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# Isolation and identification of the photodegradation products of the photosensitizing antidepressant drug clomipramine. Phototoxicity studies on erythrocytes

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The isolation and identification of the photodegradation products of clomipramine (CIP) in phosphate buffered saline (PBS pH 7.4 and 6.0) solution and methanol under aerobic conditions were studied. Six compounds were identified and four of them were isolated and characterized by spectroscopic methods. A radical mechanism with the participation of the solvent is proposed for the photodegradation of CIP which undergoes homolytic cleavage of the carbon-chlorine bond and also photooxidation of the amine group. CIP was able to induce photohemolysis when it was irradiated in PBS pH 7.4 and in PBS pH 6.0 containing a suspension of human red blood cells (RBCs). The photohemolysis experiments in the presence of additives DABCO and GSH showed nearly total inhibition of drug-induced photohemolysis. The efficient inhibition of photohemolysis by the radical scavenger GSH compared with the inhibition show by DABCO suggests a moderate effect by singlet oxygen. Clomipramine-N-oxide was the unique photoproduct able to induce hemolysis and photohemolysis when it was incubated and irradiated with RBCs for 1 h. A mechanism involving singlet oxygen, radicals and photoproducts is suggested for the reported phototoxicity.

## 1. Introduction

Tricyclic dibenzocicloheptadienes are an important group of antidepressants, which have been reported to induce contact allergy and skin photosensitization in humans [1, 2]. With the intent to find connection between the photosensitization process and the photofragmentation mechanism, some studies have been made about the photodegradation of protriptilyne (PTR), imipramine (IMP), amitriptiline (AMT), nortriptilyne (NTR) and clomipramine (CIP) [3–8]. The photodegradation of PTR has been extensively studied and photohemolytic assays on red blood cells have been done [3].

The photofragmentation of CIP (**1**) has been studied in phosphate buffered saline solution (PBS, pH 7.4) in the absence and the presence of β-cyclodextrins and the photoproducts were analyzed by liquid chromatography/mass spectroscopy (LC/MS) [8].

To the best of our knowledge, no reports on the isolation and characterization of the photodegradation products of CIP by spectroscopic methods appeared in the literature up to now. A case of photosensitivity and contact allergy to CIP was reported by Junggren and Bojs and a photoproduct of CIP was hypothetically proposed as the original sensitizer [1]. The mechanism of the cutaneous photosensitivity induced by CIP has not been determined yet.

The aim of this study was to isolate and to identify the products of CIP photolysis under mild conditions and oxygenated media (PBS pH 7.4). It was also our purpose to determine the *in vitro* phototoxicity on erythrocytes both of CIP and its photoproducts.

## 2. Investigations, results and discussion

The photodegradation of CIP was followed by monitoring the disappearance of the 230 nm band at 20 min intervals as shown in Fig. 1 for PBS (pH 7.4) solution. The Fig. shows the appearance of a new band at 315 nm. The same results were observed in PBS (pH 6.0) solution (data not shown). Nevertheless the photodegradation of CIP (pH 7.4) was faster than the photodegradation observed for CIP hydrochloride (pH 6.0). The rate of photolysis of CIP (**1**) as free amine (pH 7.4) agrees with the photostability of IMP (**2**) and AMT as free amines under oxygen atmosphere [16]. The photolability decreased when nitrogen was bubbled through the solutions before irradiation (data not shown). The presence of oxygen enhances the photodegradation and it should be involved in the degradation mechanism of CIP. The photolysis of CIP in methanol solution is shown in Fig. 3 and no appearance of a new band in 315 nm was observed. This suggests us that a different photodegradation mechanism is involved in methanol. Irradiation with UV-B and UV-A (290–400 nm) radiation of the PBS (pH 7.4) solution of CIP under oxygen atmosphere affords four photoproducts, whose formation is explained in the Scheme. The products were identified by their spectral properties; the structure of photoproduct **3**, the main reaction product (40% yield), was assigned by comparison to similar compounds reported earlier as 3-hydroxyimipramine [8, 16]. Since no positive test to KI in TLC during the course of the reaction was detected, we propose that the formation of photoproduct **3** is not compatible with the formation of a per-

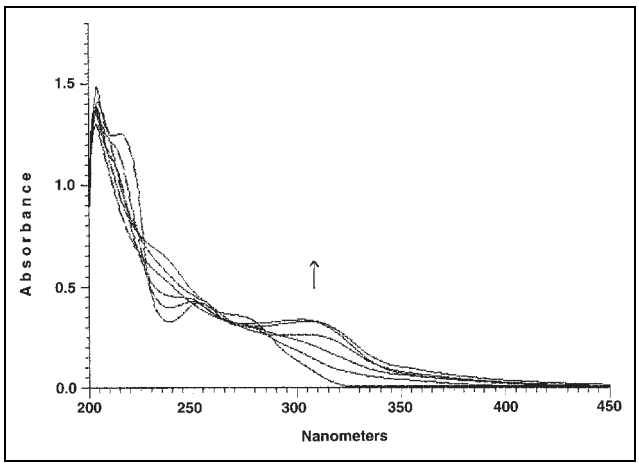
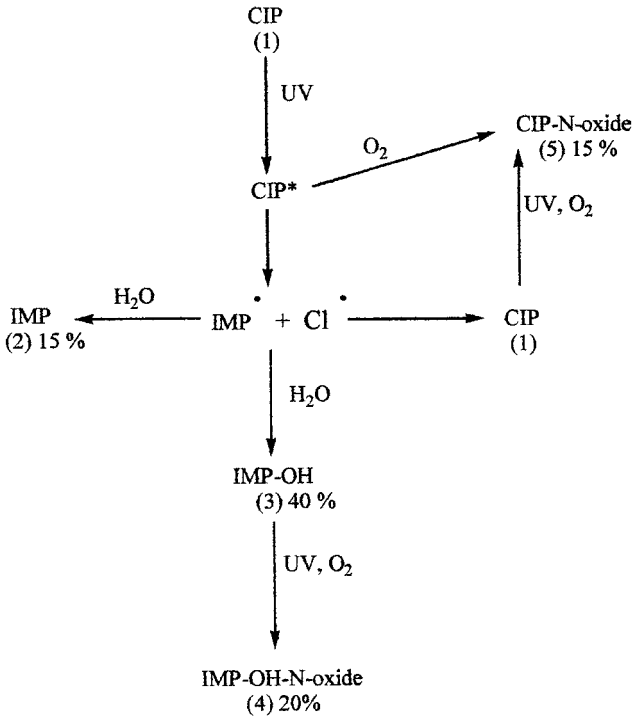



Fig. 1: UV-Vis monitoring of the photolysis of clomipramine ($10^{-6}$ M) in PBS (pH 7.4) under aerobic conditions at regular intervals of 20 min of irradiation

**Scheme**




oxy-intermediate and reduction to **3**. An interesting observation was the absence of product **3** in the reaction mixture, when the photodegradation in methanol solution was followed by TLC or HPLC chromatography. Instead, IMP was the major photoproduct observed under the same irradiation conditions (data not shown). The structure of product **4** (20% yield), was assigned by comparison to similar compounds (photoproduct **3** and clomipramine-N-oxide) as 3-hydroxyimipramine-N-oxide (**4**) and it was absent (1% yield) when the photodegradation was done under nitrogen atmosphere conditions. The formation of product **4** is compatible with the participation of oxygen. As no formation of imipramine-N-oxide was detected, the participation of oxygen was proposed by reaction with CIP and with 3-hydroxy-imipramine (**3**). Upon irradiation of photoproduct **3** in PBS (pH 7.4) solution under fully oxygenated conditions, the formation of small amounts of

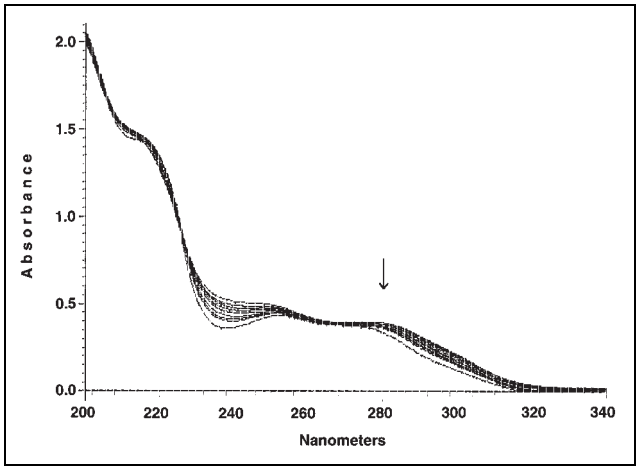



Fig. 2: UV-Vis monitoring of the photolysis of clomipramine ($10^{-6}$ M) in methanol under aerobic conditions at regular intervals of 20 min of irradiation

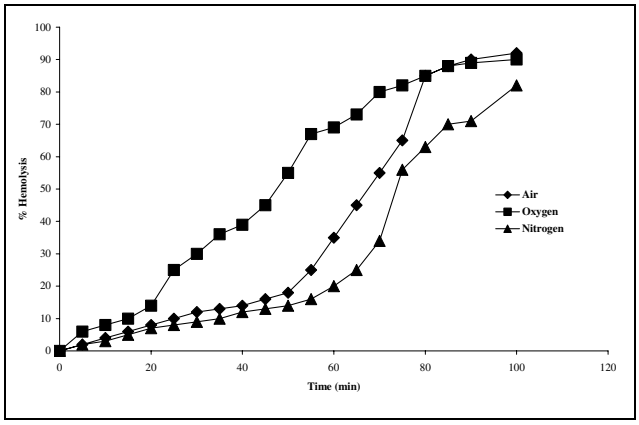



Fig. 3: Photohemolysis of RBCs ($3.3 \times 10^6$ cells ml$^{-1}$) sensitized by CIP ($4.2 \times 10^{-5}$ M) in PBS (pH 6.0) under aerated, oxygen and nitrogen conditions

product **4** was observed (data not shown). Moreover, Epling et al. mentioned that irradiation of IMP in methanol under the same conditions and chromatographic separation of the mixture led to the identification of iminodibenzyl and desipramine as the unique photoproducts [7]. IMP (15% yield) was identified in the reaction mixture obtained by irradiation in PBS (pH 7.4) solution under oxygen and in methanol solution, too. These results suggest that dechlorination was the predominant photodegradation pathway under aerobic and anaerobic conditions. Cleavage of the carbon-chlorine bond upon UV irradiation has been proposed as the predominant photodegradation pathway for chlorpromazine and other chloroaromatic compounds [18, 19]. The resulting imipraminyl radical can react with the solvent yielding 3-hydroxyimipramine (**3**) as the major product in PBS (pH 7.4) or IMP (**2**) as the major product in methanol under oxygenated conditions. The photoproduct 5 (15% yield) was identified as clomipramine-N-oxide and was obtained only in fully oxygenated conditions.

CIP ($2.8 \cdot 10^{-5}$ M) was able to induce photohemolysis when it was irradiated in PBS pH 7.4 and in PBS pH 6.0 ($4.2 \cdot 10^{-5}$ M), containing a suspension of human red blood cells (RBCs) (Fig. 3, 4). The hemolysis induced by CIP as free amine (pH 7.4) under aerobic conditions was faster than the hemolysis induced by CIP hydrochloride (pH 6.0). This agrees with the rate of photolysis observed for CIP as free amine compound with that observed for CIP hydrochloride. 50% hemolysis was obtained at 25 min of irradiation as is shown in Fig. 4 (pH 7.4). No

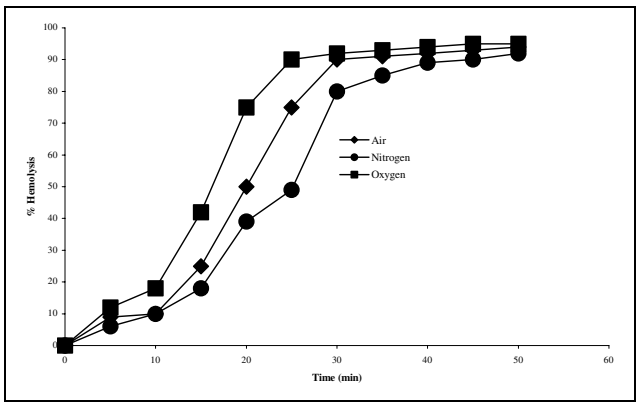



Fig. 4: Photohemolysis of RBCs ($3.3 \times 10^6$ cells ml$^{-1}$) sensitized by CIP ($2.8 \times 10^{-5}$ M) in PBS (pH 7.4) under aerated, oxygen and nitrogen conditions

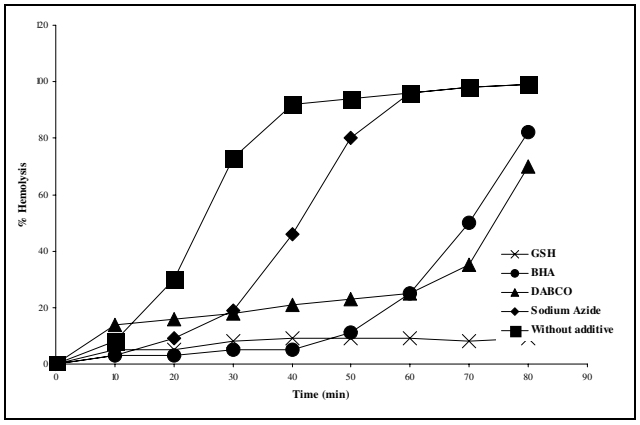



Fig. 5: Photohemolysis of RBCs ($3.3 \times 10^6$ cells ml$^{-1}$) sensitized by CIP ($2.8 \times 10^{-5}$ M) in PBS (pH 7.4) under oxygenated conditions in the presence of additives ($10^{-6}$ M)

lysis was observed when cells were irradiated for 1 h in the absence of CIP and when red blood cells were incubated with CIP in the dark. CIP induced photohemolysis also occurs when the samples were bubbled with nitrogen before adding the RBCs (Fig. 3, 4). The photohemolysis experiments were repeated in the presence of additives and we found that DABCO and GSH showed nearly total inhibition of drug-induced-photohemolysis. In the presence of additives BHA, and sodium azide, CIP showed delayed photohemolysis. The results are shown in Fig. 5. These results suggest that free radicals are involved in the process since GSH (as radical trapping) showed the best photohemolysis inhibition. The inhibition of photohemolysis in the presence of a singlet oxygen quencher (DABCO) can be attributed to the involvement of a type II mechanism in the aerobic photodecomposition process. Singlet oxygen was observed by trapping it with 2,5-dimethylfuran and detecting the oxidation products by GC-MS [11]. Incubation of preirradiated (10 h irradiation) solution of CIP in PBS (pH 7.4) with RBCs was able to induce hemolysis in the dark (data not shown). This revealed the presence of toxic photoproducts involved in the damage to the cellular membrane. Clomipramine-N-oxide ($10^{-6}$ M in PBS pH 7.4) was the unique photoproduct able to induce hemolysis and photohemolysis when it was incubated and irradiated with RBCs for 1 h (data not shown).

A radical mechanism with the participation of the solvent is proposed for the photodegradation of CIP which undergoes homolytic cleavage of the carbon-chlorine bond and also photooxidation of the amine group (Scheme). The participation of the solvent explains the formation of photoproducts **2** and **3**. The formation of singlet oxygen detected by photohemolysis assays and trapping experiments with 2,5-DMF, can explain the photooxidation reactions that produce N-oxide compounds. In this sense only those photoproducts with substituents in the aromatic ring can react with singlet oxygen since no formation of imipramine-N-oxide was observed. The involvement of radicals and singlet oxygen explains the cellular membrane damage. The efficient inhibition of photohemolysis by the radical scavenger GSH compared with the inhibition showed by DABCO suggests a moderate effect by singlet oxygen. The formation of IMP can be explained as a result of hydrogen abstraction by imipraminyl radical from the solvent. The formation of compound **3**, the major photoproduct can be explained as dechlorination of CIP and reaction with the solvent. The formation of compounds **4** and **5** most likely proceeds from **3** and **1**, respectively, both by reaction with oxygen.

The results indicate that photodegradation of CIP *in vitro* proceeds by dechlorination and reaction with oxygen and singlet oxygen to obtain N-oxide compounds. CIP is able to induce photosensitising reactions by a type I mechanism. The structure of clomipramine-N-oxide (**5**) that we found as a photoproduct under *in vivo* similar con{\textstyle\vint\limits^{}_{}}ditions, is reported as a metabolite of clomipramine, too [12].

The results obtained may be useful to complete the previous photochemical studies about the structure of the photoproducts and the possibility of a toxic photoproduct [7, 8].

## 3. Experimental

### 3.1. Chemicals

Clomipramine HCl was provided by Ciba-Geigy and was extracted from Anafranil® by means of a soxhlet extractor with acetone and recristallized from ethyl ether/methanol mixture (m.p.: 189–190 °C). The purity was 99% determined by GC-MS (Hewlett Packard 5972, MS 5890; Carlo Erba Kratos MS 25RFA, 25 m cross linked 5% phenylmethyl silicone), $^1$H NMR (Bruker Aspect 3000, 300 MHZ; Jeol Eclipse 400 MHZ), TLC (general purpose silica gel on polyester and C18-silica gel on glass 1000 μm), HPLC (Waters Delta prep 4000, μBondapack C18; 10 μm, 125 Å, 19 X150 mm ID column) and UV-Vis spectrophotometry (Milton Roy 3000). Reduced glutathione (GSH) and butylated hydroxyanisol (BHA) were purchased from Sigma. 2,5-Dimethylfuran, sodium azide ($NaN_3$) and 1,4-diazabicyclo[2.2.2]octane (DABCO) were purchased from Aldrich Chemical Company (Milwaukee USA). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany). Human red blood cells (RBCs) concentrate was supplied by QUIMBIOTEC, C.A. (Venezuela). Phosphate buffered saline pH 7.4 and pH 6.0 (PBS) was used for photolysis and *in vitro* assays. Clomipramine-N-oxide was prepared in our laboratory following the general procedure found in the literature [13].

### 3.2. Photolysis

The course of the photodegradation of CIP (3-chloro-10,11-dihydro-*N,N*-dimethyl-5H-dibenz[*b,f*]azepine-5-propanamine) was observed by TLC until 100% of CIP was consumed and it was also followed by UV-Vis spectrophotometry using a Milton Roy 3000 instrument. The measure of optical density (OD) for *in vitro* assays was followed by UV-Vis spectrophotometry using a Shimadzu UV-160 instrument. CIP ($10^{-6}$ M) was irradiated at room temperature in PBS (pH 7.4 and pH 6.0) and methanol under aerobic and anaerobic conditions with an OSRAM HQL 250 W medium pressure Hg lamp located inside a pyrex immersion-well photoreactor (Applied photophysics parts no. 3230 and 3307) for $\lambda \geq 290$ nm irradiation. The samples (2–3 ml) were irradiated in quartz cuvettes of 1 cm optical path and monitored by UV-Vis irradiation intervals of 20 min. The photon flux incident on samples was $1.75 \cdot 10^{-4}$ J · S$^{-1}$ · cm$^{-2}$ measured as described previously [11]. The temperature inside the samples during irradiation was between 25 and 28 °C. Preparative irradiations ($10^{-3}$ M solutions) in PBS (pH 7.4 and pH 6.0) were monitored by TLC during 21 h until the disappearance of 100% of CIP was detected [10]. On completion of irradiation, the PBS solution was extracted with methylene chloride. A second extraction was done after reaching a value of pH 8 by adding aqueous NaOH solution. The organic phase was evaporated under reduced pressure (14 torr) at room temperature. The residue was purified by reverse phase TLC and preparative HPLC. The liquid chromatograph was equipped with a semi-preparative μNovapack C18 column. The samples were eluted with 70% aqueous sodium perchlorate solution, pH 2.5 as eluent, at a flow-rate of 1.8 ml/min and a column temperature of 30 °C, with monitoring at the wavelength of 220 nm [12]. The isolated products were analyzed by $^1$H NMR and $^{13}$C NMR and GC-MS. These exhibited the following spectroscopic features:

Product **2** (15% yield): the spectroscopic data agree with those found for imipramine [16].

Product **3**, the main product of the reaction (40% yield): $^1$H NMR ($CD_3COCD_3$, 300 MHz), $\delta = 7.12–6.40$ (m, 7 H, aromatic-H), 5.40 (s, 1 H, OH), 3.77 (t, 2 H, $CH_2$), 3.05 (m, 4 H, $-CH_2CH_2-$), 2.45 (s, 6 H, $CH_3$), 2,75 (t, 2 H, $CH_2$), 1.82 (m, 2 H, $CH_2$); $^{13}$C NMR ($CD_3COCD_3$, 70 MHz), $\delta = 156.3$ (s, aromatic-C3), 149.7 (s, aromatic-C4a), 149.3 (s, aromatic-C6a), 135.8 (s, aromatic-C9a), 131.3 (s, aromatic-c1), 130.3 (s, aromatic-C9), 127,1 (s, aromatic-C7), 124,9 (s, aromatic-C1a), 123.6 (s, aromatic-C8), 120.9 (s, aromatic-C6), 110.3 (s, aromatic-C2), 107.5 (s, aromatic-C4), 57.7 (s, aliphatic-Ca), 48.6 (s, aliphatic-Cc), 44.8 (s, alipha-

tic-$CH_3$), 32.8–32.2 (m, aliphatic-C10-C11), 25.5 (s, aliphatic-Cb); MS: m/e (%) = 296 (1, $M^+$), 269 (1), 251 (1, $M^+$-$N(CH_3)_2$), 65 (100), 58 (40), 43 (72).

Product **4** (20% yield): $^1H$ NMR ($CD_3COCD_3$, 300 MHz), δ = 7.13–6.40 (m, 7 H, aromatic-H), 5.36 (s, 1 H, OH), 3.84 (t, 2 H, $CH_2$), 3.40 (t, 2 H, $CH_2$), 3.09 (m, 4 H, $-CH_2CH_2-$), 3.00 (s, 6 H, $CH_3$), 2.08 (m, 2 H, $CH_2$); $^{13}C$ NMR ($CD_3COCD_3$, 70 MHz), δ = 156.8 (s, aromatic-C3), 149.4 (s, aromatic-C6a), 149.1 (s, aromatic-C4a), 135.8 (s, aromatic-C9),131.5 (s, aromatic-C1), 130.4 (s, aromatic-C7), 127.2 (s, aromatic-C9a), 125.1 (s, aromatic-C1a), 123,9 (s, aromatic-C8), 120.7 (s, aromatic-C6), 110.6 (s, aromatic-C2), 107.6 (s, aromatic-C4), 57.3 (s, aliphatic-Cc), 47.8 (s, aliphatic-Ca), 43.9 (s, $CH_3$), 32.7–32.0 (m, C10-C11), 23.7 (s, aliphatic-Cb). MS: m/e (%) = 296 (7, $M^+$-16), 251 (25, $M^+$-$N(CH_3)_2$), 211 (15, $M^+$-$C_5H_{12}N$), 65 (33), 58 (100).

Product **5** (15% yield): the spectroscopic data agree with those found for clomipramine-N-oxide synthesised in our laboratory, $^1H$ NMR ($CD_3COCD_3$, 300 MHz), δ = 7.22–6.89 (m, 7 H, aromatics), 3.92 (t, 2 H, $CH_2$), 3.47 (t, 2 H, $CH_2$), 3.11 (m, 4 H, aliphatic), 3.02 (s, 3 H, $CH_3$), 2.05 (m, 2 H, $CH_2$); $^{13}C$ NMR ($CD_3COCD_3$, 70 MHz), δ = 149.9 (s, aromatic-C4a), 148.3 (s, aromatic-C6a), 136.1 (s, aromatic-C9a), 132.8 (s, aromatic-C1a), 132.5 (s, aromatic-C1), 132.0 (s, aromatic-C3), 127.6 (s, aromatic-C9), 124.6 (s, aromatic-C7), 123.1 (s, aromatic-C8), 120.6 (s, aromatic-C6), 110.6 (s, aromatic-C2), 107.4 (s, aromatic-C4), 57.2 (s, aliphatic-Ca), 48.1 (s, aliphatic-Cc), 43.9 (s, $CH_3$), 32.6–31.9 (m, C10-C11), 23.7 (s, aliphatic-Cb).

### *3.3. Photoinduced hemolysis assays*

RBCs were prepared by diluting samples of fresh (no more than four days) packed human erythrocytes in phosphate-buffered saline solution pH 7.4 (PBS) until the resultant suspension had an OD of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponds to $3.3 \cdot 10^6$ cells · ml $^{-1}$. For the hemolysis experiment the RBCs suspensions with CIP ($2.8 \cdot 10^{-5}$ M) were irradiated and the hemolysis percentage was determined by measuring the decreasing OD at 650 nm, since the optical density is linearly proportional to the number of intact RBCs [14]. Similar experiments were carried out without irradiation, with a preirradiated solution of CIP and with isolated photoproducts in concentrations of $10^{-5}$ M. Experiments have been performed under aerobic and anaerobic conditions. In the case of anaerobic conditions PBS solution was enriched with $N_2$ by bubbling it previous to the addition of RBCs. The photohemolysis test was repeated with solutions of CIP in the presence of DABCO ($10^{-5}$ M) and $NaN_3$ as singlet oxygen quenchers, BHA and GSH as radical scavengers. Experimental details were similar to previous works [14, 15]. All the experiments were repeated three times and the average (arithmetic mean) of these experiments is reported.

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