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Solar radiation exposure of dihydrobiopterin and biopterin in aqueous solution

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Abstract

Dihydrobiopterin (H_2Bip) and biopterin (Bip) accumulate in the skin of patients suffering from vitiligo, whose lack of melanin causes a deficient protection against UV radiation. H_2Bip and Bip undergo photooxidation upon UV irradiation and the former acts as a photosensitizer of biomolecules. The aim of this work is to study the photochemical reactions of these compounds triggered by sunlight under outdoor conditions. H_2Bip and Bip in aqueous solutions were exposed to sunlight in La Plata city (34.90°S, 57.92°W) from Spring equinox to near Summer solstice and the spectral solar irradiance was recorded with a high-resolution spectrometer. The photochemical reactions were followed by HPLC and UV-Vis spectrophotometry. Upon sun exposure, excitation of H_2Bip leads to the formation of dimers and to its oxidation to Bip, which, in turn, is photooxidized into 6-formylpterin (Fop). Further excitation induces the oxidation of Fop to 6-carboxypterin, which is much more photostable than Bip and Fop and then it is accumulated in the solution. Rates of reactant consumption were determined under different weather conditions and the corresponding quantum yields were also calculated. We have demonstrated that solar radiation causes significant oxidation of the pterin derivatives investigated within a few minutes, even in cloudy days. Finally, the biological implications of our results are discussed.

Keywords: Dihydrobiopterin; Biopterin; Solar-exposure; Photooxidation

1. Introduction

Many living organisms are naturally exposed to sunlight. Ultraviolet (UV) solar radiation effects have been widely studied mainly because of the profound influence on human health and the effectiveness to start photochemical processes. Any increase in the UV intensity is therefore expected to produce significant changes in biological systems, which have a spectral sensitivity in this interval. The UVA (320–400 nm) and UVB (280–320 nm) radiation can reach Earth's surface, the latter range being strongly dependent on the ozone layer depletion in the stratosphere. Human skin has a significant damage risk under UV radiation. The consequence of the radiation below 320 nm can range from a slight erythema (sunburn) to skin cancer by a long-term exposure (de Gruijl, 1999; Hermann, 1998).

Furthermore, UVA irradiation can induce modifications in DNA through photosensitized reactions (Cadet and Spaull, 2005). The melanin of the epidermis, is the main protection against the harmful effects of UV solar radiation. The vitiligo is a chronic depigmentation disorder (Glassman,

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2011), characterized by loss of melanin, where the deactivation of enzymes necessary for melanin biosynthesis takes place, causing melanin's photo-protection failure.

Pterins are heterocyclic compounds present in living systems and, depending on the oxidation state, they can be classified as: oxidized (or aromatic) and reduced pterins (Fig. 1). Dihydrobiopterin (H_2Bip) belongs to the latter group and is a naturally occurring pterin derivative involved in the metabolism of amino acids (Nichol et al., 1985). Biopterin (Bip) and other oxidized pterins, which are not present in mammalians under physiological conditions, accumulate in the skin of patients suffering from vitiligo (Schallreuter et al., 2001). Among them, 6-carboxypterin (Cap), a product of Bip photolysis (Vignoni et al., 2009; Lyudnikova et al., 2009), has been isolated from the affected tissues (Rokos et al., 2002), suggesting that photooxidation of Bip takes place in vivo. Oxidized pterins are photochemically reactive upon UVA radiation exposure in aqueous solution and undergo photooxidation to produce several photoproducts and reactive oxygen species such as singlet molecular oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Lorente and Thomas, 2006; Neverov et al., 1996). In addition, it has been demonstrated that Bip and its photoproducts are able to photosensitize the oxidation of biomolecules (Serrano et al., 2013; Serrano et al., 2012). Therefore, the photochemistry of pterins is of vital importance for the knowledge of the mechanism of vitiligo disease.

The photochemistry of Bip and H_2Bip upon UVA exposure has been described in detail (Fig. 1) (Vignoni et al., 2010; Vignoni et al., 2009; Lyudnikova et al., 2009). Briefly, UVA excitation of H_2Bip in the presence of O_2 leads to two photochemical pathways: the formation of isomeric dimers with molecular masses equal to exactly twice the molecular mass of the reactant and the oxidation to its aromatic analogue, Bip. This latter reaction has been considered as a potential source of Bip in the skin (Vignoni et al., 2010). This compound, under aerobic conditions, is photochemically converted into 6-formylpterin (Fop), which, in turn, undergoes photooxidation to Cap, which is much more photostable than Bip and Fop. However, those studies have been performed using quasi-monochromatic sources under controlled laboratory conditions.

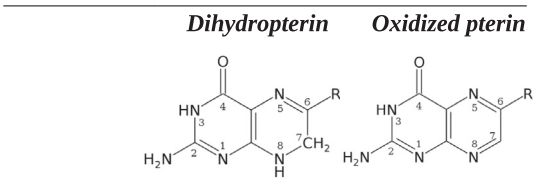

Fig. 1. Chemical structures of the pterin derivatives studied in this work and photochemical reaction pathway.

On the other hand, the effect of sunlight on Bip and H_2Bip has not been investigated. The aim of this work is to find out if the energy of the sun under different conditions is enough to cause significant chemical changes in Bip and H_2Bip and to compare those reactions triggered by sunlight to the photochemistry of these compounds already described in the literature. Therefore a study under outdoor conditions was performed to characterize the photochemical behavior of H_2Bip and Bip in a natural environmental context. In particular, we have identified the photoproducts, analyzed the kinetics and determined the quantum yields, simultaneously with the measurement of solar energy and the determination of atmospheric variables. The results are analyzed in the context of the general photochemical behavior of pterins in aqueous solution and the biological implications are discussed.

2. Materials and methods

2.1. 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. As a control, the electronic absorption spectrum of each solution was recorded on a Shimadzu UV-1800 spectrophotometer.

2.2. Solar spectral irradiance measurements

Irradiance (E) is defined as the radiant power incident on a small element of surface containing the point under consideration divided by the area of the element. Spectral irradiance (E_λ) is the derivative from E with respect to wavelength (λ) and is expressed in W m^{-2} nm^{-1}. The photon irradiance ($E_{n,p}$) is the number of *einstein* (mol of photons) per time interval (photon flux, $q_{n,p}$), incident on a small element of surface containing the point under consideration divided by the area of the element Braslavsky, 2007. Spectral photon irradiance ($E_{n,p,\lambda}$) is the derivative from ($E_{n,p}$) with respect to λ and is expressed in einstein m^{-2} s^{-1} nm^{-1}. E_λ can be converted into $E_{n,p,\lambda}$ by means of the Planck relation:

$$E_{n,p,\lambda} = E_\lambda \cdot \left(\frac{\lambda}{N_A \cdot hc}\right) \quad (1)$$

where N_A is the Avogadro constant, h is the Planck constant and c is the speed of light.

Systematic measurements of solar spectral irradiance (E^S_λ) were carried out with a high-resolution Avantes spectrometer (AvaSpec-ULS3648 model). The equipment is executed with an option of Irradiance Mode, using dark and reference data. Avantes spectrometer includes a fiber optic sensor and computer connection through USB2 interface cable and all devices are portable. It also has a 3648 pixel CCD detector linear array, grating (200–1100 nm) and slit-25. The instrument works in several integration times (from 10 microseconds to 10 min) and its signal extends up to 59,000 counts for 16bit ADC. The fiber optic sensor was placed on a flat, horizontal position to perform data acquisition. During the measurement routine, software was configured to the interval 247–812 nm with an integration time of 19 μs to achieve good stability and resolution. Solar spectral photon irradiance ($E^S_{n,p,\lambda}$) was calculated from E^S_λ (measured *in situ*) using Eq. (1).

2.3. Tropospheric ultraviolet and visible radiative transfer model

The solar spectrum was also calculated using the Tropospheric Ultraviolet Visible radiation model (TUV) developed by Madronich, 1993. The source code (Available at http://acd.ucar.edu/UV/) solves the radiative transfer equation by applying the discrete-ordinates algorithm (DISORT) Stamnes et al., 1988. The optical properties of the atmosphere depend on its altitude and composition features (aerosols, ozone profile, cloud cover, water vapor and other gases). TUV algorithm divides the atmosphere in homogeneous layers and assumes constant scattering and absorption in each of them. The intensity solar radiation able to reach the Earth's surface varies depending also on the seasons, geographic location, soil reflectivity, etc.

The most important inputs to TUV model are: date, local time, location (latitude, longitude and height above sea level), total ozone column, aerosol optical depth at 550 nm, the Ångström coefficient, surface albedo, single-scattering albedo, interval wavelength and spectral resolution (Madronich, 1993). In order to determine E^S_λ in clear sky days, all parameters mentioned above were considered into the code.

Ozone values were taken from measurements of the Ozone Monitoring Instrument (OMI) on board NASA's EOS-AURA satellite. OMI was designed by NASA in collaboration with the Netherlands's Agency for Aerospace Programs (NIVR) and the Finnish Meteorological Institute (FMI). The data are available from Goddard Space Flight Center (GSFC/DAAC). We have considered the data over a satellite pixel to La Plata city coordinates. At the same time, the data base corresponding to the aerosol was consulted. However, the lack of data for this region, makes it impossible to consider aerosol measurements provided by OMI. As an alternative, we searched the records of a station of AErosol RObotic NETwork (AERONET) located 45 km far from the city. The aerosol measurements, could be used as a first approximation into the model.

2.4. Solar radiation exposure

Aqueous solutions containing Bip and H_2Bip in quartz cells (0.5 cm optical path length) were exposed to outdoor conditions on a horizontal platform alongside the fiber optic sensor of the Avantes spectrometer (see Section 2.2). The measurement routine was mainly performed for clear sky days at solar noon, being the moment of maximum intensity and a minimal optical path crossed by light. The quartz cells were aligned toward sun's axis, until achieving the minimum shadow.

The attenuation of the solar intensity is strongly dependent on zenith angle, latter related to local time. Accordingly, we carried out an identical procedure using ensembles of samples exposed along the hours for a clear sky day. Also the temporal variation of the intensity was taken into account month by month. The quartz cells position were always aligned following the sun trajectory during the day. This system of sun tracking was designed so that the non-exposed volume of a given sample was always negligible with respect to the total volume.

2.5. High-performance liquid chromatography

A high-performance liquid chromatography Prominence from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP and photodiode array detector SPD-M20A) was employed for monitoring the reaction. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 A, 4.6 mm, 4 μm, Phenomenex) was used for product separation. Solutions containing 4% of methanol and 96% of ammonium acetate (pH = 6.5) were used as mobile phase.

In particular, H_2Bip chromatographic peak appears in the same retention time as the corresponding to its oxidized product (Bip). Thereby, integrations of the peaks at different wavelengths were performed such as reported in the literature Vignoni et al., 2009. 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 system of linear equations:

$$Area_{\lambda_1} = m^R_{\lambda_1}[R] + m^P_{\lambda_1}[P] \quad (2)$$

$$Area_{\lambda_2} = m^R_{\lambda_2}[R] + m^P_{\lambda_2}[P] \quad (3)$$

where $Area_{\lambda_1}$ and $Area_{\lambda_2}$ are the values resulting from integration of the chromatogram peaks at analysis wavelengths λ_1 and λ_2, $[R]$ and $[P]$ are the concentrations of the reactant and the product, $m^R_{\lambda_1}, m^P_{\lambda_1}, m^R_{\lambda_2}$ and $m^P_{\lambda_2}$ are the factors obtained from the calibration curves for the reactant and the product at λ_1 and λ_2. Another system of equations can be constructed using the above criteria, but at different

wavelengths, and thus the results obtained can be corroborated. On the other hand, in the case of Bip, the peak of the reactant could be well separated from those corresponding to its products.

2.6. Actinometry

Given a radiation source and a sample, the incident photon flux density ($q^{0,V}_{n,p}$) is the amount of incident photons per time interval ($q^{0}_{n,p}$) and divided by the volume of the sample:

$$q^{0,V}_{n,p} = \left(\frac{q^{0}_{n,p}}{V}\right) \quad (4)$$

Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of $q^{0,V}_{n,p}$ from Rayonet RPR lamp (as an artificial source), at the excitation wavelength. The method for the determination of $q^{0,V}_{n,p}$ has been described in detail elsewhere (Schuster et al., 1991; Kuhn et al., 2004).

3. Results

3.1. Solar irradiance determination

La Plata city is located in a mid-latitude region in Argentina, near sea level (Latitude 34.90°S, Longitude 57.92°W and 25 m asl) and the local time is GMT-3 h. Data acquisitions were obtained in two opposite conditions, clear sky and cloudy days. The experimental period was between Spring equinox and Summer solstice. From August 29th to December 4th, 2013, the solar zenital angle at noon was gradually reduced from 44.02° to 11.68°. The solar noon times at the beginning and end of the period were at 12:52 and 12:42, respectively. In one hour around noon, E^S_λ measured was practically constant as well as the ambient temperature. The average temperature at noon for all experimental days was (19.2 ± 5.4) °C, while the humidity and pressure were (48.2 ± 7.7)% and (1021.5 ± 11.7) hPa respectively.

The TUV model (Section 2.3) was applied to estimate E^S_λ. We took into account the surrounding topography and also several parameters provided by satellites and previous studies (Luccini et al., 2006; Cabrera et al., 2012). However, we estimated a higher aerosol value than recorded by AERONET because an industrial area and an oil refinery are located just outside the La Plata city. Additionally, the mean surface reflectivity value of 0.05 for typical grass soil was considered (Koelemeijer et al., 2003). Lastly, a single scattering albedo of 0.95 (Luccini et al., 2006) was included.

The Avantes spectrometer (Section 2.2), mounted in a black flat platform on grass, allowed us to easily measure the E^S_λ (composed by a Sun-Earth direct line of sight incident beam plus a diffuse one) coming from the whole celestial sphere (Fig. 2). As soon as the parameters were

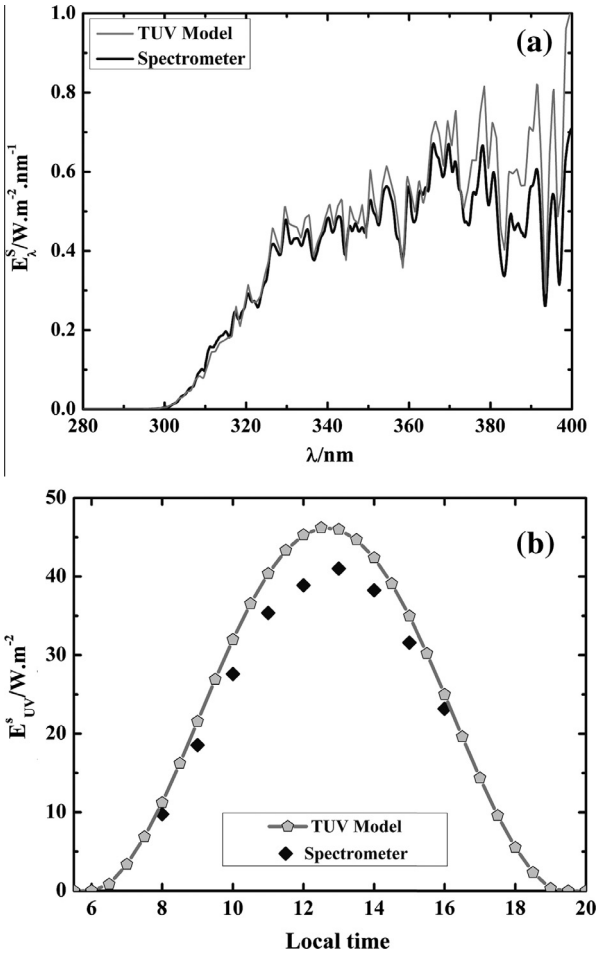

Fig. 2. (a) Solar spectral irradiance recorded with the spectrometer (black curve) and calculated with the TUV model (gray curve) at 13:00 using O_3 data from OMI/KNMI/NASA. (b) Solar UV irradiance integrated from 280 nm to 400 nm, derived from the spectrometer measurements (diamond black) and from the TUV model calculations (gray pentagon) along a clear sky day on October 7th, 2013 in La Plata city.

according to the environment, multiple comparisons were performed between E^S_λ values calculated applying the TUV model and those recorded with the spectrometer. Both sets of spectra showed a good agreement, resulting a mean relative difference of about 14% among them. As an example, in Fig. 2a the E^S_λ registered by the spectrometer in a clear sky day is compared to that calculated with the TUV model under the same environmental conditions.

Further, the solar UV irradiance at Earth's surface (E^S_{UV}) was obtained by integrating E^S_λ from λ_1= 250 nm to λ_2 = 400 nm:

$$E^S_{UV} = \int_{\lambda_1}^{\lambda_2} E^S_\lambda d\lambda \quad (5)$$

The hourly E^S_{UV} values, calculated from E^S_λ values measured at different times during the day using the spectrometer, showed a typical gaussian behavior and matched very well with the estimations obtained by the TUV model (Fig. 2b).

To find out if the solar radiation is enough to photolyze pterin derivatives of biological relevance, the values of E^S_{UV} (Fig. 2), obtained in our experiments under outdoor conditions, were compared to the intensity of a source used in previous studies (Vignoni et al., 2009, 2010). In this way, a Rayonet RPR lamp was employed as an artificial source and its corresponding $q^{0,V}_{n,p}$ values was measured by means of an actinometer (detailed in Section 2.6) (Terazima et al., 1992), for a sample placed in front of the lamp, as described in literature (Vignoni et al., 2009, 2010).

Taking into account that the lamp emits quasi-monochromatic radiation, $q^{0,V}_{n,p}$ values were converted into the UV irradiance of the lamp (E^L_{UV}) with the following equation:

$$E^L_{UV} = q^{0,V}_{n,p} \cdot N_A \cdot h\nu \left(\frac{V}{S}\right) \quad (6)$$

where $N_A h\nu$ is the energy of a mol of photons emitted by the lamp ($\lambda_{em} = 350$ nm) and V and S are, respectively, the volume and the area exposed to irradiation of the cell used. A value of 41 $W \cdot m^{-2}$ was obtained for E^L_{UV}, which is of the same order of magnitude as those determined for E^S_{UV} measured in La Plata city (Fig. 2b). These results provide evidence that the photooxidation of pterins such as Bip and H_2Bip should take place at a significant rate under solar exposure.

In addition, as a control, the Avantes spectrometer was employed to measure E^L_{UV}, using an equation equivalent to Eq. (5) value of 36 W m^{-2} was obtained, which is in good agreement with that calculated using the chemical actinometer. Finally, this comparison confirms the proper operation of the fiber optic spectrometer.

3.2. Oxidation of Bip and H_2Bip upon solar exposure

Aqueous solutions of Bip and H_2Bip were exposed to solar radiation at noon in clear sky days in La Plata city and then they were analyzed using our HPLC device (see Section 2.5). For both compounds a significant decrease in their concentrations was observed within a few minutes of exposure, which indicates that solar radiation is able to cause the degradation of the pterin derivatives. By means of HPLC analysis, carried out using commercial standards and considering published chromatographic data (Vignoni et al., 2009; Vignoni et al., 2010), dimeric products, Bip, Fop and Cap were detected in irradiated solutions of H_2Bip and Fop and Cap were detected in irradiated solutions of Bip.

To further investigate the photochemical reactions, the concentration profiles of reactants and products were determined upon solar exposure of the corresponding solutions. Fig. 3a clearly shows that Bip was converted into Fop and, subsequently, this compound underwent oxidation to Cap. The sum of the concentrations of Bip, Fop and Cap remained constant during the experiments, which indicates that Fop and Cap are the main photoproducts, with no other substances being formed in significant amounts. Fig. 3b, in turn, shows that H_2Bip was converted into Bip and other photoproducts with the same retention times and spectral features as those reported for the dimers of H_2Bip (Vignoni et al., 2010, 2012).

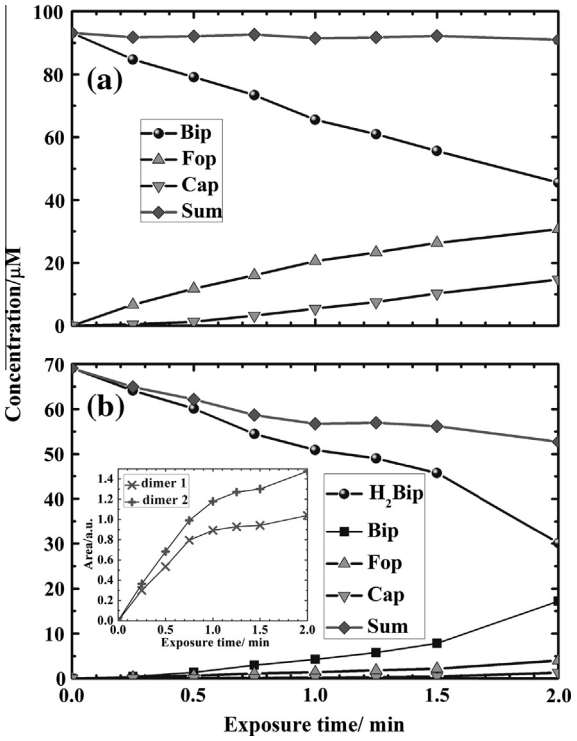

Fig. 3. Time evolution of reactants and photoproducts concentrations in air-equilibrated aqueous solutions irradiated under sunlight in clear sky days in La Plata city. The exposures were carried out within one hour around solar noon for two consecutive days near spring equinox (on September 23rd and 24th 2013, for solutions of Bip and H_2Bip, respectively). Concentrations were determined by HPLC analysis. (a) $[Bip]_0 = 93$ μM; (b) $[H_2Bip]_0 = 70$ μM, inset: time evolution of the areas of chromatographic peaks corresponding to the dimers of H_2Bip.

These results are in good agreement with the pathways depicted in Fig. 1. Therefore in this Section we have presented evidence that indicates that solar exposure of Bip and H_2Bip solutions, under outdoor conditions, leads to the exactly the same photochemical transformations as those reported for photolyses carried out under laboratory conditions using quasi-monochromatic light sources (Vignoni et al., 2009, 2010). In addition, it is worth mentioning that, as expected taking into account the comparison of sources described in the previous section, the photochemical reactions took place very fast, i.e. the sun is able to photoinduce significant chemical changes of pterins in a few seconds.

In diseased skin cells micromolar concentrations of pterins have been determined, which is much higher than concentrations found in healthy cells; e.g., in human keratinocytes and cell cultures from suction blister roofs the concentration of total biopterin was determined to be in the range 4–93 μM (41–950 pmol/mg of protein), depending on the cell type (Schallreuter et al., 2001); concentrations higher than 35 μM (360 pmol/mg of protein)

were determined for Cap (Rokos et al., 2002). In addition, fluorescence studies have determined that the concentration of pterins is not homogeneous inside the cells. Therefore the local concentrations could be even much higher than those noted above. Our experiments were performed with solutions of pterins at concentrations of the same order of magnitude as those reported for in vivo determinations. Consequently, our study strongly suggests that photodegradation of pterins can take place in cells upon solar irradiation.

In tissues affected by vitiligo non-enzymatic oxidation of H_4Bip by O_2 or other oxidants leads to the formation of H_2Bip (Nixon et al., 1980). It is accepted that Bip is formed in vivo from H_2Bip by a non-enzymatic oxidation. However, the specific chemical process that leads to this conversión in human skin under pathological conditions is still unknown. It had been proposed that the formation of Bip results from the oxidation of H_2Bip by H_2O_2 (Moore et al., 2002), but it was later demonstrated that this reaction yields dihydroxanthopterin (H_2Xap) as a product (Dántola et al., 2008). The same main product was obtained for the "autooxidation" of H_2Bip (reaction with O_2 in an air-equilibrated solution) Dántola et al., 2008. Therefore the results presented so far suggest that the photochemical oxidation of H_2Bip upon solar radiation can be the pathway responsible for the formation of Bip in the affected tissues. Likewise, Cap found in the skin (Rokos et al., 2002) very likely is produced by the oxidation of H_2Bip by solar radiation. It is worth mentioning that artificial sources with a significant UV component might also contribute to the oxidation of pterins in vivo. Moreover, if photochemistry of pterins occurs, reactive oxygen species, such as H_2O_2 and $^1O_2$ should be formed and photosensitized reactions and oxidative stress could be exacerbated in vivo, which, in turn, means that photochemistry of pterins could contribute to inactivation of enzymes and unspecific damages in other biomacromolecules.

3.3. Quantum yield determinations

The absorption factor is a function of λ that represents the fraction of the incident radiation absorbed by the sample (Braslavsky, 2007) and is defined as follows:

$$f_\lambda = 1 - T_\lambda = 1 - 10^{-A_\lambda} \tag{7}$$

where T_λ is the solution transmittance when the reflection and scattering are negligible, and A_λ is the absorbance spectrum. Therefore, the absorbed spectral photon irradiance of a sample exposed to sunlight is given by:

$$E^a_{n,p,\lambda} = f_\lambda \cdot \left(\frac{E^S_\lambda}{N_A h\nu}\right) \tag{8}$$

As an example, Fig. 4 shows the functions f_λ and $E^S_{n,p,\lambda}$, as well as their product $E^a_{n,p,\lambda}$, for an experiment carried out with a H_2Bip solution in a clear sky day. The absorbed

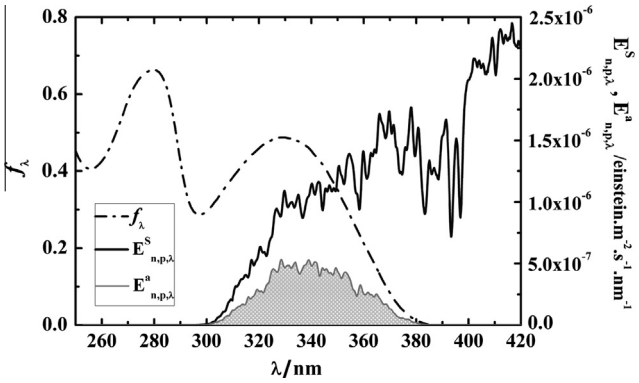

Fig. 4. Solar spectral photon irradiance ($E^S_{n,p,\lambda}$) and, absorption factor (f_λ) and absorbed spectral photon irradiance ($E^a_{n,p,\lambda}$) from a H_2Bip solution (95 μM). Experiment carried out in La Plata for a clear sky day at solar noon (on September 24th, 2013).

photon irradiance of the sample ($E^a_{n,p}$) is obtained by integration of $E^a_{n,p,\lambda}$ (gray area in Fig. 4) and mathematically can be expressed by:

$$E^a_{n,p} = \int_{\lambda_1}^{\lambda_2} E^a_{n,p,\lambda} d\lambda \tag{9}$$

Finally, the absorbed photon flux density ($q^{a,V}_{n,p}$) of a given aqueous solutions during the solar exposure can be calculated from the $E^a_{n,p}$ as follows:

$$q^{a,V}_{n,p} = \frac{S}{V} \cdot E^a_{n,p} \tag{10}$$

where V and S are the volume and the area of the cell containing the sample exposed to the irradiation, respectively.

Therefore combining Eqs. (7)–(10), the complete equation to calculate $q^{a,V}_{n,p}$ is obtained:

$$q^{a,V}_{n,p} = \frac{S}{V} \int_{\lambda_1}^{\lambda_2} \left(1 - 10^{-A_\lambda}\right) \frac{E^S_\lambda}{N_A h\nu} d\lambda \tag{11}$$

The quantum yields of reactant disappearance (Φ_R) can be calculated as follows:

$$\Phi_R = -\frac{(d[R]/dt)_0}{q^{a,V}_{n,p}} \tag{12}$$

where $(d[R]/dt)_0$ is the initial rate of reactant consumption. For determining the initial rates, the experiments were carried out during periods of time within which the change of $q^{a,V}_{n,p}$ was lower than 20 %. In our experiments, this condition was fulfilled at exposure times lower than 60 s. The initial rates were obtained from the slope of the corresponding plots of concentration vs. irradiation time within such time windows, where the time evolution of the concentrations of reactants and products followed a zero-order rate. Φ_R values of 0.05 ± 0.01 and 0.037 ± 0.004 were obtained for H_2Bip and Bip, respectively. These values are equal, within experimental error, to those reported in literature, calculated using a lamp (Vignoni et al., 2009, 2010).

3.4. The effect of several solar exposure conditions

The intensity of the solar radiation depends on many factors, such as the time of the day, the date and the presence of clouds. Therefore, to investigate the effect of the mentioned factors on the photodegradation of pterin derivatives, aqueous solutions of Bip and H_2Bip were exposed to solar radiation under different conditions.

In a first study, in order to characterize the consumption rate as a function of local time, a set of samples were exposed for different times (10, 20, 40 and 60 s) to sun's rays for a clear sky day. This process was repeated every hour for a part of the day, centered at solar noon. For each time, $(d[R]/dt)_0$ was obtained from the HPLC analysis and $q^{a,V}_{n,p}$ was calculated combining the E^S_λ value registered with the spectrometer and the corresponding absorption spectrum of the sample (as shown in Fig. 4), as explained in Section 3.3. $(d[R]/dt)_0$ was proportional to $q^{a,V}_{n,p}$ with a maximum at noon (Fig. 5a). For each photolysis the corresponding Φ_R value was calculated and no variation of this parameter was registered during the day (Fig. 5b). The mean quantum yield along the day was $\Phi_R = 0.037 \pm 0.003$ consistent with the values obtained in Section 3.3. Moreover, the mean hourly value of temperature was increased towards the afternoon from 24.8 °C (at 11:00) to 29.5 °C (at 17:00). It is worth mentioning that, although there was a variation of temperature during the day, Φ_R did not change significantly.

On the other hand, the current investigation involved sampling and analyzing for four months to evaluate monthly changes in the consumption rates. The relative concentration of Bip ([Bip]/[Bip]$_0$) as a function of irradiation time is plotted in Fig. 6 for different experiments

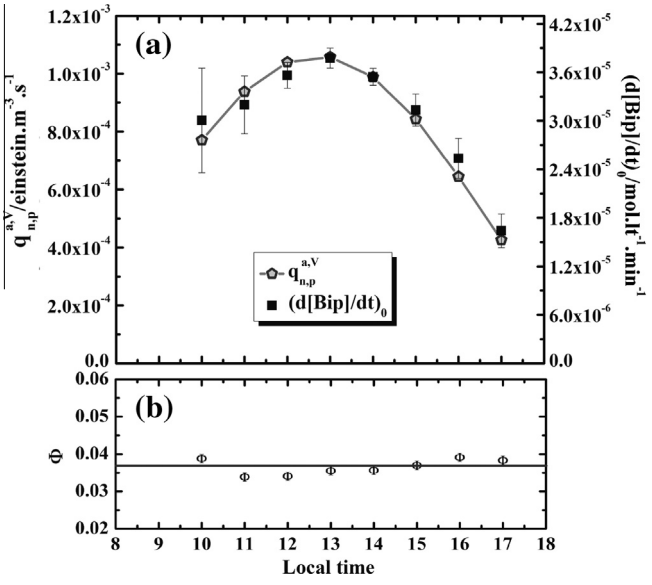

Fig. 5. (a) The incident photon flux density (black square) during a clear sky day (on December 4th,2013) and the corresponding initial rate consumption of Bip (gray pentagon). (b) Quantum yield (Φ) as a function of the local time.

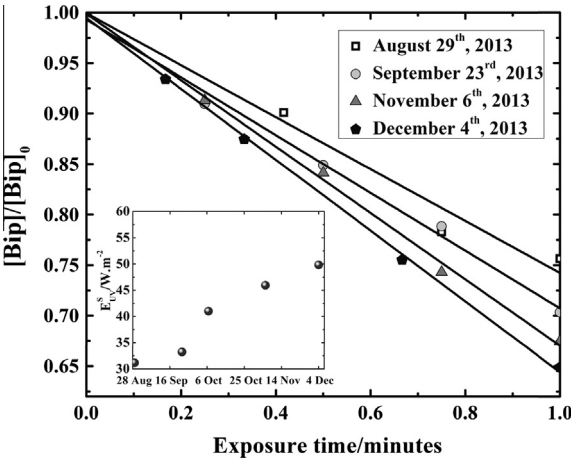

Fig. 6. Monthly variation of the initial rates of Bip consumption at solar noon for clear sky days. Inset: E^S_{UV} as function of date in year 2013.

carried out in clear sky days at noon. As expected taking into account the increase in the values of E^S_{UV}, the $(d[Bip]/dt)_0$ increased from August to December.

Up to now, all the results presented correspond to experiments carried out in cloud-free weather conditions. Clouds are one the most important parameters to influence the solar radiation. For the purpose of assessing the energy required to trigger the studied reactions, a new set of experiments, similar to those described for clear sky conditions, were performed in cloudy days. Nevertheless, the variability in form and height of cloud is very large and can hardly be determined. A cloud coverage is a challenge to perform an exposure of serial samples. It would be very difficult to proceed ensuring a relatively constant intensity (as interposing a 'uniform filter') in a long time.

Therefore, the exposures were carried out in short times at noon in a cloudy day, in which the cloud coverage was a homogeneous layer distributed over La Plata city and its surroundings, about 100 km away (minimizing atmospheric fluctuations). Measurements of $E^S_{n,p,\lambda}$ were performed on November 11th, 2013 as detailed in Fig. 7. Additionally, we added for comparative purposes a measure recorded in a clear sky day (on November 6th, 2013). The values of E^S_{UV} for the compared cloudy and clear sky days were 24.22 and 45.94 W m^{-2} respectively. It is clear that the solar energy of the cloudy day is significantly lower than that corresponding to the clear sky day, but it is not negligible. In fact, E^S_{UV} was enough to cause the oxidation of half of the initial Bip in 2 min (inset Fig. 7), which means that even in cloudy weather conditions the photolysis of pterins was significant.

To consider the feasibility of these reactions in the skin, the solar radiation reaching the cells at different depths and the localization of pterins should be taken into account. Earlier studies have identified an accumulation of H_2O_2 and pterins oxidation products at the epidermis level, in patients affected by vitiligo (Schallreuter et al., 2001; Rokos et al., 2002). The evidence that significant quantities of UV light transverse the stratum corneum and reaching

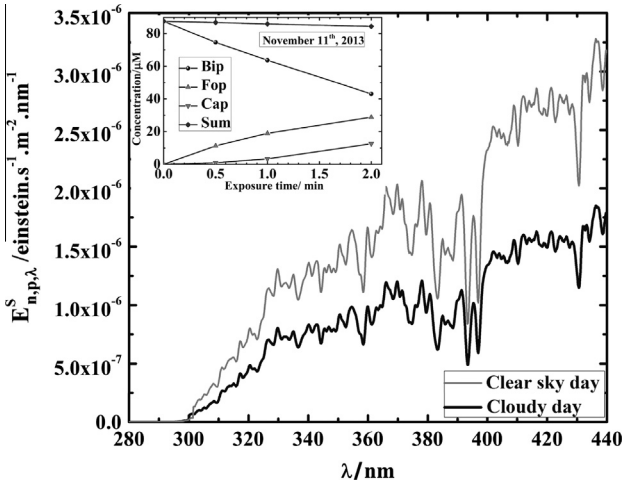

Fig. 7. Solar exposure of Bip in a cloudy day (November 11th, 2013) and clear sky day (November 6th, 2013). Inset: time evolution of reactants and photoproducts concentrations in Bip aqueous solutions irradiated for a cloudy sky day in La Plata city.

the epidermis have been provided by in vivo experiments (Philp and Allcock, 1989; Everett et al., 1966; Diffey, 1980). Moreover, is well known that in the epidermis, the major absorber of radiation is the melanin (Anderson and Parrish, 1981) and if absent, the protection against UV radiation fails. Therefore, this rough analysis suggests that pterin photochemistry can take place in the affected tissues upon solar exposure.

4. Conclusions

In the present work, we have investigated the photochemistry of dihydrobiopterin (H_2Bip) and biopterin (Bip) in aqueous solution upon solar irradiance and outdoor conditions. Sun exposure of H_2Bip leads to the formation of dimers and to its oxidation to Bip, which, in turn, is photooxidized into 6-formylpterin (Fop). Further excitation induces the oxidation of Fop to 6-carboxypterin, which is much more photostable than Bip and Fop and then it is accumulated in the solution. This reaction scheme is in agreement with that previously proposed for the photolysis of H_2Bip and Bip carried out using artificial UV radiation sources (Vignoni et al., 2009, 2010). The quantum yields of reactant disappearance (Φ_R) were determined to be 0.05 ± 0.01 and 0.037 ± 0.004 for H_2Bip and Bip, respectively. The reactions were carried out under different conditions and the results reveal that the intensity of the sun is enough to efficiently oxidize H_2Bip into Bip and Bip into its oxidized derivatives within a few minutes. In particular, we have demonstrated that these photochemical reactions take place when the solar irradiance is small in comparison to that corresponding to the noon in clear sky day, e.g. during the afternoon and in cloudy days.

Taking into account the solar radiation reaching the skin cells at different depths and the localization of pterins, the results presented in this work suggest that sunlight might be responsible for the generation of oxidized pterins, which are the photochemically active derivatives, under outdoor conditions. In particular, the energy of the sun under different environmental conditions is enough to oxidize the biologically occurring H_2Bip to Bip, which is photochemically active and, in turn, generates other oxidized derivatives and reactive oxygen species.

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