = 7.0) were irradiated at 350 nm (radiation source I, Experimental). The spectral changes registered were very different from those observed in the absence of O_2 (Fig. 2b). In this case, whereas the higher energy band of H_2Bip, centered at 230 nm, rapidly decreased in intensity, an absorbance increase at $\lambda > 340$ nm, where oxidized pterins show a typical band, was observed (Fig. 2). In addition, no isosbestic points could be detected, thus indicating the presence of several products.

HPLC analysis of the irradiated H_2Bip solutions revealed the presence of 6 photoproducts. Comparison of retention times and spectra showed that 3 of them are the same photoproducts as those detected in anaerobic experiments (P1, P2 and P3, Fig. 3). The retention times and the spectra of the other 3 additional products are identical to those obtained for standard solutions of Bip, Fop and Cap. The formation of these compounds explains the increase in the absorbance above 340 nm and reveals the existence of an oxidative pathway. Taking into account that Cap is a photooxidation product of Fop,[29] and Fop is, in turn, formed by photooxidation of Bip,[10,11] this latter compound can be proposed as the primary photoproduct in the oxidative pathway.

The concentration profiles of the reactant and the photoproducts, showed that the rate of H_2Bip consumption, as well as the rate of Bip production, increased with irradiation time (Fig. 4a). In the experiment shown in Fig. 4a, Bip concentration reached a maximum and then decreased due to its own photooxidation yielding Fop. Accordingly, Fop formation occurred later. Similarly to Bip, Fop concentration reached a maximum and then decreased due to its photooxidation yielding Cap. Cap was the last oxidized pterin appearing in the irradiated solution. On the other hand, the area profiles of chromatographic peaks of P2 and P3 showed a steady increase until reaching a plateau. Therefore, (i) the production of P2 and P3 does not undergo any acceleration,

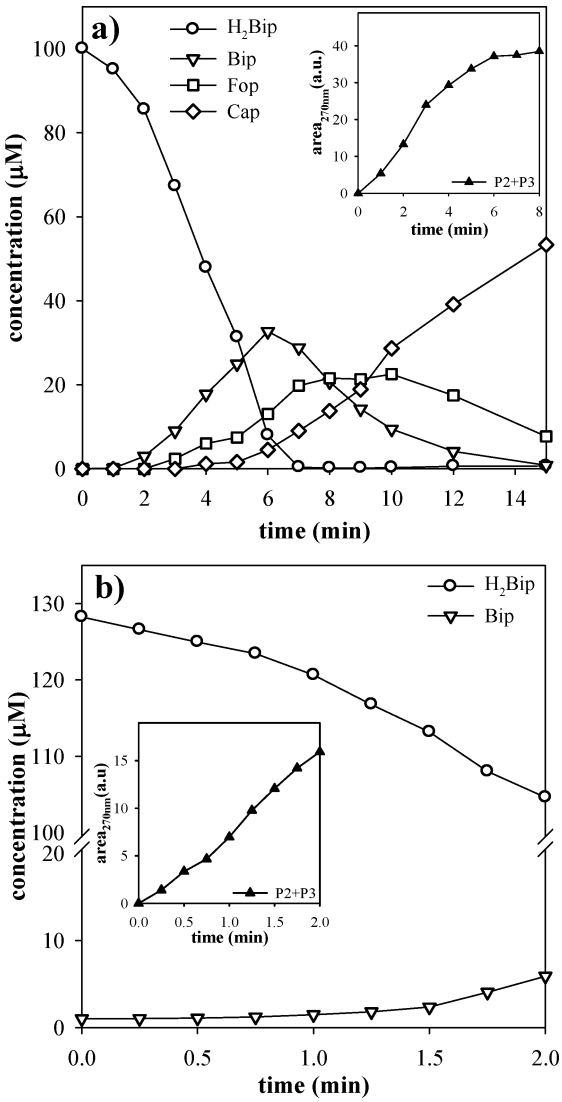


Fig. 4 Time evolution of reactant and photoproduct concentrations in air-equilibrated aqueous solutions under UV-A irradiation. (a) Total irradiation time = 15 min, $[H_2Bip]_0$ = 100 μM. (b) Total irradiation time = 2 min, $[H_2Bip]_0$ = 128 μM. Insets: time evolution of the areas of chromatographic peaks corresponding to photoproducts P2 and P3. Experiments performed at pH 7.0 using irradiation source I (350 nm) and chromatographic system I (Experimental).

(ii) after the reactant is completely consumed, their formation stops and (iii) these compounds do not participate in subsequent reactions. The same behavior was observed independently of the initial H_2Bip concentration.

We performed a more detailed HPLC analysis within the first 2 min of irradiation, where the secondary photolysis of Bip was still negligible. The results (Fig. 4b) clearly showed that, whereas the reaction forming Bip underwent an autocatalytic effect, the rate of reactions yielding P2 and P3 remained constant, as observed under anaerobic conditions. The initial quantum yield of H_2Bip consumption was evaluated within the time window where the rate of reactant disappearance is constant (*ca.* 1 min). In spite of the relatively large error in the calculation due to the small conversion, the value obtained, $(5 \pm 1) \times 10^{-2}$, was equal within the experimental error to that determined under anaerobic conditions. On the other hand, the estimation of the quantum yield of Bip formation led to a value at least one order of magnitude lower ($\Phi_{Bip} < 5 \times 10^{-3}$). This fact is logical considering that, as previously pointed out (Fig. 4b), the initial rate of Bip production is negligible in comparison with the H_2Bip consumption to yield P2 and P3.

H_2O_2 was detected in irradiated air-equilibrated H_2Bip solutions, its concentration increasing with irradiation time. As shown previously, H_2O_2 is also produced during the photooxidation of Bip[11] and Fop[14] in a 1 : 1 stoichiometry (one molecule of H_2O_2 generated for each molecule of substrate consumed). However, during the photolysis of H_2Bip, H_2O_2 was detected during the first minutes of irradiation, when photolysis of Bip was still negligible, thus suggesting that H_2O_2 was generated together with Bip.

In addition, the mass balance was calculated in larger time windows (Fig. 5). If H_2O_2 present in the irradiated H_2Bip solutions were generated only during the photooxidations of Bip and Fop, its

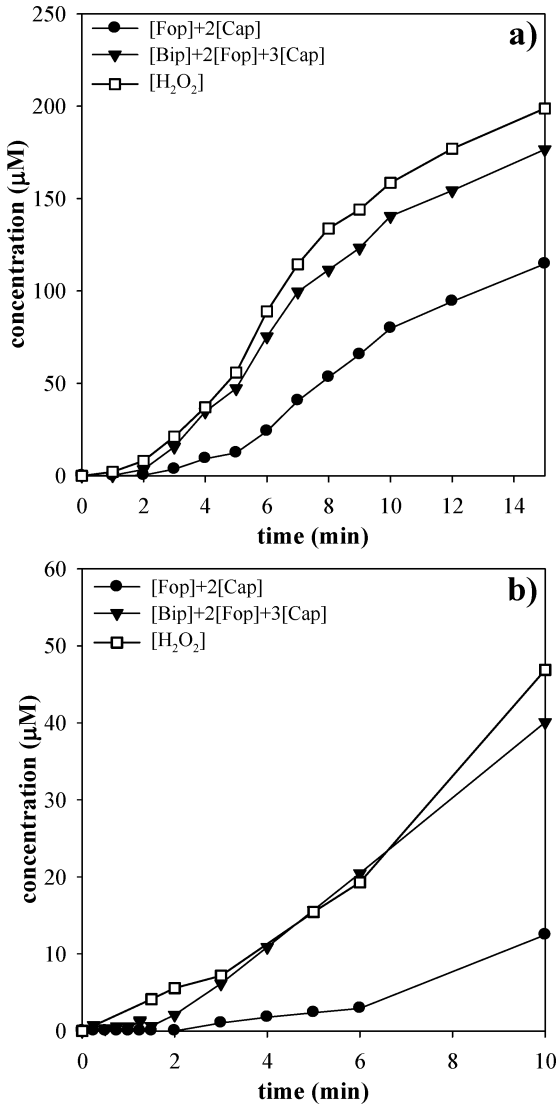


Fig. 5 Time evolution of H_2O_2 concentration in H_2Bip solutions under UV-A irradiation and comparison with oxidized product formation. (a) Air-equilibrated solutions. $[H_2Bip]_0$ = 100 μM. (b) O_2-saturated solutions, $[H_2Bip]_0$ = 150 μM. Experiments performed at pH 7.0 using irradiation source I (350 nm) and chromatographic system I (Experimental).

concentration at a given time should be equal to [Fop] + 2[Cap]. On the other hand, if H_2O_2 were also produced with the same stoichiometry in the photochemical conversion of H_2Bip into Bip, its concentration, at a given time, should be equal to [Bip] + 2[Fop] + 3[Cap]. The concentration profiles shown in Fig. 5 show that the latter hypothesis is the correct one, and confirm that H_2O_2 was also generated in the same reaction in which Bip was formed from H_2Bip.

The results presented so far reveal that upon irradiation in air-equilibrated solutions, two main pathways of H_2Bip consumption exist. The first one is O_2-independent and is the same as that occurring under anaerobic conditions. The second one only takes place in the presence of O_2, undergoes acceleration after some time, and is very interesting from a biological point of view, because it proves that Bip can be generated photochemically from H_2Bip. In addition, H_2O_2, in part responsible for the inhibition of enzymes of the melanin biosynthesis in vitiligo[30,31] (Introduction), is released in this process.

Photolysis of H_2Bip in aqueous solution in the presence of Bip and O_2

The acceleration of the O_2-dependent process deserves a deeper analysis. It seems that when Bip accumulates in the solution, in some way this compound causes an increase in the rate of its own production. Since a solution containing H_2Bip and Bip is stable in the dark, this effect must be photochemical. Therefore, excited states of Bip seem to be needed for the acceleration. In order to investigate this issue in more detail, solutions containing H_2Bip and Bip at different concentrations were irradiated and analyzed by HPLC.

In the first group of experiments, solutions were irradiated at 350 nm. Fig. 6a shows an example of results obtained using equimolar initial concentrations of H_2Bip and Bip. An initial increase in Bip concentration was observed as a consequence of a fast oxidation of H_2Bip. After almost all of the H_2Bip was consumed, the photooxidation of Bip itself was dominant and its concentration started to decrease. The initial rate of H_2Bip consumption ($(-d[H_2Bip]/dt)_0 = (11 \pm 1)$ μM min^{-1}) was higher than that calculated from the initial quantum yield of H_2Bip consumption in the absence of Bip ((4.6 ± 0.9) μM min^{-1}). Moreover, the initial rate of H_2Bip oxidation, measured as $(d([Bip]+[Fop]+[Cap])/dt)_0$ ((7.2 ± 0.6) μM min^{-1}), instead of being negligible as in the absence of Bip (Fig. 4), was of the same order of magnitude as $(-d[H_2Bip]/dt)_0$. In addition, the initial proportion of H_2Bip converted into products P2 and P3, assessed through the areas of the corresponding peaks, was much lower than that registered in the experiments performed in the absence of an initial concentration of Bip.

Another set of experiments was performed using the irradiation source II (Experimental section), under conditions where the proportion of radiation absorbed by H_2Bip was very low (Fig. 1), *i.e.* only Bip was excited (wavelengths of irradiation >370 nm). The results obtained in one of these experiments are illustrated in Fig. 6b. The initial rate of H_2Bip consumption was much higher than those obtained in experiments performed in the absence of Bip. Moreover, the proportion of H_2Bip converted into Bip was very high (>80%). Accordingly, the amounts of products P1, P2 and P3 formed were undetectable or very low. These results clearly

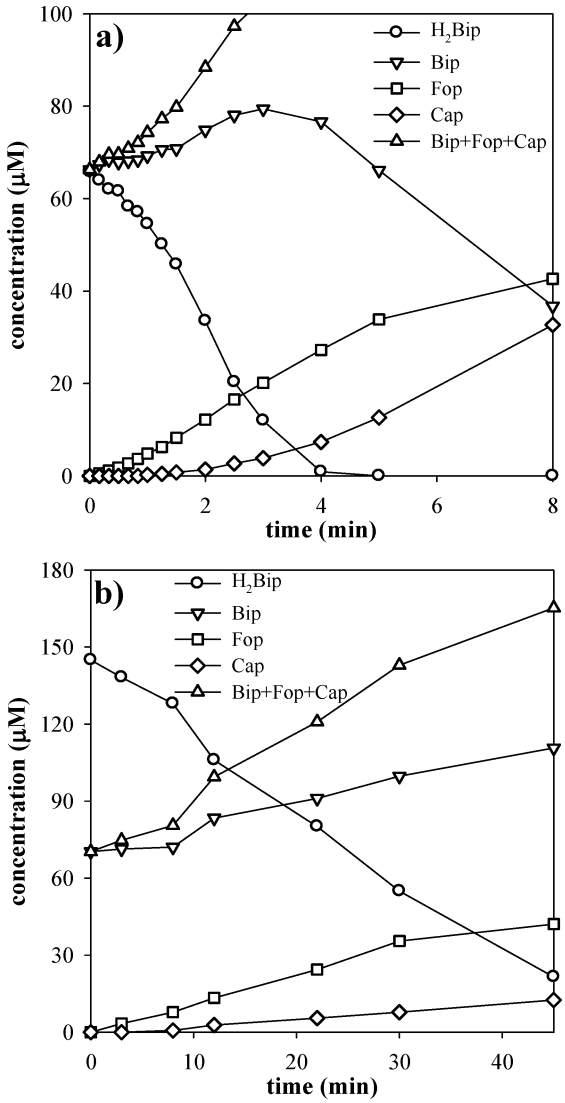


Fig. 6 Concentration profiles of reactants and photoproducts during irradiation of air-equilibrated aqueous solutions containing initially H_2Bip and Bip. pH = 7.0. (a) $[H_2Bip]_0 = 65$ μM, $[Bip]_0 = 65$ μM, experiments performed using irradiation source I (350 nm) and chromatographic system I. (b) $[H_2Bip]_0 = 145$ μM, $[Bip]_0 = 70$ μM, experiments performed using irradiation source II (380 nm) and chromatographic system II (Experimental).

indicate that when Bip is excited, the oxidation of H_2Bip into Bip takes place even in the absence of radiation absorbed by the reactant (H_2Bip), which means that this is a photosensitized process. A control experiment was performed under the same conditions, but in the absence of air. After irradiation, the solutions were aerated and then analyzed. As expected, the consumption of H_2Bip was negligible in comparison to that observed in the presence of air. These results demonstrated that O_2 is needed for the photosensitized formation of Bip, *i.e.* no reaction occurs between excited Bip and H_2Bip under anaerobic conditions.

Coming back to the general analysis of the H_2Bip photolysis, we have shown that an O_2-dependent process yields Bip as the main product. This oxidation involves, as a matter of fact, two different pathways. One of them consists of the reaction of excited states of H_2Bip with dissolved O_2, does not need the presence of

excited Bip and is responsible for the very slow initial formation of Bip during the irradiation of air-equilibrated H_2Bip solutions. The other pathway is a photosensitized process, in which excited states of Bip induce the oxidation of H_2Bip to yield the same product. This second pathway explains the acceleration in the oxidation of the reactant observed in solutions initially containing only H_2Bip, as well as the high rates of H_2Bip consumption and Bip formation in experiments performed with mixture solutions containing both pterin derivatives.

In order to find out if the photosensitized oxidation of H_2Bip also takes place using other pterin derivatives as photosensitizers, air-equilibrated solutions containing H_2Bip and an oxidized pterin different from Bip were irradiated at 380 nm. In order to avoid interferences and to simplify the analysis, pterin (Ptr)[32] and 6-methylpterin (Mep)[33] were chosen for their low quantum yields of consumption under UV-A irradiation. Fig. 7 shows the results in the case of Mep (similar results were obtained with Ptr). Whereas Mep was not consumed significantly, a strong consumption of H_2Bip was observed with a significant production of Bip, and negligible amounts of P1, P2 and P3. In control experiments performed in the absence of Mep, no significant amount of Bip was detected. These results clearly demonstrate that the photosensitized conversion of H_2Bip into Bip takes place not only with Bip as sensitizer, but also with Mep, Ptr, and probably other oxidized pterins, acting as sensitizers.

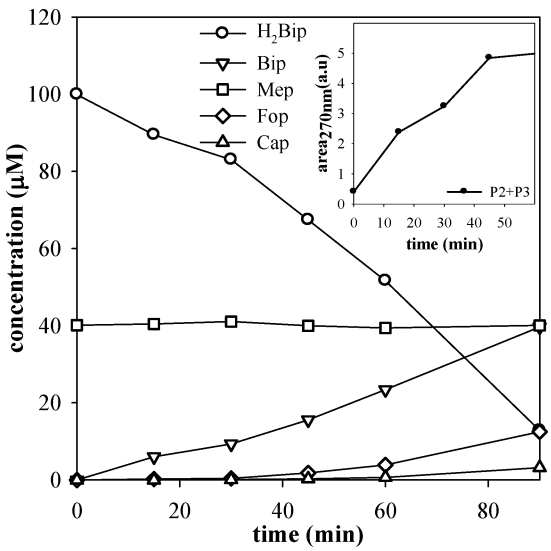


Fig. 7 Concentration profiles of reactants and photoproducts during irradiation of air-equilibrated aqueous solutions containing initially H_2Bip and Mep. Inset: time evolution of the areas of chromatographic peaks corresponding to photoproducts P2 and P3. $[H_2Bip]_0 = 100\ \mu M$, $[Mep]_0 = 40\ \mu M$, pH = 7.0, experiments performed using irradiation source II (380 nm) and chromatographic system II (Experimental).

Experiments were carried out to assess the effect of the O_2 concentration, by bubbling O_2 before irradiation (Fig. 8). HPLC analysis showed that the results were similar to those obtained under air-equilibrated solutions. The concentrations of products P1, P2 and P3 increased with irradiation time (O_2-independent photolysis), and, as expected, the oxidative pathway also occurred (formation of Bip). The corresponding mass balance (Fig. 5b)

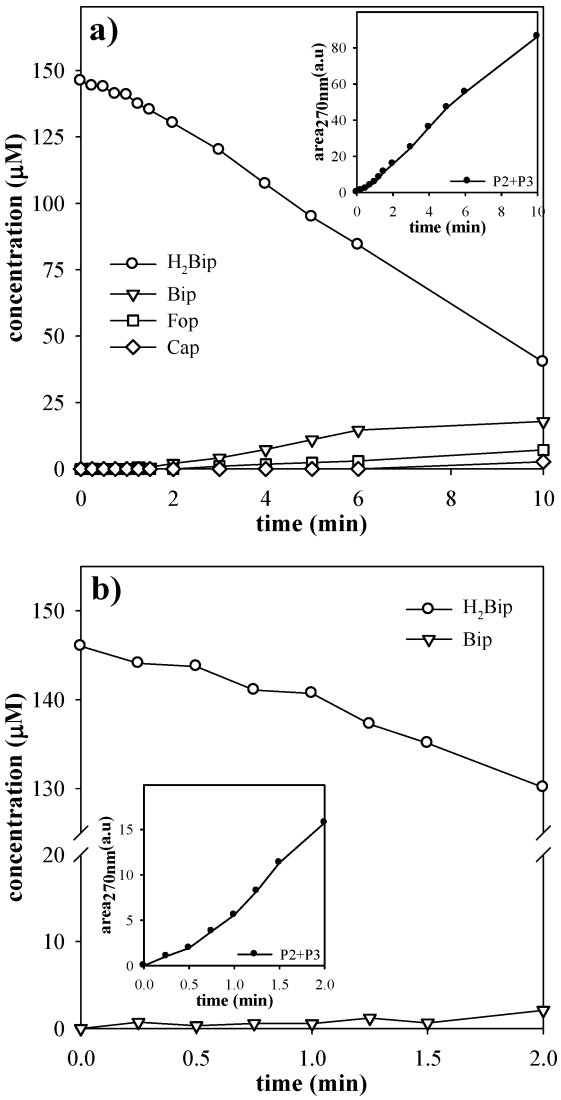


Fig. 8 Time evolution of reactant and photoproduct concentrations in O_2-saturated aqueous solutions under UV-A irradiation. (a) Total irradiation time = 10 min. (b) Total irradiation time = 2 min. Insets: time evolution of the areas of chromatographic peaks corresponding to photoproducts P2 and P3. $[H_2Bip]_0 = 146\ \mu M$, experiments performed at pH 7.0 using irradiation source I (350 nm) and chromatographic system I (Experimental).

showed that H_2O_2 was also a photoproduct of the photooxidation of H_2Bip. From the analysis of the first 2 min of irradiation (Fig. 8b), a value of $(5 \pm 1) \times 10^{-2}$ was obtained for the quantum yield of H_2Bip consumption, *i.e.* equal within experimental error to that calculated under anaerobic and air-equilibrated conditions.

However, kinetic analysis of concentration profiles showed different results from those expected. The initial rate of Bip formation was not higher than that measured in air-equilibrated solutions. Moreover, the consumption of H_2Bip did not undergo a strong acceleration and, accordingly, the rate of oxidized pterins formation hardly increased. Therefore the oxidative pathway of H_2Bip photolysis was not enhanced in O_2-saturated solutions. On the contrary, the photosensitized oxidation of H_2Bip seemed to be inhibited by O_2 at high concentrations (*vide infra*).

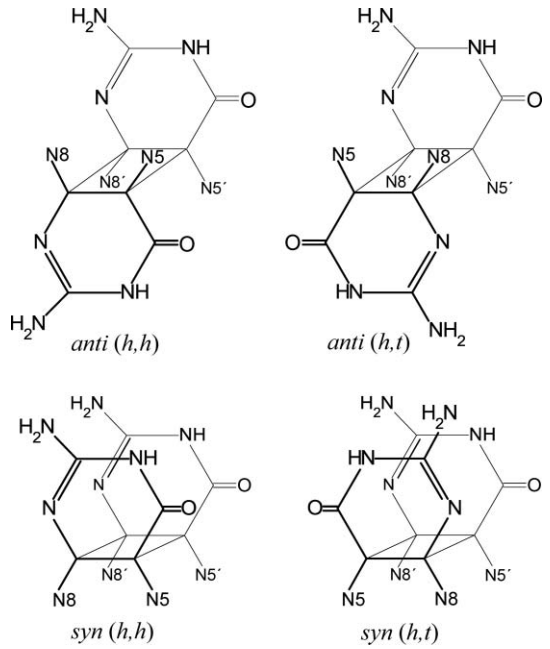
Mass spectrometry analysis of photoproducts

Solutions of H_2Bip were analyzed by HPLC coupled to electrospray ionization (ESI) mass spectrometry, before and after irradiation. The mass analysis was carried out in both positive and negative ion modes (ESI^+ and ESI^-, respectively). An H_2Bip standard solution was analyzed and, as expected, the signal corresponding to the intact molecular ion of H_2Bip as $[M - H]^-$ species at m/z 238.2 Da was observed in ESI^- mode (data not shown). In addition, in ESI^+ mode the intact molecular H_2Bip ion as $[M + H]^+$ and the adduct $[M + Na]^+$ were detected at m/z 240.2 Da and 262.2 Da, respectively (Fig. S2, ESI†). The mass chromatograms of H_2Bip solutions irradiated under anaerobic conditions were registered for large mass windows and specific ion masses, corresponding to the intact molecular ion of each detected compound (H_2Bip, P1, P2 and P3, data not shown). Whereas the signals of photoproducts P2 and P3 were intense, the signal of P1 was very weak, thus suggesting again that P1 is a minor photoproduct.

The mass spectra of P2 and P3 obtained in ESI^+ mode are shown in Fig. S2 (ESI†). Notice that the more intense peaks of the spectra of H_2Bip, P2 and P3 are identical ($m/z = 240.2$ Da), and correspond to the mass of the intact ion of H_2Bip ($[M + H]^+$). However, both P2 and P3 have an additional peak at $m/z = 479$ Da, which corresponds to $[M_2 + H]^+ = 2 \times 239 + 1$ Da. Therefore, the molecular mass of both P2 and P3 should be equal to exactly twice the molecular mass of H_2Bip. These results strongly suggest that P2 and P3 are dimers of H_2Bip and that the most efficient pathway for fragmentation during the electrospray ionization process is the cleavage of the dimers to yield the corresponding monomers. P2 and P3 have the same molecular weight and similar absorption spectra, but their structures are different enough to produce a variation in the retention time in the chromatographic runs, which allows their separation and independent analysis. Most probably, photodimerization occurs through the enone-type C=C bond (C9=C10) at the junction of the pyrazine and pyrimidine rings. This type of dimerization was reported in the case of thymine and uracil (pyrimidine derivatives).[34,35] In the case of the photodimerization of H_2Bip, four dimers may be formed by *syn* or *anti* combinations: for each *syn* or *anti* dimer, the *head-to-head* (*h*,*h*) and *head-to-tail* (*h*,*t*) isomers are possible (Scheme 2). The products denoted above as P2 and P3 might correspond to the *anti* and *syn* isomers.

The MS/MS spectra of H_2Bip and of the dimers ($m/z = 240.2$ Da) were registered in the ESI^+ mode (Fig. S3, ESI†). In the fragmentation of H_2Bip, the main peak ($m/z = 165$ Da) corresponds to the loss of the 6-substituent ($[M - (CHOH)_2CH_3 + H]^+$) and the peak at $m/z = 222$ Da corresponds to the loss of H_2O ($[M - H_2O + H]^+$). It is difficult to speculate about the rest of the fragments and, to the best of our knowledge, this is the first time H_2Bip has been analyzed by ESI mass spectrometry. The fragmentations registered, under the same conditions, for P2 and P3 (Fig. S3, ESI†) are exactly the same as those for H_2Bip. Therefore, it can be concluded that the main fragments formed during ionization of products P2 and P3 are H_2Bip. These results confirmed the dimeric nature of P2 and P3.

Irradiated air-equilibrated H_2Bip solutions were also analyzed by HPLC-ESI mass spectrometry. As expected, besides the reactant, P1 and the dimers, products with retention times and spectra matching those of Bip, Fop and Cap were also detected. Mass spectra confirmed that the pathway yielding dimers also takes place under aerobic conditions. In the mass spectra obtained from the chromatographic peaks of Bip, Fop and Cap, the signals corresponding to the intact molecular ions were detected in both ESI^+ and ESI^- modes. Therefore, these results also confirm the formation of the oxidized pterins (Bip, Fop and Cap) in the aerobic photolysis of H_2Bip.



Scheme 2 Hypothetical structures for the dimers formed by photolysis of H_2Bip.

Mechanistic analysis of the oxidative pathway

As dihydropterins are reactive towards oxidizing species[20,23,36,37] and Bip, and other oxidized pterins produce reactive oxygen species,[11,15] one of the mechanisms to be considered is the photochemical production of such species by Bip and the subsequent reaction of these species with H_2Bip to yield more Bip. One hypothesis involves H_2O_2; however, this species can be discarded because it was already demonstrated that the product of the corresponding reaction is not Bip[20] (Introduction).

The pterins Bip, Fop and Cap are good $^1O_2$ sensitizers upon UV-A excitation,[15] and the oxidation of H_2Bip by $^1O_2$ is fast, yielding Bip and H_2O_2 as products;[23] therefore, the participation of this species was assessed more carefully. In the case of the oxidation of H_2Bip by $^1O_2$, the fraction of H_2Bip converted to Bip ($\Delta[Bip]/\Delta[H_2Bip]$) was reported to be 0.42 ± 0.03, whereas the relationship between the H_2O_2 released and the H_2Bip consumed ($\Delta[H_2O_2]/\Delta[H_2Bip]$) was equal to 0.17 ± 0.03.[23] Analysis of mass balances, such as those shown in Fig. 5, indicate that $\Delta[H_2O_2]/\Delta[H_2Bip]$ is very close to 1 for experiments performed in both air-equilibrated and O_2-saturated solutions. Similarly, calculation of $\Delta[Bip]/\Delta[H_2Bip]$ gave values significantly higher than 0.42. This is particularly evident in experiments where the H_2Bip consumption *via* the oxidative pathway is much higher than *via* the pathway yielding the dimers (Fig. 6). Hence, oxidation *via*

$^1O_2$ cannot be a significant pathway for H_2Bip consumption and is not responsible for the acceleration phenomenon.

Quenching of the triplet state of Bip by O_2 has already been studied by time-resolved[38] and steady-state methods.[15] On the other hand, although it was demonstrated that O_2 does not deactivate singlet excited states of several oxidized pterins,[7,39] no previous studies have been reported for Bip. Therefore, emission spectra of Ar-, air- and O_2-saturated Bip solutions at the same concentration were registered. No differences in the total fluorescence intensities were detected, thus indicating that O_2 does not quench the singlet excited state of Bip. For this reason, the inhibition of the photosensitized process observed at high O_2 concentrations is due to the quenching of the Bip triplet state by O_2.

In order to investigate this issue further, experiments in the presence of iodide (I^-) were performed. This anion is able to interact with both singlet and triplet excited states of organic compounds. The resulting effects on the photophysical behaviour of a given compound depend on the relative rates of the different deactivation pathways (non-radiative decays to ground state, intersystem crossing).[40] Therefore, in some cases, the presence of I^- causes an increase of the quantum yields of triplet state formation, whereas in others, a decrease is observed. The efficiency of I^- in quenching flavin triplet states is much higher than the efficiency of quenching the corresponding excited singlet states. This property has been used to investigate the role of the excited states of flavin molecules in photochemical mechanisms.[41,42] The same approach was used to evaluate the participation of triplet excited states of pterins in their photoreduction.[43] Furthermore, in studies of the phosphorescence of pterins adsorbed on paper at room temperature, it was observed that the non-radiative decay from the lowest triplet state of pterins was enhanced by I^-.[44]

We applied this methodology to our system, and evaluated the capability of I^- to deactivate the S_1 states of Bip and H_2Bip by fluorescence quenching experiments (Fig. S4, ESI†). For both compounds, only moderate quenching was registered within the range of I^- concentration used; *i.e.*, a decrease of less than 50% of the fluorescence was measured at a concentration of 10 mM of I^-. At I^- concentrations lower than 1 mM, the fluorescence quenching was negligible (Fig. S4 insets, ESI†).

Photolysis experiments performed in the presence and in the absence of different I^- concentrations were compared. The results showed that, whereas I^- did not affect the rate of dimer formation, the acceleration of the H_2Bip consumption was not observed any more and, consequently, formation of Bip was very slow (Fig. 9). Therefore, I^- inhibited the photosensitized oxidation of H_2Bip. In addition, during the first 4 min of irradiation, the rate of formation of dimers was identical to the control, thus indicating that I^- did not inhibit this pathway.

These results are in agreement with experiments performed in O_2-saturated solutions (Fig. 8) and strongly suggest that dimers are formed from the singlet excited states of H_2Bip. On the other hand, although it is difficult to assess the effect of I^- on the initial non-photosensitized oxidation of H_2Bip, it is clear that the quenching of the triplet state of Bip by I^- inhibits the photosensitized process, thus preventing the acceleration of H_2Bip consumption and the resulting fast Bip formation.

The oxidation of H_2Bip could be photoinduced *via* energy transfer or electron transfer from the triplet excited state of Bip. In the former mechanism, the triplet state of H_2Bip would be

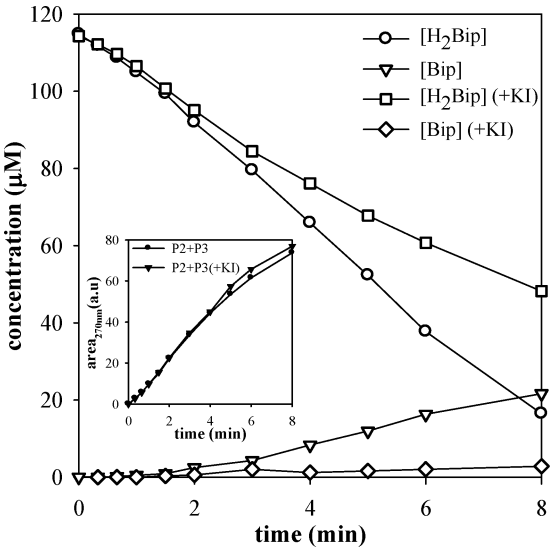


Fig. 9 Time evolution of concentrations of reactant and photoproduct during the photolysis of H_2Bip, in the absence and in the presence of KI (600 μM). Inset: time evolution of the areas of chromatographic peaks corresponding to photoproducts P2 and P3. $[H_2Bip]_0 = 115$ μM, experiments performed in air-equilibrated aqueous solution at pH 7.0 using irradiation source I (350 nm) and chromatographic system I (Experimental).

generated and would react with O_2 to yield Bip and H_2O_2. In the latter one, an electron transfer from the ground state of H_2Bip to the triplet state of Bip would take place. The capability of pterins to act as photosensitizers *via* electron transfer processes has already been proven. In the case of the oxidation of purine nucleotides photoinduced by pterin,[17,18] the photosensitizer in its triplet excited state reacts in a first step with the substrate to yield the pterin radical anion ($Ptr^{\bullet-}$) and the nucleotide radical cation. In a second step, $Ptr^{\bullet-}$ reacts with O_2 and $O_2^{\bullet-}$ is formed.

Taking into account these reactions and the results presented so far, the following mechanism can be proposed for the oxidation of H_2Bip:

$$^1H_2Bip \xrightarrow{h\nu} {}^1H_2Bip^* \xrightarrow{ISC} {}^3H_2Bip^* \quad (6)$$

$$^1H_2Bip^* + H_2Bip \rightarrow (H_2Bip)_2 \quad (7)$$

$$^3H_2Bip^* + O_2 \rightarrow Bip + H_2O_2 \quad (8)$$

$$Bip \xrightarrow{h\nu} {}^1Bip^* \xrightarrow{ISC} {}^3Bip^* \quad (9)$$

$$^3Bip^* + O_2 \rightarrow Bip + {}^1O_2 \quad (10)$$

$$^3Bip^* + O_2 \rightarrow Fop + H_2O_2 \quad (11)$$

$$H_2Bip + {}^3Bip^* \rightarrow H_2Bip^{\bullet+} + Bip^{\bullet-} \quad (12)$$

$$Bip^{\bullet-} + O_2 \rightarrow Bip + O_2^{\bullet-} \quad (13)$$

$$H_2Bip^{\bullet+} + O_2 \rightarrow Bip + O_2^{\bullet-} + 2H^+ \quad (14)$$

$$O_2^{\bullet-} + H_3O^+ \leftrightarrows HO_2^{\bullet} + H_2O \quad (15)$$

$$HO_2^{\bullet} + O_2^{\bullet-} \rightarrow HO_2^- + O_2 \quad (16)$$

Conclusions

In this work, we have studied the photochemistry of dihydrobiopterin (H_2Bip) in aqueous solution upon UV-A irradiation. Under anaerobic conditions, excitation of H_2Bip leads to the formation of isomeric dimers with molecular masses equal to exactly twice the molecular mass of the reactant. This reaction takes place from the singlet excited state of the reactant and the corresponding quantum yield of H_2Bip consumption Φ_{H_2Bip} was equal to $(5.3 \pm 0.3) \times 10^{-2}$. The formation of dimers by photolysis of H_2Bip has so far never been reported. In the presence of air, H_2Bip is initially consumed with the same quantum yield and the dimers are again the main photoproducts. However, a small proportion of the reactant is converted into its oxidized analogue, biopterin (Bip). As the reaction proceeds and a certain amount of Bip accumulates in the solution, a new pathway appears. This is a photosensitized process in which Bip photoinduces the oxidation of H_2Bip to Bip, and H_2O_2 is formed, *i.e.* the photoproduct acts as a sensitizer of its own production. As a consequence, the rates of H_2Bip consumption and Bip formation increase as a function of irradiation time, resulting in an autocatalytic photochemical reaction. In this process, in which no excitation of H_2Bip is needed, Bip in its triplet excited state reacts with the ground state of H_2Bip. The first step could involve an electron transfer from H_2Bip to the triplet excited state of Bip. Other oxidized pterins can also act as photosensitizers of H_2Bip oxidation, thus revealing a general mechanism.

The quantum yields of H_2Bip consumption are relatively high and show that this substance can be easily photolyzed. This is of particular importance from a biomedical point of view, since H_2Bip is accumulated in the skin of patients suffering from vitiligo,[5] a chronic depigmentation disorder, in which the protection against UV radiation fails. Even more important is the photooxidation of H_2Bip because it demonstrates that Bip, a compound that generates reactive oxygen species and is toxic for melanocytes,[45] can be formed photochemically from H_2Bip. Moreover, taking into account the presence of oxidized pterins, the fast photosensitized oxidation of H_2Bip should be considered as a very probable source of Bip in the skin.

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