Chemical Physics Letters 484 (2010) 330–332

Contents lists available at ScienceDirect

Chemical Physics Letters

journal homepage: www.elsevier.com/locate/cplett

^{1}H NMR characterization of the intermediate formed upon UV-A excitation of biopterin, neopterin and 6-hydroxymethylpterin in O_2-free aqueous solutions

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ARTICLE INFO

Article history:
Received 27 October 2009
In final form 2 December 2009
Available online 4 December 2009

ABSTRACT

Pterins belong to a family of heterocyclic compounds present in a wide range of living systems and participate in relevant biological functions. Under anaerobic conditions, the unstable red intermediate generated by UV-A irradiation of biopterin, neopterin and 6-hydroxymethylpterin was identified by ^{1}H NMR analysis, in alkaline D_2O solutions, as 5,8-dihydro-6-formylpterin.

1. Introduction

Biopterin (Bip), neopterin (Nep) and their 7,8-dihydro and 5,6,7,8-tetrahydro reduced derivatives are the most important unconjugated pterins in living systems. 5,6,7,8-Tetrahydrobiopterin (H_4Bip) is present in the skin of human beings and acts as a coenzyme in hydroxylation reactions of the metabolism of some amino acids [1] and is also relevant in nitric oxide metabolism [2]. Nep, a metabolite in the biosynthesis of H_4Bip, is synthesized mainly in activated macrophages [3] and the determination of its concentration in body fluids has proven its clinical value [4].

In the tissues affected by vitiligo, a chronic depigmentation disorder, 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 the lack of melanin. Patients suffering this disease express a characteristic fluorescence in their white skin patches upon Wood's light examination (320–400 nm with a peak at 365 nm) caused by the accumulation of oxidized unconjugated pterins [5]. Therefore, the photochemistry of pterins is of particular interest to this disease. Moreover, 6-carboxypterin (Cap), a product of photolysis of Bip (*vide infra*) that is not synthesized in the skin cells, has been isolated from the affected skin [6], thus proving that photooxidation of pterins occurs *in vivo* under pathological conditions.

It is known since many years ago that Bip is photosensitive and its photolysis under aerobic conditions leads to the formation of Cap [7,8]. In the late seventies, the photochemistry of Bip and Nep in alkaline aqueous solution (phosphate buffer, pH 10) was studied [9,10] and it was demonstrated that under anaerobic conditions a 'red intermediate' (RI) is formed (Scheme 1). This compound reacts very fast with O_2 to yield 6-formylpterin (Fop). In addition, it was demonstrated that photolysis of Bip and Nep in air-equilibrated solutions leads to Fop as the first product, which is transformed into Cap on further photooxidation. These reactions release H_2O_2 as product and could be an additional source for generation of reactive oxygen species in vitiligo [6].

RI is a compound with a longwave absorbing band of low molar absorption coefficient centered at 480 nm. This compound would be an intermediate in the photooxidation of Bip in air-equilibrated solutions. It has been recently suggested that the reaction of RI with dissolved O_2 produce superoxide anion ($O_2^{\cdot-}$) [11] (Scheme 1), which is very important from a biomedical point of view because this radical is implicated in the etiology of many pathological conditions. Moreover, the photoinduced generation of H_2O_2 by Bip could be *via* the reaction of RI with O_2. In recent works, it was proposed that RI is also generated during photolysis of 6-hydroxymethylpterin (Hmp) in the absence of O_2 [12,13] (Scheme 1).

Taking into account the comparison of its spectral features and reactivity with those of 1,4-dihydropirazines [9], RI was proposed to be 5,8-dihydro-6-formylpterin. However, despite the biological interest of photochemistry of Bip, to the best of our knowledge, this compound has never been identified. Technical reasons are probably responsible for this fact. In first place, due to the low solubility of Bip, in both polar and nonpolar solvents, no high RI concentrations can be reached. Besides RI can only be obtained, manipulated and analyzed under anaerobic conditions. Finally, even in the absence of O_2 and light, RI is not stable: this compound undergoes a slow thermal reaction, its lifetime being of about only a few hours [11].

In this work alkaline D_2O solutions of Bip, Nep and Hmp exposed to UV-A radiation were analyzed by means of Proton Nuclear

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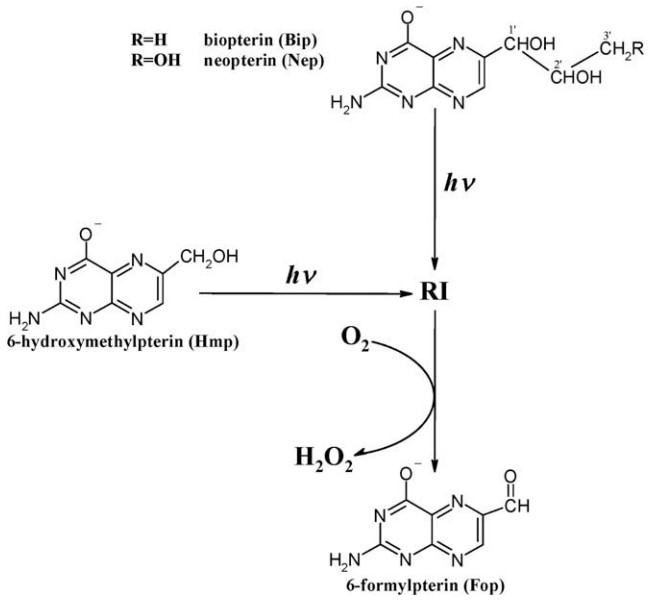
doi:10.1016/j.cplett.2009.12.008



Scheme 1. Photochemistry of Bip, Nep and Hmp in aqueous solution.

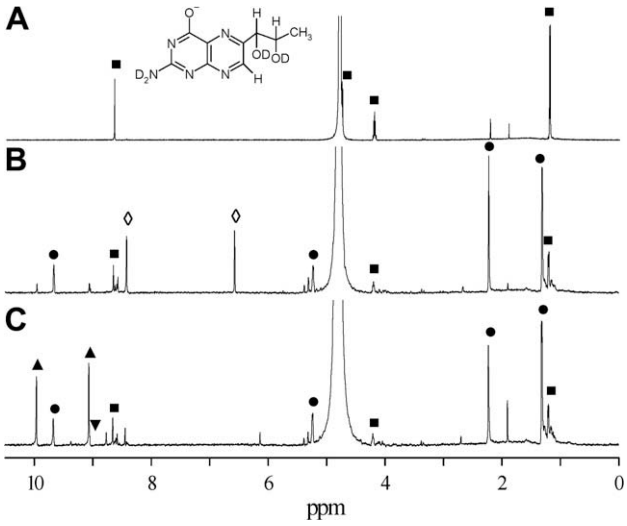


Fig. 1. 500-MHz ^{1}H NMR spectra of alkaline D_2O solutions of Bip (2 mM, pD = 10.3), recorded at room temperature. (A) Non-irradiated air-equilibrated solution (2 mM), (B) Ar-saturated solution irradiated for 20 min, (C) Ar-saturated solution irradiated for 20 min and then bubbled with air for 2 min. (■) Bip, (◇) RI, (●) acetaldehyde and its aldol, (▲) Fop, (▼) Cap.

Magnetic Resonance (^{1}H NMR) spectroscopy. The results presented herein provide, for the first time, direct evidence of the chemical nature of the RI.

2. Experimental

Pterins were purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. D_2O (Sigma, minimum isotopic purity of 99.9%) was used as solvent and solutions of DCl (Aldrich, 99.5% D) and NaOD (CEA) in D_2O were employed for adjusting the pH (pD) value to 10.

Steady-state irradiation of D_2O solutions was carried out in NMR tubes at room temperature, using 8W Rayonet RPR lamps emitting at 350 nm (Southern N.E. Ultraviolet Co.). 500-MHz ^{1}H NMR spectra were recorded on a Bruker AM-500 spectrometer. Chemical shifts in D_2O are referenced to tetramethylsilane.

3. Results and discussion

The ^{1}H NMR spectrum of the non-irradiated Bip solution shows three signals at low δ values with relative intensities of 1:1:3 (δ = 4.75, 4.19, 1.19 ppm) corresponding to aliphatic protons and one signal at high δ value (δ = 8.65 ppm) corresponding to the chemically equivalent aromatic protons (Fig. 1A). H_2O broad signal at ~4.8 ppm is observed.

After *ca.* 20 min of UV-A irradiation under anaerobic conditions, the solution became deep red, indicating the formation of RI, and several new signals appeared in the ^{1}H NMR spectrum (Fig. 1B), apart from those corresponding to the residual Bip (■). As soon as air was let into the NMR tube, the red color vanished, revealing that, as expected, RI was consumed in a fast thermal reaction in the dark. The corresponding ^{1}H NMR spectrum (Fig. 1C) shows that, as a consequence of this latter process, two signals (◇) disappeared (δ = 8.42 and 6.58 ppm), while three new signals appeared: two major ones (δ = 9.95, 9.05) and another very small (δ = 9.03 ppm).

These results suggest that a cleavage of the Bip molecule takes place upon irradiation under anaerobic conditions and part of the photoproducts undergoes further oxidation upon admission of air in the NMR tube. Assuming that during the thermal reaction RI is oxidized by O_2, the signals that disappeared (◇) as a consequence of this process must correspond to RI. On the other hand, the signals that appeared in the photolysis and did not disappear with the admission of air must correspond to phoproducts stable in the presence of O_2. Finally, the signals that appeared as a consequence of the thermal reaction must correspond to products of oxidation of RI.

The ^{1}H NMR spectra obtained from standard alkaline D_2O solutions of Fop and Cap are shown in Fig. 2A and B, respectively. Comparison of these spectra with that obtained after admission of air to the irradiated Bip solution (Fig. 1C) indicates that the signals that appeared after the oxidation of RI belong to Fop (δ = 9.95 and 9.05 ppm) and to Cap (δ = 9.03 ppm). These results are in agreement with those previously reported in the literature [9–11] (Scheme 1).

If the cleavage of the Bip molecule takes places in the C1′–C2′ bond the stable product should be a compound with two carbon atoms. ^{1}H NMR spectrum of a standard solution of acetaldehyde

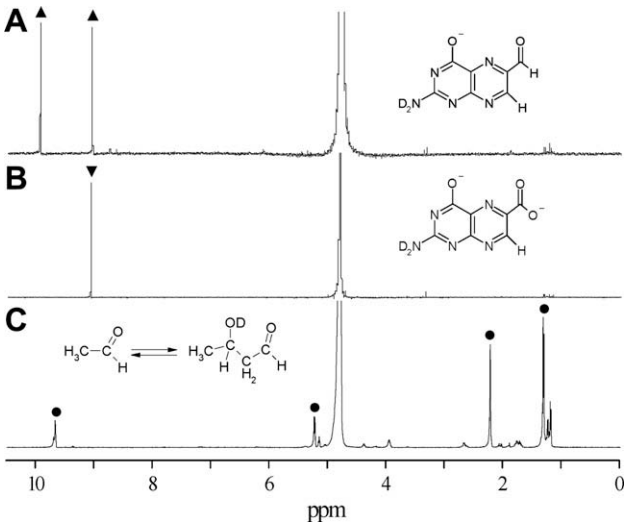


Fig. 2. 500-MHz ^{1}H NMR spectra of standard alkaline D_2O solutions (pD = 10.3) recorded at room temperature: (A) Fop (▲) (2 mM), (B) Cap (▼) (2 mM) and (C) acetaldehyde (●) (1 mM), which in part is as its corresponding aldol.

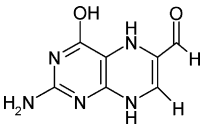


Fig. 3. Chemical structure of 5,8-dihydro-6-formylpterin (RI).

is shown in Fig. 2C. The main signals observed correspond to the acetaldehyde molecule itself (δ = 9.66 and 2.22 ppm) and to the product of its aldol addition, 3-hydroxybutanal (δ = 5.23 and 1.32 ppm). These signals are present in the spectra of irradiated Bip solutions before and after admission of air. Therefore these results confirmed the photochemical cleavage of Bip and the formation acetaldehyde as a product.

Results presented so far indicate that RI has the pterin moiety and a substituent with only one carbon atom. The values of the signals assigned to RI suggest that no aromatic protons are detected. Taking into account that the loss of aromaticity of the ring causes a shift of the aromatic H signal to lower δ values, the signal δ = 6.58 ppm may be attributed to the presence of olefinic proton. This fact in turn suggests that one of the rings of the pterin moiety is reduced. Therefore, considering the absence of O_2, the substituent should be oxidized. This is in agreement with the value of the signal δ = 8.42 ppm, which may be assigned to a proton of a carbon aldehyde that show the extension of conjugation with an olefin. Therefore, the previous analysis correlate very well with the chemical structure of 5,8-dihydro-6-formylpterin (RI) (Fig. 3), previously proposed by Pfleiderer and co-workers [9]. In addition, the mechanism hypothesized for the photodegradation of Bip in [9] is also supported by the results obtained in the present study.

To find out what the chemical nature of the RI produced under anaerobic irradiation of Nep and Hmp are, a similar set of experiments was carried out with these two pterin derivatives, under the same experimental conditions as in the case of Bip. The ^{1}H NMR spectra obtained after irradiation in the absence of O_2 are shown in Fig. 4, together with that corresponding to Bip. The spectra of the irradiated solutions were also registered after admission of air (Supplementary material). For the three compounds the values of the signals corresponding to RI (◇) were equal and disappeared when O_2 was added. These results strongly suggest that for the three pterin derivatives the same RI is formed upon UV-A irradiation in the absence of O_2, which is in good agreement with the hypothesis suggested by our group in recent works [11,12].

For Nep the signals of the product corresponding to the substituent (containing the atoms C2′ and C3′), as expected, did not change upon admission of air (Supplementary material) and were different from those corresponding to acetaldehyde and its aldol, the product formed from Bip (Fig. 4A). This is logical taking into account that Bip and Nep differ in the substituent. Accordingly, no signal corresponding to these products were registered for Hmp (Fig. 4C).

In addition, in the spectra registered after admission of O_2 the signals corresponding to Fop and Cap were also observed for Nep and Hmp (Supplementary material). Therefore, these results confirmed that Fop and Cap are the main final products of RI oxidation.

Finally, the values obtained for the signals assigned to RI were compared to those expected for different possible compounds.

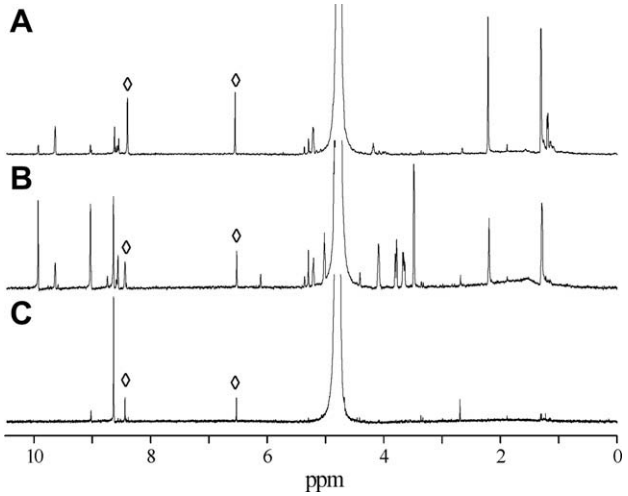


Fig. 4. 500-MHz ^{1}H NMR spectra, recorded in alkaline D_2O solutions (pD = 10.3) at room temperature of: (A) 20 min irradiated Ar-saturated Bip solution (2 mM), (B) 20 min irradiated Ar-saturated Nep solution (2 mM), and (C) 60 min irradiated Ar-saturated Hmp solution (2 mM). (◇) RI.

However, the results presented in this study are only compatible with the chemical structure shown in Fig. 3: 5,8-dihydro-6-formylpterin.

Acknowledgements

This work was partially supported by CONICET (PIP 5443 and 6301/05), ANPCyT (PICT 06-615, 01482/06 and 33919) and UBA (X072). M.V. thanks CONICET for doctoral research fellowships. M.L.S., R.E.B., A.H.T. and F.M.C. are research members of CONICET.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cplett.2009.12.008.

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