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Photochemical reaction of a dye precursor 4-chloro-1,2-phenylenediamine and its associated mutagenic effects

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Abstract

4-Chloro-1,2-phenylenediamine (4-Cl-*o*-PDA) is an aromatic diamine used as a precursor for manufacture of hair dyes and dyes of other purposes. 4-Cl-*o*-PDA has been found to be photomutagenic in bacteria when concurrently exposed to simulated sunlight irradiation. Irradiation of 4-Cl-*o*-PDA by either outdoor sunlight or indoor lamp, one main photoproduct appeared and it was found to be 2,3-diamino-7-chlorophenazine, a dimerized product through the excited state reaction of 4-Cl-*o*-PDA in the presence of oxygen. The isolated yield of 13% for 2,3-diamino-7-chlorophenazine is far better than the oxidation reaction of 4-Cl-*o*-PDA by H_2O_2 and may therefore be used as a synthetic method. The half live of transformation for 4-Cl-*o*-PDA in water (100 μM) is 39 min when exposed to outdoor sunlight. The photomutagenicity of 4-Cl-*o*-PDA and its photoproduct were tested in *Salmonella typhimurium* TA102. Under the same conditions, both compounds are photomutagenic. In addition, 2,3-diamino-7-chlorophenazine is both phototoxic and mutagenic.
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Keywords: 4-Chloro-1,2-phenylenediamine; Dye intermediate; Photochemical reaction; 2,3-Diamino-7-chlorophenazine; Photomutagenicity

1. Introduction

The potential carcinogenicity of hair dye ingredients has attracted the attention of toxicologists and epidemiologists for years [1–4]. Some aromatic amines were recognized as early as the late 19th century to produce an increased incidence of bladder cancer in occupationally exposed workers of the dye industry [5]. To find a possible correlation between consumer use of hair dyes and increased risk of bladder cancer, the guidelines for assessment of the genotoxic potential of hair dyes have been developed by the European Scientific Committee on Cosmetics and Non-Food Products [6,7]. Similarly in the United State, the Cosmetics, Toiletry and Fragrance Association established an independent expert panel that reviewed and published safety information on 650 ingredients including hair dyes during the past 25 years [8]. The U.S. Environmental Protection Agency reported that about 15 million people are potentially exposed to hair dye ingredients as a result of personal use or through application of hair dyes to other people [9]. Hair dyes are classified into three categories: oxidative or permanent coloring, semi-permanent coloring, and temporary coloring [10,11]. Approximately 80% of the hair dyes in the US and the EU belong to the oxidative or permanent coloring dyes [7,12]. 4-Chloro-1,2-phenylenediamine (4-Cl-*o*-PDA) is a halogenated aromatic diamine used in permanent hair dye formulations and is therefore worthy of investigation. 4-Cl-*o*-PDA itself is found to be mutagenic [13–15] and genotoxic through standard *in vitro* assays [16,17]. It is mutagenic and a liver carcinogen in big blue mice [18–20], and induces hepatocellular carcinomas in both sexes of mice and tumors of the urinary bladder [14]. 4-Cl-*o*-PDA is not mutagenic in *Salmonella typhimurium* bacteria strain TA98 in the presence of the S9 mix, but its oxidation products show mutagenicity [21]. Since it is unavoidable that people using hair dyes and hairdressers are exposed to both the hair dyes and light at the same time, the photoreaction and phototoxicity of the hair dyes should be taken into consideration for risk assessment. In this research, we report the photochemical reaction of 4-Cl-*o*-PDA, purification and structural elucida-

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tion of its main photoproduct, and the associated mutagenic effects.

2. Materials and methods

2.1. Materials

S. typhimurium strain TA102 was provided by Dr. Bruce Ames from the University of California (Berkeley, CA). 4-Cl-*o*-PDA, methanol, hexane, ethyl acetate, dimethyl sulfoxide-d6 (d6-DMSO), DMSO, and 8-methoxypsoralen were from Sigma–Aldrich (St. Louis, MO). All solvents used were spectroscopic grade. Thin layer chromatography (TLC) and silica (60 Å, 40–75 μm) were from Fisher Scientific (Houston, TX). Other solvents and chemicals were in their highest purity grade. The water used (18 MΩ) was deionized by Barnstead Nanopure Infinity water purification system (Dubuque, IA, USA).

2.2. Methods

2.2.1. Photoreaction of 4-chloro-1,2-phenylenediamine and isolation/structure elucidation of its main photoproduct

4-Cl-*o*-PDA was dissolved in deionized water containing 1% methanol to reach a final concentration of 0.33 g/l and irradiated in an open beaker with a SUNTEST® CPS+ lamp system (1500 W Xe Lamp with light output intensity of 250 J/cm^2). The solution was vortexed every 30 min during the irradiation. After 2 h of irradiation, the initial 4-Cl-*o*-PDA was mostly disappeared and a main photoproduct was formed as shown by HPLC (Simadzu Corp., Kyoto, Japan) and TLC. The solvent was evaporated at below 30 °C and the residue column chromatographed on silica gel eluting with 100% ethyl acetate. The collected ethyl acetate fractions were separated based on TLC analysis and dried on rotavapor (Brinkmann Instruments Inc., Westbury, NY) at below 30 °C. The purified photoproduct was weighed and ready for structural analysis and toxicity tests.

MS analysis was carried out with an EMD 1000 Mass Spectrometer (Waters Corp., Milford, MA). The purified photoproduct was infused into the mass detector with full scan ESI$^+$ mode. MS conditions were as follows: cone voltage 70 V, source extractor voltage 4 V, RF lens voltage 0.2 V, source temperature 99 °C, desolvation temperature 150 °C. The molecular ion is at *m/z* 245 and 247 $(M+H)^+$ with a relative intensity of 3:1.

The ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker 300 MHz spectrometer in d6-DMSO. Chemical shifts were given in ppm with tetramethylsilane as the internal standard. The ^{1}H NMR spectrum has five signals: 7.92 ppm (1H, d, $J = 2.3$, H_6). 7.90 ppm (1H, d, $J = 9.1$, H_9), 7.53 ppm (1H, dd, $J = 2.4$ and 9.0 Hz, H_8), 6.88 ppm (1H, s, H_1), 6.87 ppm (1H, s, H_4), 6.42 ppm (2H, s, $-NH_2$), 6.37 ppm (2H, s, NH_2). The ^{13}C NMR spectrum has 12 signals: 145.7, 145.3, 143.3, 143.1, 141.2, 139.6, 131.3, 130.5, 127.7, 127.0, 102.8 and 102.5 ppm. The ^{1}H NMR spectrum matches the previously published data for 2,3-diamino-7-chlorophenazine, $C_{12}H_9N_4Cl$, obtained through the oxidation of 4-Cl-*o*-PDA by H_2O_2 [21].

2.2.2. Photoreaction kinetics

An aqueous solution of 4-Cl-*o*-PDA (100 μM, 2 ml) was filled into two identical quartz cuvettes with 1 cm light path. One cuvette was irradiated by outdoor sunlight for a total of 40 min on a sunny day between 1:00 and 3:00 p.m. in August in Jackson, MS. The intensity of the sunlight was measured with a research radiometer equipped with UVA, UVB and visible light probes (PMA 2100, Solar Light and Co. Philadelphia PA). The average intensity of sunlight during irradiation time was 11.8 mW/cm^2 visible light, 3.6 mW/cm^2 UVA light, and 0.015 mW/cm^2 UVB light. The other sample was irradiated with a 300 W Xe lamp from ORIEL Instruments (Stratford, CT) for a total of 60 min. The lamp produces a simulated solar radiation with intensity of 7.0 mW/cm^2 visible light, 3.8 mW/cm^2 UVA light, and 0.012 mW/cm^2 UVB light. After each 5 min irradiation, an absorption spectrum was recorded on a Varian CARY 300 UV–vis spectrophotometer. Both a first-order and a second-order kinetic model were used to analyze the degradation based on standard equations: $\ln A_0/A_t = kt$ and $1/[A_t] = kt + 1/[A_0]$. A_0 and A_t are the concentration of 4-Cl-*o*-PDA at time zero and time t, respectively, and k is the rate constant.

2.2.3. Involvement of oxygen

To determine if oxygen is involved in this photoreaction, an aqueous solution of 4-Cl-*o*-PDA (40 μM) was filled into two identical Pyrex glass vials. One solution was purged with argon through sealed septa for 20 min to remove ambient air, and the other not purged. After 40 min of irradiation by the 300 W Xe lamp, the absorption of 4-Cl-*o*-PDA solution under air or argon were recorded on the CARY 300 UV–vis spectrophotometer. The experiment was repeated once and obtained similar result.

2.2.4. Mutagenicity test

The mutagenicity test was carried out with *S. typhimurium* TA102 as previously described [22–24]. The bacteria strain TA102 was selected because it was more resistant to light irradiation than strains TA98 and TA100. This histidine auxotrophic strain contains an ochre mutation in the hisG gene which enables TA102 to readily detect numerous types of mutagens such as X-rays and UV light as well as cross-linking agents such as psoralens and mitomycin C [22]. Test tubes containing the mixture of 20 mM sodium phosphate buffer, TA102, and 4-Cl-*o*-PDA or 2,3-diamino-7-chlorophenazine in DMSO with a volume ratio of 5:1:1 were pre-incubated for 20 min in the gyrorotatory incubator at 210 rpm to homogenize. Then 0.7 ml of this mixture was pipetted into the test tubes containing 2.0 ml of top agar in a Dri-bath at 45 °C. The resulting 2.7 ml mixture was vortexed and poured onto the minimal agar Petri dishes. The final concentrations for the test chemicals in the agar plates were 0, 1, 5, 25, 125 μM. Six plates were prepared at each concentration and the experiment was repeated a second time a week later to ensure quality. The positive control was 8-methoxypsoralen at 10 μg/plate irradiated for 2 min [23,25] and 2053 revertant colonies were found. There were two treatments for each concentration. One was the control without light irradiation while

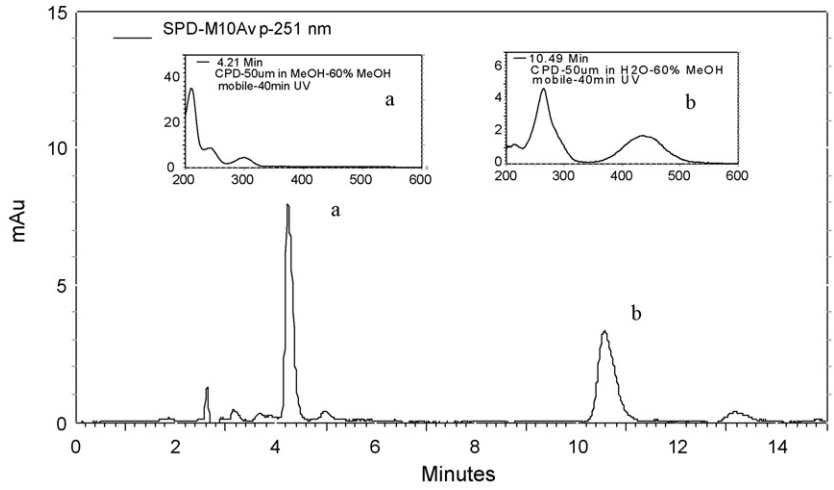

Fig. 1. HPLC chromatogram of 4-chloro-1,2-phenylenediamine after 40 min irradiation. HPLC analysis: column, Phenomenex Luna C18 (250 mm × 4 mm); flow rate, 1 ml/min; mobile phase, 60% MeOH in water. The inserts are the absorption spectra for 4-chloro-1,2-phenylenediamine (a) and 2,3-diamino-7-chlorophenazine (b) captured by the UV/vis photodiode array detector.

the other was irradiated for 15 min by the 300 W Xe lamp described above. The light dose was 3.3 J/cm^2 of UVA and 6.3 J/cm^2 of visible light. After irradiation, the control and light-irradiated Petri dishes were incubated for 48 h at 37 °C before the number of revertant colonies was counted with a colony counter (Bantex, Model 920A).

3. Results

3.1. Photoproduct isolation and structure identification

After irradiation, 4-Cl-*o*-PDA was converted into one main photoproduct as detected by both HPLC and TLC. There are two main peaks shown on HPLC chromatogram (Fig. 1), and both peaks (at 4.25 and 10.56 min) were captured by the photodiode array detector and shown as inserts in Fig. 1. The 4.25 min peak is that of 4-Cl-*o*-PDA and the 10.56 min peak is the spectrum of the main photoproduct. The photoproduct has two absorption bands with maximum absorptions at 264 and 427 nm. This shows that the photoproduct has absorption at longer wavelengths than its parent compound.

The peak at 10.56 min was isolated through silica gel column chromatography and its mass spectrum was obtained. The molecular ion $(M+H)^+$ is at *m/z* 245 and 247 with a relative intensity of 3:1. The two isotopic peaks indicate there is a chlorine atom in the molecule since the natural abundance for chlorine 35 and 37 is 3:1. Thus the molecular mass for the photoproduct is 244/246, indicating that the photoproduct is likely a dimerized 4-Cl-*o*-PDA since 4-Cl-*o*-PDA's molecular weight is 142/144. The ^{1}H NMR spectrum matches the previously published spectrum for 2,3-diamino-7-chlorophenazine, $C_{12}H_9N_4Cl$, obtained through oxidation of 4-Cl-*o*-PDA by H_2O_2 [21]. From an irradiation of 100 mg of 4-Cl-*o*-PDA, 11.4 mg of pure 2,3-diamino-7-chlorophenazine was isolated. This translates into an isolated yield of 13%, and in comparison, the isolated yield is less than 1% for the oxidation of 4-Cl-*o*-PDA by H_2O_2.

3.2. Photoreaction kinetics

Natural sunlight or simulated solar light (300 W Xe) can initiate the photochemical reaction of 4-Cl-*o*-PDA in water and the progress of the reaction was monitored by absorption spectral changes. Fig. 2 shows the absorption spectra of 100 μM 4-Cl-*o*-PDA in water irradiated by sunlight. It can be seen that the absorption band at 209 nm gradually decreases due to light irradiation. Meanwhile, a broad but relatively weak band appears between 400 and 500 nm as well as a new band near 260 nm. As a result, relatively well-defined isobestic points at 226, 248, 295, and 309 nm emerged. Under the same conditions, the absorption changes of 4-Cl-*o*-PDA irradiated by the Xe lamp are similar (not shown).

Assuming first-order degradation kinetics, the plots obtained for $\ln A_0/A_t$ versus time *t* for the initial 40 min of sunlight irradiation or the initial 60 min of Xe lamp irradiation are both curved (Fig. 3A). However, a linear plot was obtained for $1/[A_t]$ versus irradiation time *t*, assuming the reaction follows the second-order kinetic (Fig. 3B). This indicates that the photoreaction is second-order and the reaction rate depends on the concentration of 4-Cl-*o*-PDA to the second power. Two molecules of 4-Cl-*o*-

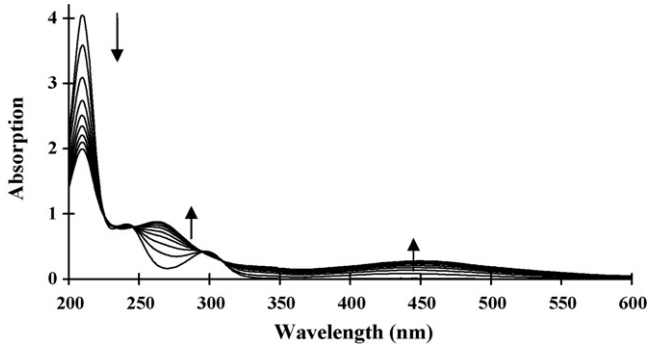

Fig. 2. Photodegradation of 100 μM 4-Cl-1,2-phenylenediamine in aerated aqueous solution by outdoor sunlight for 0, 5, 10, 15, 20, 25, 30, 35, and 40 min, respectively.

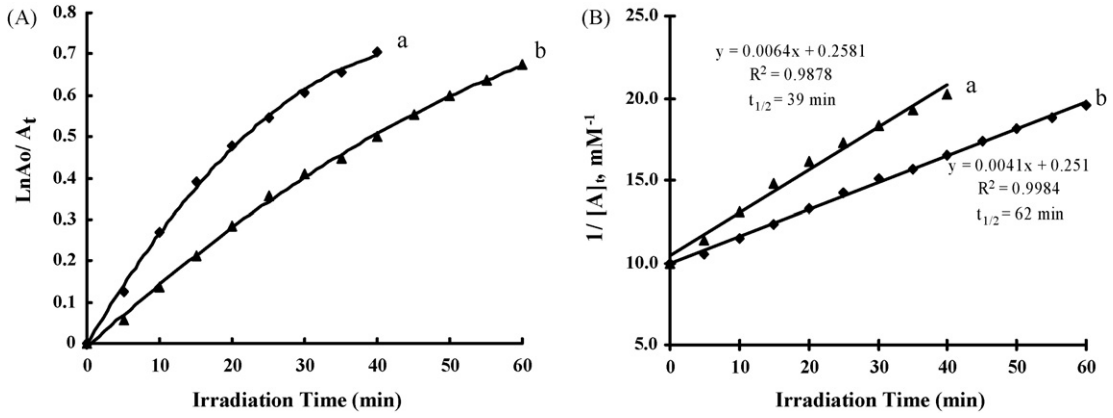

Fig. 3. (A) Plot of $\ln A_0/A_t$ vs. irradiation time (first-order kinetic); (B) plot of $1/[A_t]$ vs. irradiation time (second-order kinetic). Line a: irradiated by sunlight and line b: irradiated by the 300 W Xe lamp.

PDA are involved in forming the photoproduct, 2,3-diamino-7-chlorophenazine, agreeing with the structure of the photoproduct being a dimer of 4-Cl-*o*-PDA. The slope of the plot was obtained and from which the rate constant k and the half-life time ($t_{1/2}$) were calculated, $t_{1/2} = 1/k[A_0]$. For the sunlight irradiation, $k = 0.0064$, $R^2 = 0.9878$, $t_{1/2} = 39$ min. For the Xe lamp irradiation, $k = 0.0041$, $R^2 = 0.9984$, $t_{1/2} = 62$ min. This indicates that the photochemical reaction of 4-Cl-*o*-PDA is faster under natural sunlight than the simulated solar light due to the higher intensity of the natural sunlight in the visible spectrum. Sunlight is composed of 91% visible (>400 nm), 8.7% UVA (320–400 nm), and 0.3% UVB (280–320 nm) light [26]. Comparing with sunlight, the Xe lamp has a weaker visible light output.

3.3. Involvement of oxygen

The solutions of 4-Cl-*o*-PDA in water with 1% methanol, either purged with argon or not purged, were irradiated for 40 min simultaneously. The absorption spectra after irradiation are shown in Fig. 4. The final absorption after irradiation were 1.77, 1.67, and 1.36 at 209 nm, respectively, for the control (peak 1 without light irradiation), argon purged (peak 2), or not purged (peak 3) solutions. Comparing to the absorption of 4-Cl-*o*-PDA not irradiated by light (peak 1), the one purged by argon decreased 5.6% while the one with ambient air decreased 23%. There is a new absorption band near 427 nm for the ambient air sample. This demonstrates that degassing with argon can effectively stop the photoreaction. Therefore, oxygen in ambient air must be involved in the photoreaction to form 2,3-diamino-7-chlorophenazine.

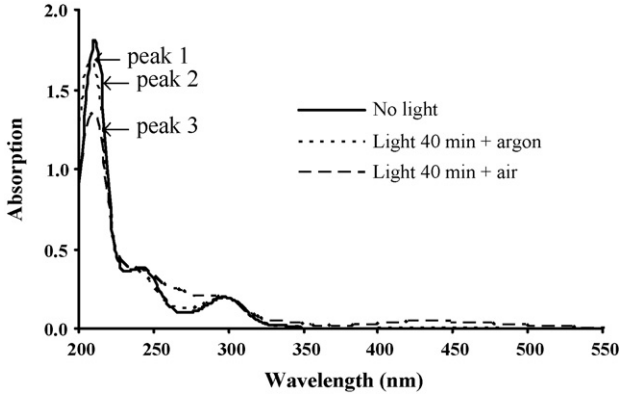

Fig. 4. The UV–vis absorption spectra of 4-chloro-1,2-phenylenediamine solution (40 μM) after 40 min of irradiation (300 W xenon). Peak 1: light irradiation; peak 2: argon purged; peak 3: ambient air.

3.4. Mutagenicity of 4-chloro-1,2-phenylenediamine and 2,3-diamino-7-chlorophenazine

The mutagenicity and photomutagenicity of 4-Cl-*o*-PDA and its photoproduct 2,3-diamino-7-chlorophenazine were tested with *S. typhimurium* TA102. The concentrations of the test compounds were 0, 1, 5, 25, 125 μM. After mixing the bacteria with the chemicals in the agar plates, the plates were irradiated with the 300 W Xe lamp to test for photomutagenicity or kept in the dark to test for mutagenicity. Afterwards, the plates were incubated for 48 h and the number of revertant bacteria colonies was counted. The results from the average of two experiments are plotted in Fig. 5. If more than twice the number of revertant colonies was observed than the negative control, a positive mutagenic response is scored. The positive control, 8-methoxypsoralen, is used for indication of proper experimental conditions for a positive mutagenic response.

It can be clearly seen from Fig. 5 that neither 4-Cl-*o*-PDA alone without light irradiation nor light irradiation alone (light-irradiated experiments at concentration 0 μM) was mutagenic to *S. typhimurium* TA102 under the conditions used in our experiment. Concomitant exposure to 4-Cl-*o*-PDA and light irradiation is photomutagenic to TA102. The number of revertant colonies is 2.7, 3.8, 3.9 and 2.8 times of the control (with light irradiation) at 1, 5, 25 and 125 μM, respectively. Between the concentrations of 5 and 25 μM, the number of revertant colonies reaches a plateau, but decreases at 125 μM to the same level as the concentration of 1 μM. This indicates that 4-Cl-*o*-PDA is photomutagenic between the concentrations of 5 and 125 μM and acutely toxic at the highest concentration. Under the same conditions, 2,3-diamino-7-chlorophenazine is mutagenic either with or without light irradiation. The number of revertant colonies

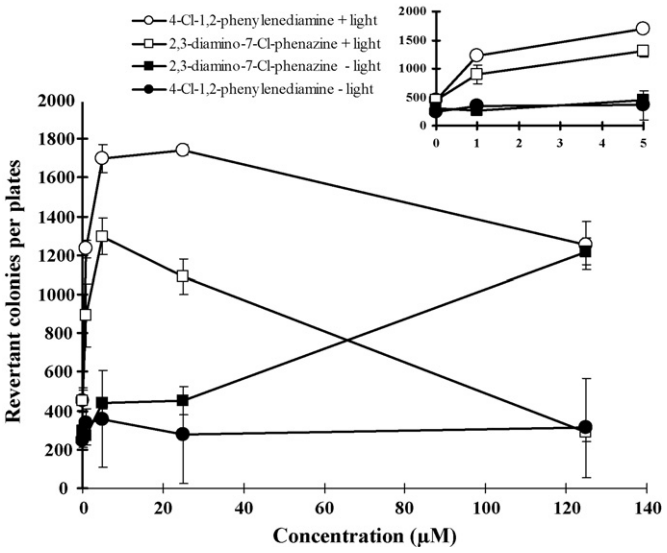

Fig. 5. Photomutagenicity test of 4-Cl-1,2-phenylenediamine and 2,3-diamino-7-Cl-phenazine with *S. typhimurium* TA102. Samples were irradiated with the 300 W Xe lamp. The 8-methoxypsoralen positive control produced 2053 revertant colonies. Insert plots the concentration range of 0–5 μM.

due to concomitant exposure to 2,3-diamino-7-chlorophenazine and light irradiation reaches the highest at 5 μM, 2.9 times of the negative control, but decreases at the next two higher concentrations and reaches the level of the negative control at 125 μM. This means that the concomitant exposure to 2,3-diamino-7-chlorophenazine and light causes not only photomutagencity, but also relatively strong phototoxicity in TA102. In addition, 2,3-diamino-7-chlorophenazine is mutagenic to TA102 even in the dark at the highest concentration. The number of revertant colonies is 4 times of the negative control at 125 μM. This indicates that 2,3-diamino-7-chlorophenazine is photomutagenic at lower concentrations, and phototoxic and mutagenic at the highest concentration tested.

4. Discussion

4-Cl-*o*-PDA as a precursor for manufacturing of dyes may come in contact with hair, skin, and eyes of humans. Although there is no study on skin absorption of 4-Cl-*o*-PDA, there have been reports on skin absorption of aromatic amines as well as phenylenediamines (PDA) including *o*-PDA [27–29]. *o*-PDA can easily penetrate the human skin. We can assume that 4-Cl-*o*-PDA would penetrate the skin similar to that of *o*-PDA. Once in the skin, 4-Cl-*o*-PDA is unavoidably exposed to light irradiation, and light-induced chemical reaction and toxicity are possible. Solar light is composed of about 90% visible light and 10% UV light. Visible light can penetrate all the way through the skin into the circulating blood, while the UVA component can penetrate into the dermis [30]. It is no doubt that those compounds in the human skin can receive light irradiation. 4-Cl-*o*-PDA is a compound that is very light sensitive and the photoreaction rate under sunlight is rapid, producing a dimeric product 2,3-diamino-7-chlorophenazine. The dimerization is initiated through photo-excitation of 4-Cl-*o*-PDA and oxidation by molecular oxygen in ambient air. Although oxygen is not in the final photoproduct, it is involved in the dimerization process that removes four protons plus one HCl from the two 4-Cl-*o*-PDA molecules. This photochemical reaction of 4-Cl-*o*-PDA is similar to that of *o*-PDA, which forms 2,3-diaminophenazine upon direct or sensitized photoreaction [31–34] or enzymatic oxidation [35]. It is proposed for *o*-PDA that the formation of the diimine is the first step toward the photoproduct. Use of tetramethylphenylenediamine or *N*,*N*′-diphenyl-*p*-PDA, the diimine intermediate was detected [36,37]. The step leading to the diimine is through oxidation by oxygen, either by singlet oxygen [33,37] or radical ion pair between oxygen anion radical and the 4-Cl-*o*-PDA cation radical [34]. Chemical/enzymatic oxidation of *o*-PDA leading to 2,3-diaminophenazine is also proposed to be via the diimine intermediate [21,35,38]. Thus

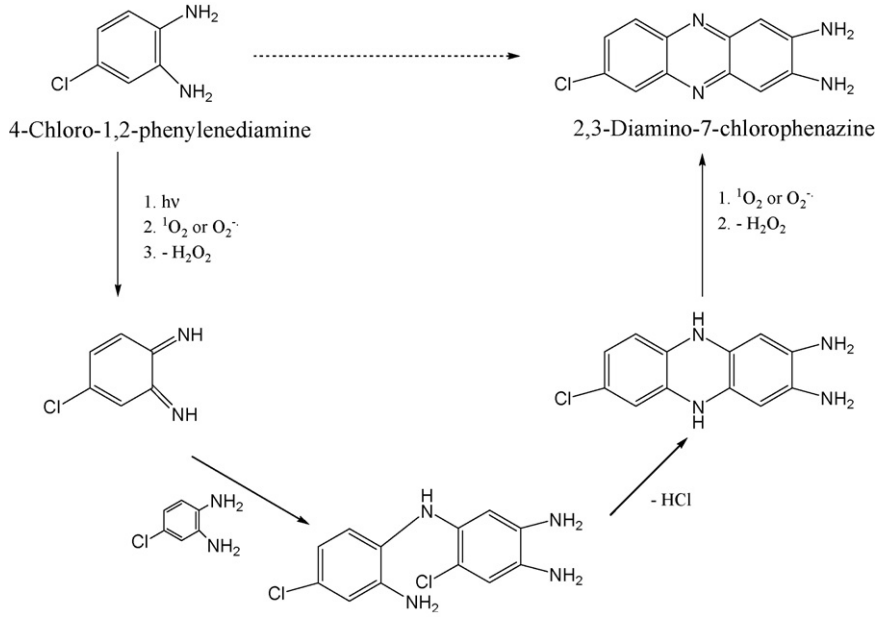

Fig. 6. Photo-oxidation of 4-chloro-1,2-phenylenediamine in aerated aqueous solution.

the formation of 2,3-diamino-7-chlorophenazine is proposed in Fig. 6. In short, 4-Cl-*o*-PDA absorbs a photon and either transfers the energy to an oxygen molecule to form singlet oxygen, $^1O_2$, or transfers an electron to form an oxygen anion radical, $O_2^{\bullet -}$. Either $^1O_2$ or $O_2^{\bullet -}$ oxidizes 4-Cl-*o*-PDA to form the diimine. The diimine would react quickly with another 4-Cl-*o*-PDA to dimerize. The dimer further loses one HCl molecule and cyclizes to form 7-chloro-2,3-diamino-5,10-dihydrophenazine, which is further oxidized to form the phenazine final product.

The photoproduct 2,3-diamino-7-chlorophenazine is more stable under light irradiation. Hence, exposure of 4-Cl-*o*-PDA to sunlight can quickly covert it into 2,3-diamino-7-chlorophenazine and the toxicity and photochemical properties of 2,3-diamino-7-chlorophenazine must be considered when evaluating the risk of 4-Cl-*o*-PDA as it is done for other aromatic compounds [39–41].

Both 4-Cl-*o*-PDA and 2,3-diamino-7-chlorophenazine are photomutagenic to *S. typhimurium* TA102. The number of revertant colonies caused by 4-Cl-*o*-PDA between 5 and 25 μM is 1.3 times of that caused by 2,3-diamino-7-chlorophenazine at 5 μM. 2,3-Diamino-7-chlorophenazine also exhibits mutagenicity without light irradiation and strong phototoxicity at higher doses (25 and 125 μM). Similarly, 2,3-diaminophenazine was mutagenic [21,42] and can cause light-induced DNA strand cleavage [43].

2,3-Diamino-7-chlorophenazine was first isolated and reported by Watanabe et al. as one of the many oxidation products of 4-Cl-*o*-PDA by H_2O_2 in ethanol [21]. Under that condition, the isolated yield is less than 1% for the two-day reaction. Under our experimental conditions, the isolated yield for 2,3-diamino-7-chlorophenazine is 13%. Comparing with the H_2O_2 oxidation reaction, the photochemical reaction has a much shorter reaction time, less complex products, uses water as solvent instead of ethanol, and has much higher isolated yield (13% versus <1%). Therefore, it is possible to use the photoreaction of 4-Cl-*o*-PDA in an aqueous solution as a synthetic method for 2,3-diamino-7-chlorophenazine.

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