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Mini-Review

OXIDATION OF BIOMOLECULES PHOTOSENSITIZED BY PTERIN DERIVATIVES

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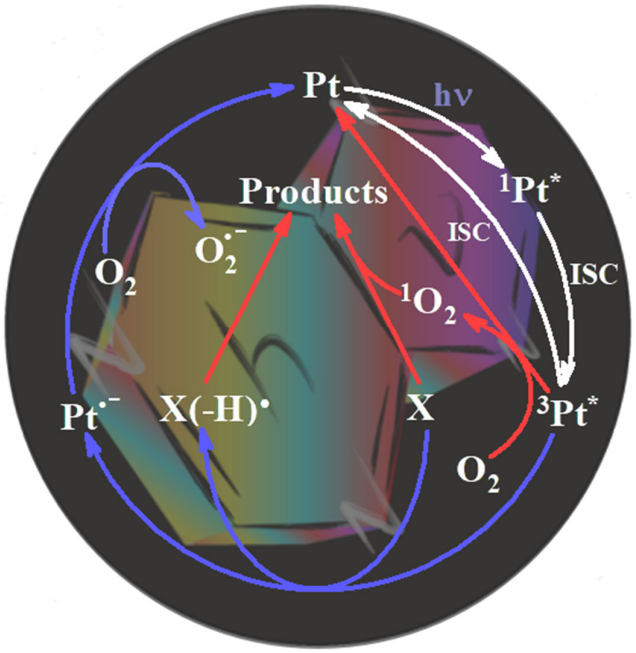
Graphical abstract



Depending on the target molecule (X), pterin-photosensitized oxidations may be purely *type I* (electron transfer) or *type II* (singlet oxygen, $^1O_2$), or a combination of both.

Resumen

La importancia biológica y médica de las reacciones fotosensibilizadas se relaciona principalmente con su participación en los procesos involucrados en el desarrollo de cáncer de piel y de terapias fotodinámicas para el tratamiento del cáncer e infecciones. Las pterinas, son una familia de compuestos heterocíclicos derivados de la 2-amino-4-pterinidinona, que se encuentran ampliamente distribuidas en los sistemas vivos

participando de importantes funciones biológicas. En condiciones patológicas, como es el caso del vitiligo, pterinas oxidadas se acumulan en las manchas blancas de la piel de los pacientes que sufren este desorden de la pigmentación. Estas moléculas son fotoquímicamente activas, bajo radiación UV-A (320 – 400 nm), fluorescen, producen radicales orgánicos y especies reactivas de oxígeno, y también sufren reacciones de fotooxidación. Nuestro grupo de investigación, ha estudiado desde hace más de 20 años, la degradación fotosensibilizada por pterinas de biomoléculas tales como ADN, proteínas, lípidos y sus componentes. Los resultados experimentales incluyen análisis cinético, evaluación de la interacción entre los estados excitados de las pterinas con diferentes sustratos, detección de especies radicalarias, y la identificación de productos bajo diferentes condiciones experimentales. Dependiendo de la molécula blanco, las pterinas pueden fotosensibilizar la oxidación de las mismas por mecanismo *tipo I* (transferencia de electrones) o *tipo II* (oxígeno singlete, $^1O_2$), o una combinación de ambos. En este artículo, presentamos un resumen de los cambios químicos fotosensibilizados por pterinas bajo irradiación UV-A en diferentes biomoléculas y los mecanismos involucrados.

Abstract

The biological and medical importance of photosensitized reactions is mainly related to their participation in processes involved in the development of skin cancer and in photodynamic treatments against cancer and infections. Pterins, a family of heterocyclic compounds derived from 2-aminopteridin-4(1H)-one, are widespread in living systems and participate in important biological functions. In pathological conditions, such as vitiligo, oxidized pterins accumulate in the white skin patches of patients suffering this depigmentation disorder. These molecules are photochemically active and, under UV-A excitation (320–400 nm), they can fluoresce, produce organic radicals and reactive oxygen species and undergo photooxidation. Our research group has investigated for more than 20 years the degradation of biomolecules such as DNA, proteins, lipids, and their components, photosensitized by pterins under UV-A irradiation. The experimental results include kinetics analysis, evaluation of interaction between pterins excited states with different substrates, detection of radical species, and identification of products, under different experimental conditions. Depending on the target molecule, pterin-photosensitized oxidations may be purely *type I* (electron transfer) or *type II* (singlet oxygen, $^1O_2$), or a combination of both. In this article, we present a summary of the chemical changes photoinduced by pterins upon UV-A irradiation in different biomolecules and the mechanisms involved.

Palabras Clave: *pterinas, fotosensibilización, ADN, proteínas, lípidos*

Keywords: *pterins, photosensitization, DNA, proteins, lipids*

1. Introduction

Pterins and their derivatives belong to a family of heterocyclic compounds which were uncovered over a century ago, with the isolation of its first members from the pigment of the wings of a butterfly (Pteridae) [1, 2]. Nowadays, it is known that these compounds are present in different biological systems and play various roles. Besides acting as pigments, pterins participate in other relevant biological functions which includes being the light- harvesting antenna of DNA photolyases, enzymes involved in DNA repair processes after UV irradiation [3-5], as well as behaving as coenzymes [6, 7] and enzymes inhibitors [8].

These compounds, structurally related to 2-aminopteridin-4(1*H*)-one or pterin (Ptr), can exist in living systems in different redox states and may be classified into three classes according to this property: fully oxidized (or aromatic) pterins, and dihydro and tetrahydro derivatives (Figure 1). Finally, they behave as weak acids in aqueous solution. The dominant equilibrium at pH > 5 involves the lactam group (pyrimidine ring) (Figure 1). The pK_a of this equilibrium is *ca.* 8 for the aromatic pterins and *ca.* 10 for dihydropterin derivatives. Other functional groups of the pterin moiety (*e.g.*, the 2-amino group or ring N-atoms) have pK_a values < 2 [9].

The most common pterin derivatives are 6-substituted compounds (Table 1). According to the molecular weight and the functional groups of these substituents, pterins can be divided into two groups: (i) unconjugated pterins, containing substituents with one carbon atom or a short hydrocarbon chain, and (ii) conjugated pterins, with larger substituents containing a *p*-aminobenzoic acid (PABA) moiety. In Table 1 the molecular structures of the most common substituents are shown together with the names of the corresponding oxidized pterin derivatives. Analogous derivatives can be found for dihydro and tetrahydro pterins.

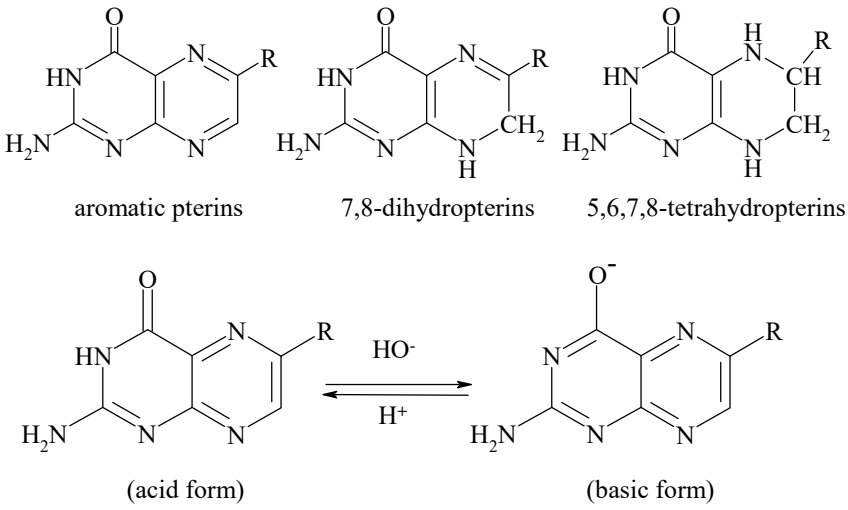
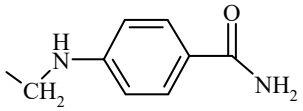
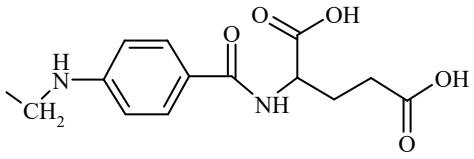


Figure 1. Molecular structures of pterins and the acid–base equilibrium in aqueous solution.

Table 1. Molecular structures of the substituents of the most common aromatic pterin derivatives.

R	Compound
Unconjugated pterins	
-H	pterin (Ptr)
-CH_3	6-methylpterin (Mep)
-CH_3	6,7-dimethylpterin (Dmp)
-CHO	6-formylpterin (Fop)
-COOH	6-carboxypterin (Cap)
-$(CHOH)_2$-CH_3	biopterin (Bip)
-$(CHOH)_2$-CH_2OH	neopterin (Nep)
Conjugated pterins	
-CH_2-PABA	pteroic acid (Pte)
-CH_2-PABAGlu	folic acid (PteGlu)


-CH_2-PABA


-CH_2-PABA-Glu

Pterins are present in human epidermis given that 5,6,7,8-tetrahydrobiopterin (H_4Bip) is an essential cofactor for aromatic amino acid hydroxylases [10] and participates in the regulation of melanin biosynthesis [11]. In vitiligo, a skin disorder characterized by a defective protection against UV radiation due to the acquired loss of constitutional pigmentation [12], H_4Bip metabolism is altered [13, 14]. Dihydro and oxidized pterin derivatives, such as biopterin (Bip) and 6-carboxypterin

(Cap), accumulate in the affected tissues at concentrations significantly higher than those reported for healthy cells [15].

The photochemistry and photophysics of pterins is relevant to understand the harmful effects of radiation on skin, particularly in pigmentation disorders, where these compounds are present in higher concentrations. Pterins are photochemically reactive in aqueous solutions and, under UV-A excitation (320 – 400 nm), they can fluoresce, undergo photooxidation to produce different products, and generate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\bullet-}$) [13, 16, 17]. Different studies have demonstrated or suggested that pterins are involved in photochemical processes that take place in biological systems. Just to mention some relevant examples: (i) excited states of oxidized pterins are photogenerated in the skin of patients suffering from vitiligo [6,11], (ii) the photodegradation *in vivo* of folic acid has been demonstrated[18], (iii) folic acid derivative 5,10-methenyltetrahydrofolate is the light-harvesting antenna in DNA photolyases [3], enzymes involved in DNA repair after UV irradiation.

Most of the solar UV energy incidence on Earth's surface corresponds to UV-A radiation, which can induce damage mostly through photosensitized reactions [19]. A photosensitized reaction is defined as a photochemical alteration occurring in one molecular entity as a result of the initial absorption of radiation by another molecular entity called photosensitizer [20]. These processes may be mediated by endogenous or exogenous photosensitizers and can take place through different mechanisms: the generation of radicals, *e.g.*, *via* electron transfer or hydrogen abstraction (*type I* mechanism), and the production of $^1O_2$ (*type II* mechanism) [21].

In 1997 Ito and Kawanishi demonstrated for the first time that upon excitation with UV-A radiation pterins are able to photoinduce DNA damage [22]. More recently, the mechanism involved in the photosensitization of biomolecules by pterins were investigated in a series of studies carried out with free nucleotides [23, 24] and amino acids [25, 26]. It was shown that pterins can act as photosensitizers through both *type I* and *type II* mechanisms and that the predominant one depends on a combination of many factors, such as quantum yields of $^1O_2$ production by the

photosensitizer, reactivity of the substrate towards $^1O_2$, target molecule redox potential and presence of selective scavengers in the media.

Considering the studies mentioned in the previous paragraph, after UV-A excitation of a given pterin derivatives (Pt) and formation of its triplet excited state ($^3Pt^*$, Reaction 1), two competitive mechanisms can be summarized to explain the photooxidation of different biological substrates (S) (Reactions 1-7). *Type I* mechanism is initiated by an electron transfer from S to $^3Pt^*$, yielding the corresponding pair of radical ions (pterin radical anion ($Pt^{\bullet-}$) and radical cation of the biological substrate ($S^{\bullet+}$), Reaction 2). $Pt^{\bullet-}$ reacts by ground state oxygen (O_2) to produce $O_2^{\bullet-}$ and regenerate Pt (Reaction 3). The spontaneous disproportionation of $O_2^{\bullet-}$ in aqueous solution leads to the formation of hydrogen peroxide (H_2O_2) (Reaction 4). The radical $S^{\bullet+}$ (or its deprotonated form, $S(-H)^{\bullet}$) may react with O_2, H_2O or $O_2^{\bullet-}$ to yield oxidized products (Reaction 5) or with other organic radicals ($R^{\bullet}$) (Reaction 6). On the other hand, in the *type II* mechanism the process starts with an energy transfer from $^3Pt^*$ to O_2 leading to the production of singlet molecular oxygen ($O_2(^1\Delta_g)$, denoted as $^1O_2$) (Reaction 7), one of the main activated species responsible for the damaging effects of light on biological systems (photodynamic effects) [27]. In this case the oxidation of S occurs by reaction with $^1O_2$ (reaction 8).

$$Pt \longrightarrow {}^1Pt^* \longrightarrow {}^3Pt^* \qquad (1)$$

Type I mechanism

$$^3Pt^* + S \longrightarrow Pt^{\bullet-} + S^{\bullet+} \qquad (2)$$

$$Pt^{\bullet-} + O_2 \longrightarrow Pt + O_2^{\bullet-} \qquad (3)$$

$$2\ H^+ + 2\ O_2^{\bullet-} \longrightarrow H_2O_2 + O_2 \qquad (4)$$

$$S^{\bullet+}/S(-H)^{\bullet} \xrightarrow{O_2/H_2O/O_2^{\bullet-}} S(ox) \qquad (5)$$

$$S^{\bullet+}/S(-H)^{\bullet} + R^{\bullet} \longrightarrow S\text{-}R \qquad (6)$$

Type II mechanism

$$^3Pt^* + {}^3O_2 \longrightarrow Pt + {}^1O_2 \qquad (7)$$

$$S + {}^1O_2 \longrightarrow \longrightarrow S(ox) \qquad (8)$$

In the context of our investigations on the photochemistry and photosensitizing properties of pterins, we present in this article an overview of pterin-photosensitized damage in peptides and proteins, DNA and nucleotides, and lipids. We have focused our attention on the mechanisms involved and on the chemical modifications undergone by the different biomolecules used as substrates. The most relevant results, from a biological point of view, are summarized and discussed in this review.

2. Nucleotides, oligonucleotides and DNA

Photosensitization processes introduces mutagenic lesions on DNA molecules, which leads to carcinogenic processes at cellular level [28, 29]. Pteridines induce the oxidation of DNA and its component after absorption of UV-A radiation, and the mechanisms involved depends on the type of the nucleobase, purine or pyrimidine.

Photosensitized oxidation of purines by Ptr takes place only in aerated aqueous solution. Purines nucleotides, 2′-deoxyadenosine 5′-monophosphate (dAMP) and 2'-deoxyguanosine 5'-monophosphate (dGMP), are both oxidize during UV-A radiation in the presence of Ptr. Since dAMP is not oxidizable by $^1O_2$, its oxidation reveals a pure *type I* mechanism (Reactions 2-5). Indeed, after UV-A absorption by Ptr, dAMP undergoes a one-electron oxidation, yielding the corresponding radical ion ($dAMP^{\bullet+}$), which reacts with O_2 and H_2O, to form the products 8-oxo-7,8-dihydro-2'-deoxyadenosine 5'-monophosphate (8-oxo-dAMP) and a tetracyclic compound (8-P-dAMP) with a –OP(=O)(OH)O– bridge formed between the deoxyribose phosphate substituent and the C-8 of the adenine moiety (Figure 2) [30, 31].

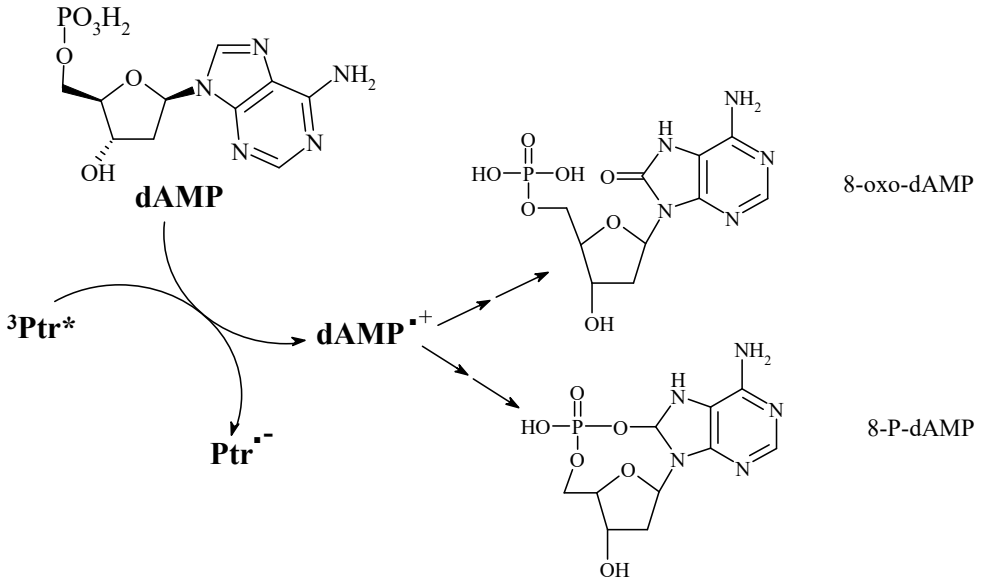


Figure 2. Products of Ptr photosensitized oxidation of dAMP (from Reference 31).

In contrast, the oxidation of dGMP photosensitized by Ptr occurs through two competing mechanisms: (1) electron transfer between dGMP and ^{3}Ptr* (*type I*) (Reactions 2-5) and (2) reaction of dGMP with $^1O_2$ produced by Ptr (*type II*) (Reactions 7-8) [23, 24]. The electron transfer reaction between ^{3}Ptr* and dGMP, yields the corresponding radical pair, and dGMP$^{\bullet+}$ immediately deprotonates to give dGMP(-H$^+$)$^\bullet$. This radical is oxidized by $O_2^{\bullet-}$ and/or O_2 and/or hydration to yield different products that have been identified: the deoxyribonucleoside 5'-monophosphate derivatives of dehydroguanidinohydantoin (dDGhMP), guanidinohydantoin (dGhMP), imidazolone (dIzMP), oxazolone (dZMP) and an adduct consisting of dGMP and dDGhMP (Figure 3) [32]. Several products, have also been described for the reaction between guanine and $^1O_2$ [33]. In the case of the photosensitization of dGMP with Ptr, the deoxyribonucleoside 5'-monophosphate derivative of spiroimidantoine (SpMP) was identified as a product of the oxidation by this ROS.

Thymidine 5'-monophosphate (dTMP), a pyrimidine nucleotide, is also oxidized by UV-A photosensitization with Ptr. In this case only mechanisms initiated by electron transfer are

involved, in contrast to that observed for purine nucleotides. Upon irradiation, consumption of the substrate is observed in the presence and in the absence of O_2 [24, 34]. These processes are relevant from a biological point of view because they demonstrate that thymine, which is frequently assumed as a non-reactive nucleobase, can be damaged under UV-A radiation. Moreover, the photosensitized damages can take place at low O_2 concentration and even under anaerobic conditions, which is important since the intracellular concentration of O_2 in tissues is much lower than that corresponding to air-equilibrated aqueous solutions. Under aerobic conditions, as in the case of purine nucleotides, electron transfer from the substrate to $^3Ptr^*$ leads to the formation of a radical cation ($dTMP^{\bullet+}$), which undergoes two main competitive reactions: deprotonation and hydration (Figure 4). The resulting radicals evolve to at least 4 stable oxidized products: thymidine glycol 5'-monophophate (dTMPGly), 5-hydroxy-5-methylhydantoin 5'-monophosphate (5-OH-5MHMP), 5-formyl-2'-deoxyuridine 5'-monophosphate (5-FordUMP) and 5-(hydroxymethyl)-2'-uridine 5'-monophosphate (5-HmdUMP) (Figure 4). On the other hand, upon irradiation under anaerobic conditions, the formation of an adduct between the pterinic moiety and the nucleotide was observed, due to coupling of the radical $PtrH^{\bullet}$ (C-centered radical on the C-6 or C-7 position of the pterin moiety) and $dTMP(-H)^{\bullet}$ (neutral methylene radical), which yields the product Ptr-dTMP(-2H) when is exposed to air (Figure 4). Similar results were obtained with dCMP, but with lower efficiency.

The formation of the photoadduct Ptr-dTMP(-2H) was also investigated on a short thymine oligomer, 5-mer oligonucleotide (dT_5) [35]. When oxygen-free aqueous solutions containing dT_5 and Ptr were exposed to UV-A radiation, several products, with absorbance in the UV-A region and fluorescence around 450 nm were observed, indicating that, after the electron transfer step, the radicals ions combine to yield an adduct where the pterinic moiety is covalently attached to the oligonucleotide.

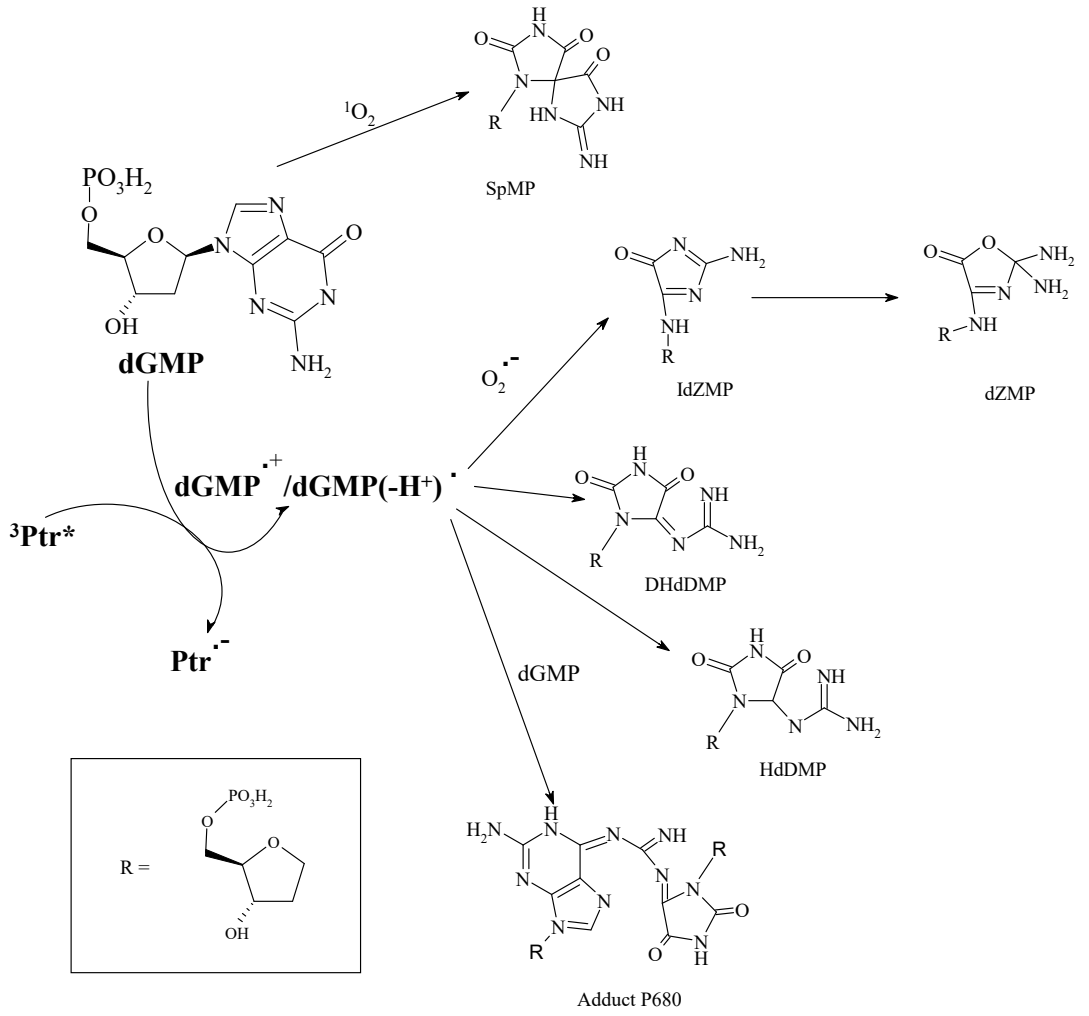


Figure 3. Products of Ptr-photosensitized oxidation of dGMP.

Additionally, at least two isomeric oligonucleotides bearing two molecules of Ptr were also detected, indicating that the covalent binding of the first Ptr moiety does not prevent the addition of a second one due to steric hindrance. On the other hand, UV-A irradiation of air-equilibrated aqueous solutions containing dT_5 and Ptr leads to degradation of the oligonucleotide, but the products do not show absorption in the UV-A region neither fluorescence upon excitation at 350 nm, indicating that in the presence of oxygen binding of the pterin moiety to the oligonucleotide does not take place, in agreement with the behavior observed using dTMP as a substrate.

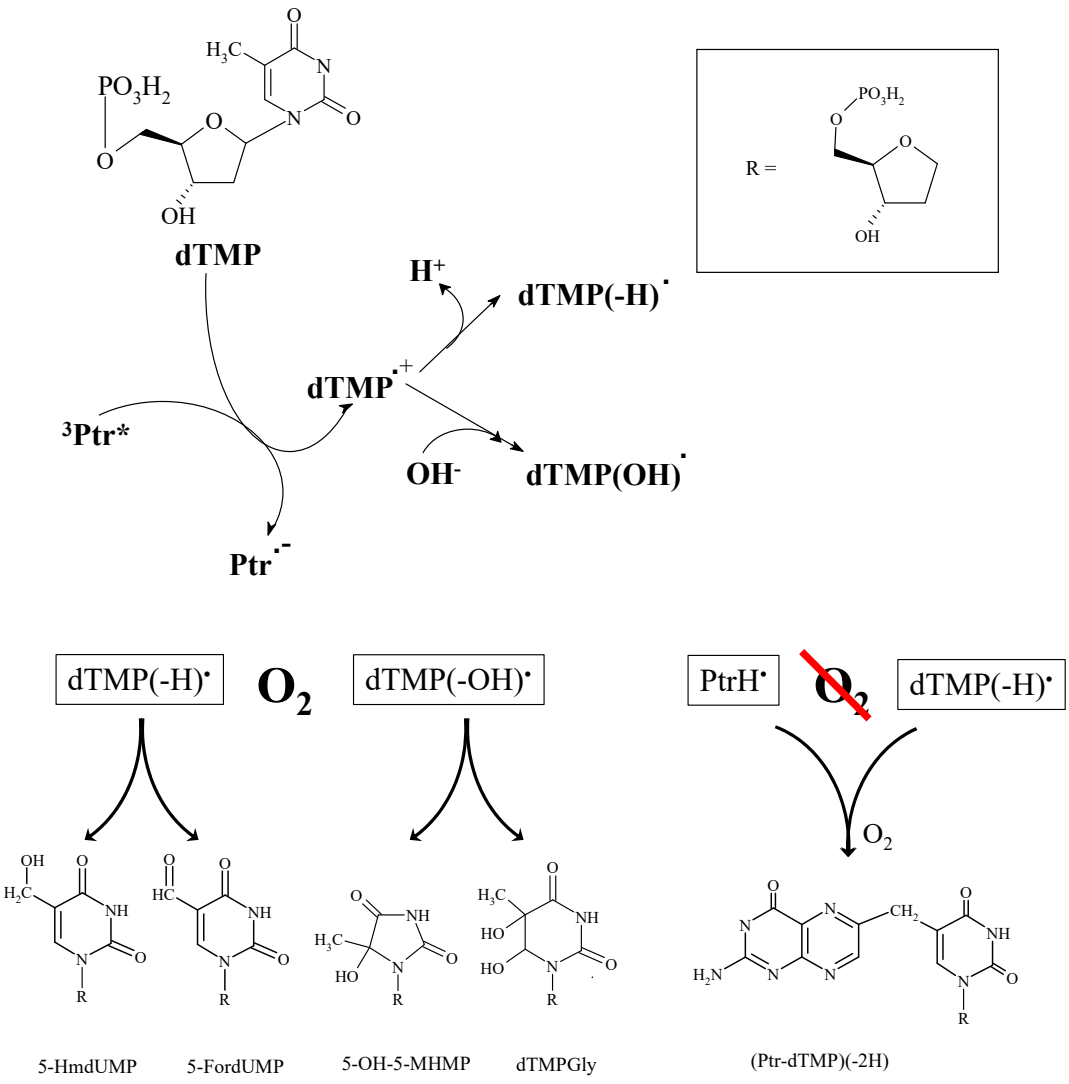


Figure 4. Products and mechanism of the degradation of dTMP photosensitized by Ptr.

The first evidence of Ptr-photosensitized degradation of DNA was in double-stranded chains in 1997 [22], and later on pUC18 plasmid [36]. In both articles it was reported that, upon excitation with UV-A radiation, pterins are able to photoinduce DNA damage. Taking into account indirect evidence, the mechanism involved in this process was proposed to be an electron transfer with the subsequent formation of the guanine radical cation and a pterin radical anion. On the contrary, an article reporting Ptr-photosensitized damage on PBR 322 plasmid [37], indicates that the main mechanism responsible for the photoinduced cleavage of plasmid DNA was a *type II* photosensitized oxidation.

Recently, we have reported damage on double stranded calf thymus DNA in UV-A irradiated aqueous solutions containing Ptr. After control experiments discarding any interaction between Ptr and DNA, the incorporation of Ptr to DNA molecules was evaluated [35]. Upon irradiation in anaerobic conditions, the treated DNA molecules show absorbance at 340 nm and fluorescence with a maximum at ~ 450 nm, suggesting the incorporation of Ptr to DNA molecules, in a relation of one molecule of Ptr every 9 base pairs. In aerobic conditions, irradiation in the presence of Ptr causes random cleavage in the DNA molecules, yielding fragments of different molecular weights. These results are in agreement with previously data reported for plasmidic DNA [36].

3. Amino acids, peptides and proteins

Proteins, due to their relatively high abundance, their ability to bind chromophores, and the reactivity of particular amino acid residues, are one of the preferential targets of the photosensitized damaging effects of UV radiation on biological systems [38]. Currently, it is accepted that the photosensitization of proteins occurs mainly through the reactions of $^1O_2$ with tryptophan (Trp), tyrosine (Tyr), histidine (His), methionine and cysteine side-chains [39]. However, in the last decade we have demonstrated that pterins are able to photoinduce damage in free amino acids, peptides and proteins through both *type I* and *type II* mechanism, being the *type I* mechanism the main contribution in the photosensitization process [25, 26, 40]. We have focused our attention on the chemical modifications of Tyr and Trp residues because these amino acids are particularly susceptible to a variety of oxidizing agents [41].

The oxidation of Trp photosensitized by Ptr takes place through both *type I* and *type II* mechanisms, the first being the predominant one. Product analysis revealed that two main products are formed in the process. One of them is hydroxytryptophan (HO-Trp) (Figure 5), which has been detected in the skin of patients affected by vitiligo [42]. The other one is N-formylkynurenine (NFK) (Figure 5). Other minor product is kynurenic acid (KA) (Figure 5), formed by several reactions steps from NFK.

Tyr is an important target in the study of the photodynamic effects of UV-A radiation in living systems, not only due to its reactivity towards $^1O_2$ [43, 44], but also because this amino acid plays a key role in polymerization and cross-linking of proteins [45, 46] *via* reactions initiated by Tyr radicals [47, 48]. Mechanistic analysis indicates that the Ptr-sensitized oxygenation/oxidation of Tyr does not involve $^1O_2$, and proceeds through an electron transfer-initiated process. In this mechanism, the one-electron oxidation of Tyr leads to the formation of the corresponding radical, $Tyr^{\bullet+}$/$Tyr(-H)^{\bullet}$, which can react with O_2 (and with $O_2^{\bullet-}$ and H_2O) (Reaction 5), to yield a series of oxidation products, such as DOPA and *o,o´*-dityrosine (Tyr_2) (Figure 6). The formation of Tyr_2 is important from a biological point of view because it implies that pterins might photoinduce the oligomerization and crosslinking of proteins in vivo.

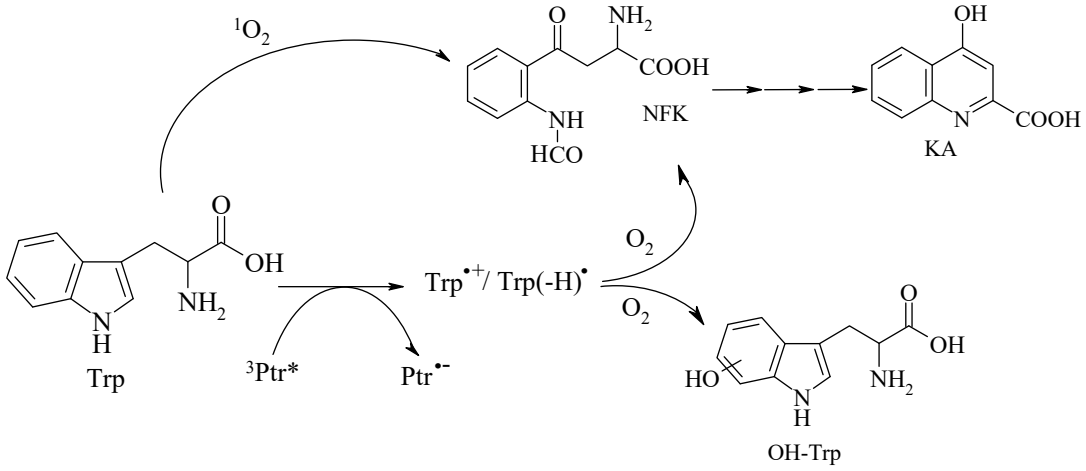


Figure 5. Products of Ptr photosensitized oxidation of Trp.

In the context of our investigations on the photosensitizing properties of pterins, we have also explored reactions that affect biomolecules involved in the pigmentation of the skin. In this way, we performed studies with α-melanocyte-stimulating hormone (α-MSH) and related peptides, tyrosinase (TYR) and albumins (bovine serum albumin (BSA) and human serum albumin (HSA)) as substrates.

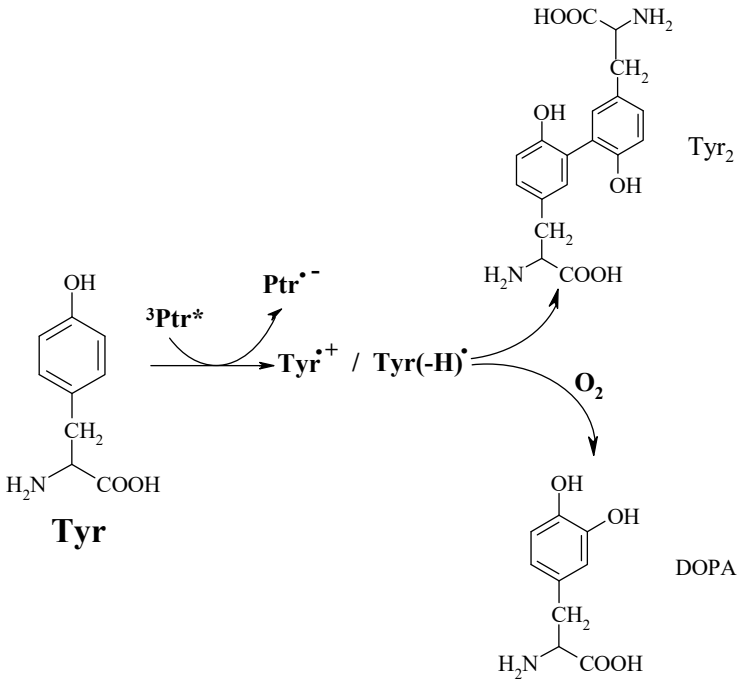


Figure 6. Products of Ptr photosensitized oxidation of Tyr

α-MSH is a short peptide, with Trp and Tyr residues in its sequence, that stimulates the production and release of melanin by melanocytes in the skin and hair. When aerated aqueous solutions containing α-MSH and Ptr were exposed to UV-A radiation, the peptide consumption and H_2O_2 generation were observed, but without significant change in the photosensitizer concentration. In this process Trp and Tyr residues are damaged and new fluorescent compounds were formed. One of these compounds exhibits an emission band with a peak maximum coinciding with that expected for Tyr_2 and the cross-linking between two peptide molecules was observed [49].

To avoid interferences in the characterization of the photoproducts, two peptides in which the amino acid sequence of α-MSH was mutated were used. In the peptide named $\alpha\text{-MSH}_{W9G}$, the Trp residue in position 9 was mutated to a glycine (Gly), whereas in the peptide named $\alpha\text{-MSH}_{Y2G}$, the Tyr residue in position 2 was mutated, also to a Gly residue. During the photosensitization of $\alpha\text{–MSH}_{Y2G}$, the Trp residue was consumed, and at least three major

products were detected. The spectroscopic characterization of these products suggested that the Trp residue was oxidized to NFK and HO-Trp. No dimeric products were observed with this peptide. However, during the photosensitization of α-MSH$_{W9G}$, two simultaneous processes occur: dimerization and incorporation of oxygen. These results confirm that the dimerization observed in α-MSH is due to the crosslinking of Tyr residues [50].

Albumin is the most abundant plasma proteins, and their main biological function is the transport of a wide variety of molecules. Besides, it is present in human skin [51], where there is an autocrine synthesis and regulation [52]. It has been reported that in patients affected by vitiligo, epidermal albumin oxidation takes place, but the mechanism of this process has not been elucidated [53]. We have studied the capability of Ptr to photoinduce chemical and structural changes in BSA and HSA. The results obtained for both proteins were very similar, as is expected due to the high structural homology between the two proteins. When air-equilibrated aqueous solutions of albumin and Ptr were exposed to UV-A radiation, the oligomerization of the protein was observed [48, 54]. For HSA, it was found that oligomers with more than 10 HSA molecules were formed. The emission spectra of the oligomer fraction revealed the presence of Tyr$_2$, suggesting that this product is, at least in part, responsible for the bonds between albumin molecules. Moreover, upon irradiation, the intensity of the Trp emission of the albumin decreased as a function of irradiation time, indicating the modification of this amino acid residue. NFK was identified as one of the photooxidation products of albumin.

To find out if the observed photoinduced chemical modifications on proteins can affect the activity of enzymes, experiments using tyrosinase (TYR, L-tyrosine, L-dopa: oxygen oxidoreductase, EC 1.14.18.1)) as a substrate were performed [55]. This biomolecule was chosen because it is an essential enzyme in the biosynthesis of melanin. A fast inactivation of the enzyme was recorded when TYR was exposed to UV-A radiation in the presence of Ptr. The mechanistic analysis suggested that the photoinactivation of TYR is initiated by an electron transfer reaction and takes place *via* a *type I* mechanism.

We extended our studies on the photoinactivation of TYR to folic acid (PteGlu) and its oxidation products (6-formylpterin (Fop) and carboxypterin (Cap)) because the photodegradation of PteGlu, an important vitamin, has been proposed as one of the reasons for the development of skin tanning in evolution. The activity of TYR decreased significantly when the enzyme was exposed to radiation in the presence of PteGlu [56]. The results suggest that PteGlu itself is unable to inactivate the enzyme, but its photoproducts, Fop and Cap, photoinduce its inactivation, being Fop the most efficient photosensitizer. These results provide evidence that processes photosensitized by pterins might affect the synthesis of melanin and, in consequence, play a key role in pigmentation disorders.

4. Phospholipids

In a study performed using cervical cancer cells (HeLa), we ascertained that pterins are readily incorporated into the cells, that cell death takes place upon UV-A irradiation of pterins, and that the integrity of the cell membrane is affected, among other alterations undergone by the cells [57]. Taking into account these results and to go further with the investigation of the photosensitizing properties of pterins, we decided to start investigating the photoinduced damage of biomembranes by pterins.

Lipid peroxidation is involved in many physiological and pathological events, and is usually due to oxidative stress [58-60]. This process can proceed by different mechanisms [61]. The most important one involves free radicals, where the initiation phase includes hydrogen atom abstraction, and the most abundant compounds of lipid membranes, phospholipids containing polyunsaturated fatty acids (PUFAs), are the main targets. Light accelerates lipid peroxidation quite substantially [62-64], and endogenous or exogenous photosensitizers can act through both *type I* and *II* mechanisms.

Therefore, we have investigated the oxidation of large unilamellar vesicles (LUVs) of soybean phosphatidylcholine (SoyPC) photoinduced by Ptr [65]. SoyPC LUVs were exposed to UV-A irradiation prepared in the presence and absence of Ptr. Conjugated dienes and trienes were detected in the treated samples, revealing that Ptr is able to photoinduced lipid peroxidation. Mechanistic studies suggested that the process would be initiated by an electron transfer step, whereas $^1O_2$ would not be involved as the main pathway. Additionally, several hydroperoxides corresponding to the oxidation of different PC derivatives were found as photoproducts, such as PC(16:0/18:2)-OOH, PC(18:1/18:2)-OOH and PC(18:2/18:2)-OOH. Moreover, when the irradiation time increases, hydroperoxides suffered cleavages in the carbon chain generating short-chain secondary oxidation products (aldehyde, ketones and carboxy products). Additionally, it was revealed that Ptr is not encapsulated in the inner fraction of vesicles, but is able to freely cross the phospholipid bilayer.

Lipophilic photosensitizers able to bind to biomembranes usually are more efficient in phototriggering lipid peroxidation than hydrophilic photosensitizers [66-69]. Therefore, new lipophilic pterin derivatives were synthesized. Four compounds were obtained with the decyl carbon chain attached to position N3 or O of Ptr (Figure 7). Conjugation of a decyl-chain to the pterin moiety dramatically increases its solubility in common organic solvents and also enables its facile intercalation in LUVs membranes [70, 71]. Constant binding were determined obtaining values comparable with other lipophilic molecules [72, 73]. In addition, decyl-pterins present more efficient intersystem crossing to the triplet excited states compared to Ptr, showing higher $^1O_2$ quantum yields, and lower fluorescence emission.

To investigate whether lipophilic pterins are better lipid peroxidation photosensitizers, the efficiency of *O*-decyl-Ptr (Figure 7) was evaluated and compared to its hydrophilic parent compound, Ptr. Investigation in SoyPC LUVs showed that the formation of conjugated dienes and trienes and lipid hydroperoxides is much faster using *O*-decyl-Ptr than Ptr.

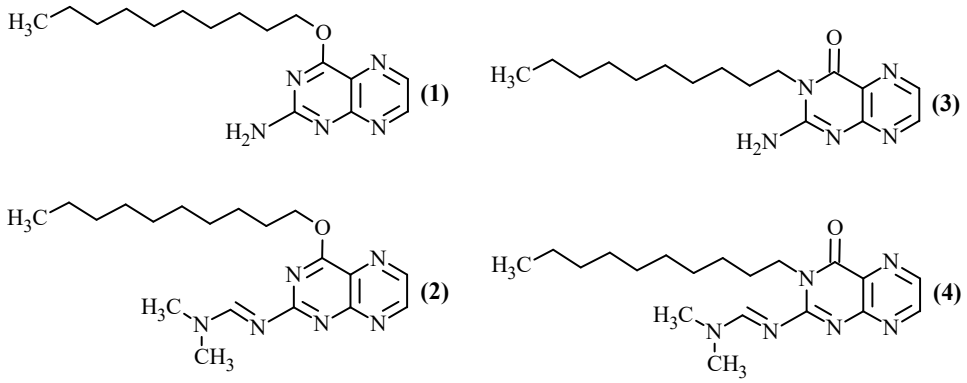


Figure 7. Synthetized lipophilic pterins (1) 4-(Decyloxy)pteridin-2-amine, (2) N′-(4-(Decyloxy) pteridin-2-yl)-N,N-dimethylformimidamide, (3) 2-Amino-3-decylpteridin-4(3H)-one and (4) N′-(3-Decyl-4-oxo-3,4-dihydropteridin-2-yl)-N,N-dimethylformimidamide.

In order to better investigate the chemical changes photoinduced by *O*-decyl-Ptr in PUFAs, products formed in the photosensitization of LUVs of DLPC (PC 18:2/18:2) were analyzed. In particular, products with the incorporation of one oxygen atom (hydroxyl derivative), two oxygen atoms (one hydroperoxide or two hydroxyl groups) [74], (Figure 8) and the incorporation of oxygen atoms with the loss of two H (keto derivatives) were characterized. In addition, short chain secondary products were identified, *e.g.*, 2-(9-carboxy-nonanoyl)−LPC and 2-(9-oxononanoyl)−LPC (Figure 8). Taking into account previous studies on lipid peroxidation[75,76], these phospholipid oxidation products are formed after rearrangement and cleavages of hydroxylderivatives or LO$^{\bullet}$ radicals, produced by reduction of hydroperoxides. A kinetic qualitative assessment of the formation of the different products, suggested that no accumulation of hydroperoxides took place before the production of hydroxy derivatives and short chain secondary products, which might indicate a fast photosensitized conversion of the former into the latter.

The alteration of the permeability of vesicles upon UV-A irradiation in the presence of pterins was also evaluated. In these studies SoyPC LUVs and DLPC giant unilamellar vesicles (GUVs) were used as substrates and Ptr and *O*-decyl-Ptr were employed as photosensitizers. Results demonstrated pterin mediated photosensitization leads to an increase in the permeability of the

membrane, revealing that the photochemical process causes damage of the structure of the membrane. It is also worth mentioning that the interaction of *O*-decyl-Ptr with the biomembranes due to the presence of the alkyl side chain, dramatically favors the photoinduced increase of permeability when compared with experiments performed with Ptr. This is in agreement with the fact that the formation of oxidized products is much faster using *O*-decyl-Ptr than Ptr.

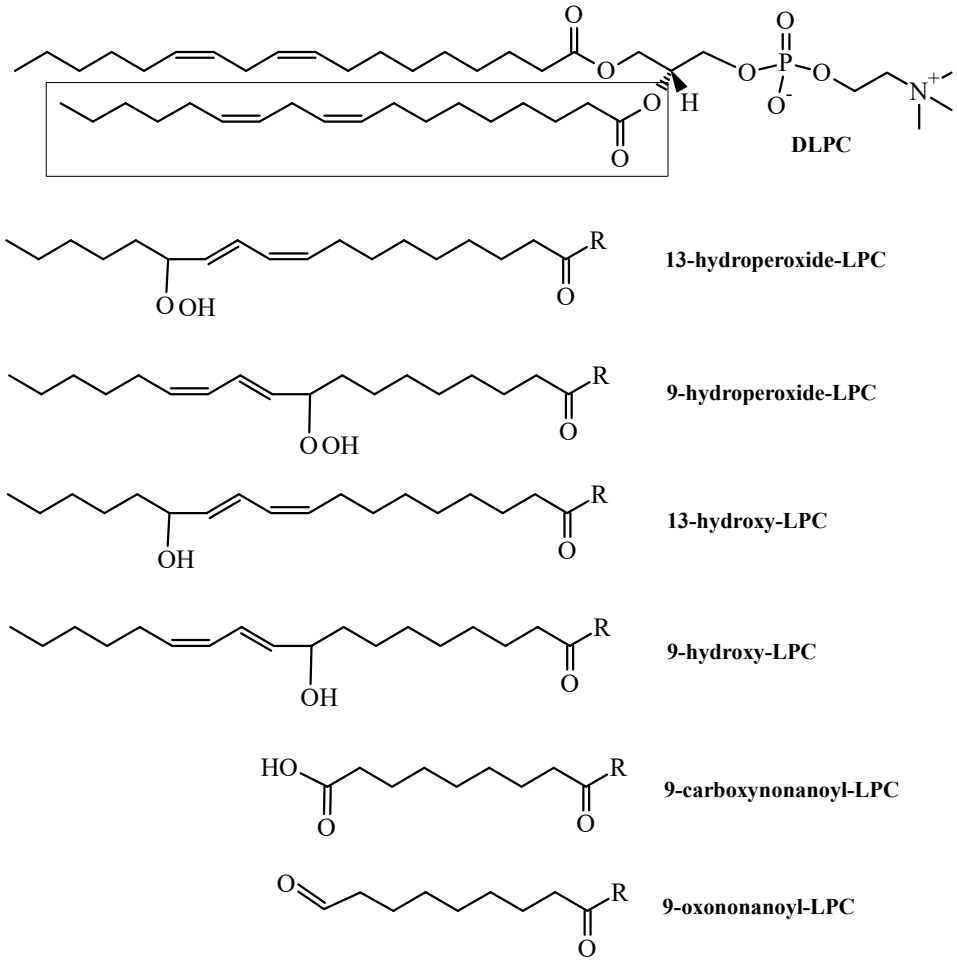


Figure 8. Several products found after photosensitized lipid peroxidation of DLPC LUVs by *O*-decyl-Ptr.

It is well known that oxidation of the double bond(s) on the acyl tails(s) of the unsaturated phospholipids promotes lipid hydroperoxidation [61]. When hydroperoxide species accumulate in the membrane, large membrane fluctuation ending in surface area increase were reported, with

no increase in membrane permeability [68, 69, 77]. In contrast, pore formation is observed when oxidized lipids with shortened chains are present in the biomembranes [78, 79]. Therefore, the significant increase in membrane permeability in LUVs and GUVs experiments are consistent with the detection of shortened oxidized lipids. However, in phase contrast optical microscopy experiments, neither membrane fluctuations nor area increase preceded membrane contrast fading, suggesting there is no hydroperoxide accumulation in agreement with our product analysis. In this way, our results furnish valuable information regarding the photodynamic mechanism of action of *O*-decyl-Ptr in biomembranes which, in turn, may perturb cell homeostasis and trigger cell death.

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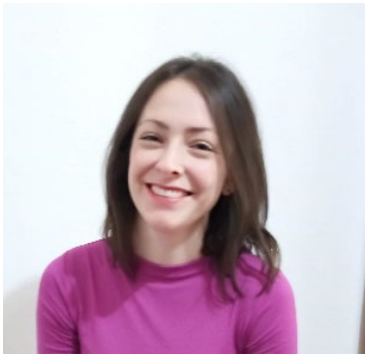
Mariana Vignoni obtained her degree in Biochemistry at the Universidad Nacional de La Plata (UNLP), Argentina, in 2007. She did her PhD in the group of Dr. Andres Thomas at INIFTA (Research Institute of Theoretical and Applied Physical Chemistry) from the UNLP. She did a post-doctoral fellowship at the University of Ottawa in Prof. Scaiano's group. She started as an Assistant Researcher in Prof. Thomas's group at INIFTA in 2013. She is currently an Adjunct Researcher and her main research activities deal with photosensitization of lipid membranes and synthesis of new lipophilic photosensitizers.



Mariana Serrano has been carrying research activities in the field of photochemistry and photosensitization processes with biological targets, specifically nucleotides, with special interest of processes related to skin diseases, such as vitiligo. The PhD thesis of Dr. Mariana Serrano (2010-2014), is an example of the approach between photochemistry and biomedical processes. Studying photosensitization process in nucleotides initiated by pterins, a group of molecules presents in the skin of patients suffering from vitiligo. Her background is related to mechanisms and chemical kinetics of reactions involving biomolecules with a broad range of analytical techniques, such as liquid chromatography, mass spectrometry and fluorescence spectroscopy. Currently, Dr. Serrano research covers aspects of photochemistry of reactions occurring in heterogenous systems.

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