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**Synthesis and *in situ* behavior of 1,4- and 2,5-<sup>13</sup>C  
isotopomers of *p*-phenylenediamine in reconstructed  
human epidermis using high resolution magic angle  
spinning NMR**

Hassan Srour,<sup>†</sup> Alexis Gosset,<sup>‡</sup> François-Marie Moussallieh,<sup>§</sup> Karim Elbayed,<sup>||</sup>

Elena Giménez-Arnau<sup>‡</sup> and Jean-Pierre Lepoittevin<sup>\*,‡</sup>

<sup>†</sup> University of Strasbourg, CNRS, Institute of Chemistry UMR 7177, F-67081 Strasbourg cedex, France. Present address: Charnwood Molecular LTD, Charnwood campus, Loughborough Leicestershire LE11 5RD, UK.

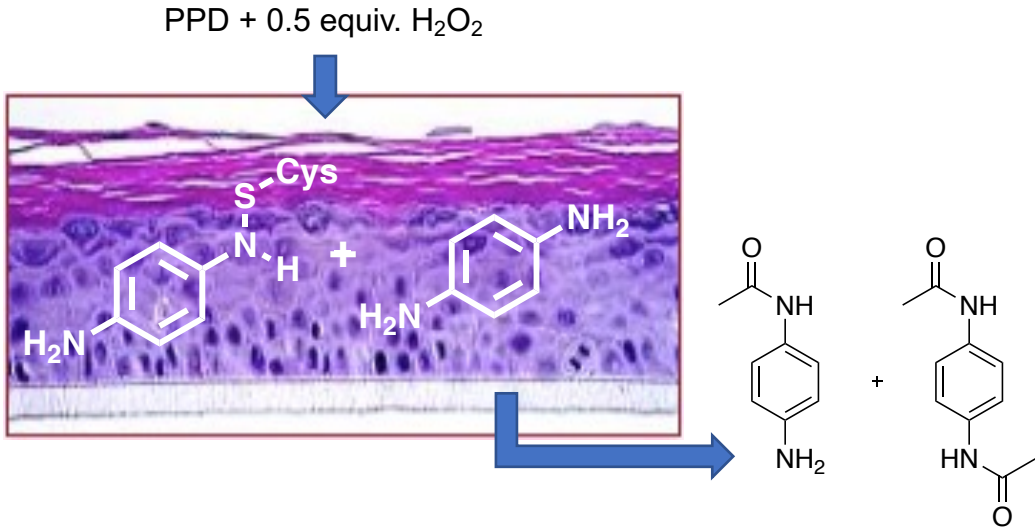
<sup>‡</sup> University of Strasbourg, CNRS, Institute of Chemistry UMR 7177, F-67081 Strasbourg cedex, France

<sup>§</sup> University of Strasbourg, CNRS, Institute of Chemistry UMR 7177, F-67081 Strasbourg cedex, France. Present address: University Paris-Saclay, CNRS, ICMMO UMR 8182, F-91405 Orsay, France

<sup>||</sup> University of Strasbourg, CNRS, ICube UMR 7357, F-67412 Illkirch cedex, France



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## Abstract

*Para*-phenylenediamine (PPD) has been classified as a strong skin allergen but when it comes to toxicological concerns, benzoquinone diamine (BQDI), the primary oxidation derivative of PPD, is frequently considered and was shown to covalently bind nucleophilic residues on model peptides. However, tests in solution are far from providing a reliable model as the cutaneous metabolism of PPD is not covered. We now report the synthesis of two <sup>13</sup>C substituted isotopomers of PPD, 1,4-(<sup>13</sup>C)*p*-phenylenediamine **1** and 2,5-(<sup>13</sup>C)*p*-phenylenediamine **2**, and the investigation of their reactivity in reconstructed human epidermis (RHE) using high resolution magic angle spinning (HRMAS) NMR technique. RHE samples were first treated with **1** or **2** and incubated for 1 to 48 h. Compared to the control, spectra clearly showed only the signals of **1** or **2** gradually decreasing with time to disappear after 48 h of incubation. However, the culture media of RHE incubated with **1** for 1 h and 24 h, respectively, showed the presence of both monoacetylated- and diacetylated-PPD as major products. Therefore, the acetylation reaction catalyzed by *N*-acetyltransferase (NAT) enzymes appeared to be the main process taking place in RHE. With the aim of increasing the reactivity by oxidation, **1** and **2** were treated with 0.5 equiv. H<sub>2</sub>O<sub>2</sub> prior to their application to RHE and incubated for different times. Under these conditions, new peaks having close chemical shifts to those of PPD-cysteine adducts previously observed in solution were detected. Under such oxidative conditions, we were thus able to detect and quantify cysteine adducts in RHE (maximum of 0.2 nmol/mg of RHE at 8 h of incubation) while no reaction with other nucleophilic amino acid residues could be observed.

# 1. Introduction

*Para*-phenylenediamine (PPD) is an aromatic amine widely used in permanent hair dye formulations but also found in temporary black henna tattoos.<sup>1</sup> While PPD is colorless, dyes containing PPD acquire their coloring potential by a complex mechanism induced either by air oxidation in the case of temporary tattoos or by using an oxidizing agent such as hydrogen peroxide in the case of permanent hair dyes. Several of these oxidation products have been shown to induce skin allergy in animals and humans<sup>2-4</sup> and based on the mouse local lymph node assay<sup>5</sup> (LLNA) and guinea pig<sup>6</sup> sensitization tests, PPD was classified as a strong skin allergen. Allergy induced by contact with PPD is characterized by clinical symptoms ranging from burning sensation, redness or rash to severe swelling of the face. The estimated percentage of women who have been using hair dye containing PPD at least one's in their life exceeds 50% and hairdressers, often subjected to daily handling of PPD, are very exposed to occupational contact dermatitis.<sup>7</sup> Therefore, the EU Cosmetics Regulation has set limit values for the maximal concentration of PPD in hair dyes to 6%.<sup>8</sup> Despite that, hair dye related skin allergy remains prevalent especially with the rapid emergence of new formulations containing PPD and the worldwide growing popularity of hair dyeing. In addition to skin allergy there are also scientific reports pointing the mutagenicity, carcinogenicity and toxicity of PPD.<sup>9</sup>

As there is no treatment for skin allergy other than symptomatic, prevention remains the only strategy. The ability of a chemical to react with epidermal proteins to form stable antigenic adducts is believed to be the molecular initiating event of skin sensitization.<sup>10-12</sup> This concept already postulated by Landsteiner and Jacobs 80 years ago<sup>13</sup> is now widely accepted by toxicologists and immunologists. However, PPD is chemically unreactive toward nucleophilic

residues in proteins and falls within the class of those compounds named pre-haptens,<sup>14</sup> which need prior oxidation to become sensitizers. During non-enzymatic oxidation, PPD is converted into reactive/electrophilic species capable of covalently binding to epidermal proteins. The oxidation process is triggered by a simple reaction with molecular oxygen or using an oxidizing agent such as hydrogen peroxide or other oxidants. In the case of PPD related skin allergy, our current chemical knowledge is limited to studies using peptides<sup>15-18</sup> containing one or more nucleophilic amino acids or proteins in solution,<sup>19-21</sup> as models for epidermal proteins. These experiments were shown to be very useful in identifying both partners *i.e.* the amino acids and the oxidation derivatives of PPD involved in these interactions. When it comes to toxicological concerns, benzoquinone diimine (BQDI), the primary oxidation derivative of PPD, is frequently evoked. Being highly electrophilic/reactive, BQDI was shown to covalently bind nucleophilic residues on model peptides<sup>15</sup> and to undergo self-conjugation with two units of PPD (Scheme 1) via sequential oxidation/addition reactions to form a trimer, namely the Bandrowski's base (BB), also classified as an extreme sensitizing chemical according to the LLNA.<sup>5,22</sup>

The reactivity of a PPD related compound, namely 2,5-(<sup>13</sup>CH<sub>3</sub>)*p*-benzoquinonediimine, toward amino acids and model peptides in solution was previously reported by Eilstein et al.<sup>23,24,25</sup> and the ability of this chemical model to bind covalently to cysteine, lysine and tryptophan residues was demonstrated by combining NMR and LC-MS/MS techniques. However, tests in solution seem to be far from providing a reliable model when compared to *in vivo* chemical proteins interactions. Another aspect which is not covered by the model experiments is the cutaneous metabolism of PPD<sup>26</sup> ensured mainly by *N*-acetyltransferase 1 (NAT1) and *N*-acetyltransferase 2 (NAT2) enzymes leading to monoacetylated and diacetylated

PPD. This detoxification pathway can indeed compete with the oxidation process of PPD<sup>27</sup> and its subsequent interaction with epidermal proteins. As a part of our efforts to better understand the chemistry taking place in the human skin at the molecular level during the sensitization process, we have developed a new method based on the use of high resolution magic angle spinning (HRMAS) NMR technique to monitor *in situ* reactions of <sup>13</sup>C substituted chemical sensitizers with nucleophilic amino acids of epidermal proteins in reconstructed human epidermis (RHE)<sup>28-30</sup> as well as their metabolism.<sup>31</sup> The use of a 3D reconstructed skin model makes it possible to test chemicals under conditions close to human use (topical application) and benefits from metabolizing enzymes and the three-dimensional microenvironment. We thus decided to further investigate the *in situ* reactivity of PPD in RHE by HRMAS NMR. Herein, we report the challenging synthesis of two <sup>13</sup>C substituted isotopomers of PPD (Chart 1), 1,4-(<sup>13</sup>C)*p*-phenylenediamine **1** and 2,5-(<sup>13</sup>C)*p*-phenylenediamine **2**, and the investigation of their reactivity in RHE used as model of human epidermis.

## 2. Materials and Methods

**Caution:** Skin contact with PPD must be avoided, as an extreme sensitizing substance, this compound must be handled with care.

### 2.1. Chemicals and reagents

2-(<sup>13</sup>C)Acetone, (<sup>13</sup>C)nitromethane and deuterated solvents were purchased from Eurisotop (Saint-Aubin, France). Acetone (99.8%, for analysis) used for the preparation of (<sup>13</sup>C)PPD solutions was purchased from Carlo Erba (Val de Reuil, France). Deionized water was obtained

by filtration on ion exchange cartridges (R3, M3 and ORC, Fisher Bioblock Scientific, Illkirch, France). Air- or moisture-sensitive reactions were carried out in flame-dried glassware under an argon atmosphere. Dichloromethane, tetrahydrofuran and diethyl ether were dried by passing through active alumina under an argon pressure using Glass Technology GTS100 devices. *tert*-Butanol was dried by refluxing with activated 3Å molecular sieves and further distillation under argon atmosphere. *N,N*-Diisopropylethylamine was refluxed with potassium hydroxide and distilled prior to use. All extractive procedures were performed using technical grade solvents. All other chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless otherwise noted and used without further purification. Analytical thin layer chromatography (TLC) was carried out on Merck 60 F<sub>254</sub> aluminium backed silica gel plates. Short-wave UV radiation (254 nm) and if necessary phosphomolybdic acid solution were used to visualise components. Compounds were purified by flash column chromatography using Merck silica gel 60 (0.040-0.063 mm). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on Bruker 300, 400 or 500 MHz spectrometers. Spectra were recorded in deuterated chloroform referenced to residual CHCl<sub>3</sub> (<sup>1</sup>H, 7.27 ppm; <sup>13</sup>C, 77.2 ppm) or in deuterated dimethyl sulfoxide referenced to residual (CH<sub>3</sub>)<sub>2</sub>SO (<sup>1</sup>H, 2.5 ppm; <sup>13</sup>C, 39.5 ppm). Chemical shifts ( $\delta$ ) are reported in part per million (ppm) and coupling constants (*J*) are reported in Hz. Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), integration and coupling constant (*J* in Hz). Assignments were determined either on the basis of unambiguous chemical shifts or coupling patterns. DEPT-135, COSY, HSQC or HMBC experiments were performed when necessary to complete spectral attribution for related compounds.

## 2.2. Synthesis of 1,4-(<sup>13</sup>C)*p*-phenylenediamine 1

2.2.1. *1,1,5,5-Tetraethoxy-[3-(<sup>13</sup>C)]pentan-3-one* **3**. A solution of BF<sub>3</sub>·OEt<sub>2</sub> (14 mL, 108 mmol, 6 eq.) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (12 mL) was added dropwise over 15 min to triethylorthoformate (13.35 g, 15 mL, 90 mmol, 4.5 eq.) at -30 °C. The reaction mixture was warmed up to 0 °C, and stirred for 20 min to give slurry of tetraethoxycarbenium fluoroborate. The mixture was then cooled to -78 °C and 2-(<sup>13</sup>C)acetone (1.1 mL, 15 mmol, 1 eq.) was added. *N,N*-Diisopropylethylamine (14.5 mL, 85 mmol, 4.5 eq.) was then added dropwise over 10 min while maintaining an efficient stirring and the resulting reaction mixture was stirred at -78 °C for 2 h. The reaction mixture was then poured into a concentrated NaHCO<sub>3</sub> solution (90 mL) and stirred vigorously for 10 min at room temperature. The resulting mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 30 mL). The combined organic layers were washed successively with ice cold 1 molar solution of H<sub>2</sub>SO<sub>4</sub> (17 mL) and cold water (2 x 30 mL) and dried over MgSO<sub>4</sub>. Removal of the solvent at reduced pressure gave the crude ketone which was purified flash chromatography on silica gel: eluent (pentane/ethyl acetate: 8/2) to give the pure product **3** (3.1 g, 12 mmol, 80% yield); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.18 (t, 12H, <sup>3</sup>J<sub>HH</sub> = 3.15 Hz, CH<sub>3</sub>), 2.78 (d, 4H, <sup>3</sup>J<sub>HH</sub> = 5.62 Hz, CH<sub>2</sub>), 3.47-3.70 (m, 8H, OCH<sub>2</sub>), 4.90 (t, 2H, <sup>3</sup>J<sub>HH</sub> = 5.57 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 15.4 (4 x CH<sub>3</sub>), 48.6 (d, <sup>1</sup>J<sub>CC</sub> = 40,4 Hz, 2 x CH<sub>2</sub>CO), 62.4 (4 x OCH<sub>2</sub>), 99.7 (2 x CH), 205.0 (<sup>13</sup>CO). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>12</sub><sup>13</sup>CH<sub>26</sub>NaO<sub>5</sub> [M+Na<sup>+</sup>] 286.1711; found: 286.1706.

2.2.2. *1-(<sup>13</sup>C)Pyran-4-one* **4**. A solution of **3** (12 g, 45.6 mmol) in ethanol (225 mL) and 10% aqueous HCl (35 mL) was heated at 80 °C for 24 h. Solvents were then removed under reduced pressure and the resulting residue dissolved in water (220 mL) was extracted with toluene (3 x 220 mL). The aqueous layers were filtered and treated with charcoal and filtered through celite. Then the solvent was evaporated at reduced pressure (at 40 °C) to give 1-(<sup>13</sup>C)pyran-4-one **4** as a

brown crystalline product (3 g, 31 mmol, 68% yield); Mp 32-32.5 °C (lit unlabeled 32.5 °C).<sup>32</sup> <sup>1</sup>H NMR (500 MHz, C<sub>6</sub>D<sub>6</sub>) δ 5.83 (d, 2H, <sup>3</sup>J<sub>HH</sub> = 6.36 Hz, CH), 6.42 (d, 2H, <sup>3</sup>J<sub>HH</sub> = 6.36 Hz, CH). <sup>13</sup>C NMR (125 MHz, C<sub>6</sub>D<sub>6</sub>): δ 118.3 (d, <sup>1</sup>J<sub>CC</sub> = 53.79 Hz, 2 x CH), 154.6 (d, <sup>2</sup>J<sub>CC</sub> = 3.31 Hz, 2 x CH), 176.5 (<sup>13</sup>CO). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>CH<sub>5</sub>O<sub>2</sub> [M+H]<sup>+</sup> 98.0323; found: 98.0324.

**2.2.3. 1,4-(<sup>13</sup>C)*p*-Nitrophenol 5.** To a suspension of **4** (3 g, 30 mmol, 1eq.) in dry *tert*-butanol (50 mL) was added (<sup>13</sup>C)nitromethane (2 g, 32 mmol, 1.1 eq.). The solution was heated to reflux under argon. Then, potassium *tert*-butoxide (30 mL, 30 mmol) (1M solution in *tert*-butanol) in an additional volume (35 mL) of dry *tert*-butanol, was added dropwise. The resulting mixture was heated for 2 h under reflux after which HCl 1M (45 mL) was added. The solution was then heated again under reflux for a further 1 h. The solvent was removed under reduced pressure and water (50 mL) was added to the residue. The aqueous phase was extracted with dichloromethane (4 x 100 mL) then the combined organic layers were washed with water (100 mL) and brine (100 mL) then dried over MgSO<sub>4</sub>. The solvent was removed at reduced pressure to give the crude product which was purified by flash chromatography on silica gel, eluent: (pentane/ethyl acetate:3/1) to give 1,4-(<sup>13</sup>C)*p*-nitrophenol **5** (2.7 g, 19 mmol, 66 % yield) as a brown solid. Mp 109.5-110°C (lit unlabeled 109-110 °C)<sup>33</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 5.72 (d, 1H, <sup>2</sup>J<sub>HC</sub> = 2.35 Hz, OH), 6.92 ((ddd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.14 Hz, <sup>2</sup>J<sub>CH</sub> = 9.27 Hz, <sup>3</sup>J<sub>CH</sub> = 2.4 Hz, CH), 8.18 ((ddd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.14 Hz, <sup>2</sup>J<sub>CH</sub> = 9.19 Hz, <sup>3</sup>J<sub>CH</sub> = 3.8 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 115.8 (dd, <sup>1</sup>J<sub>CC</sub> = 65.67 Hz, 2 x CH), 126.4 (d, <sup>1</sup>J<sub>CC</sub> = 68.37 Hz, 2 x CH), 141.9 (d, <sup>3</sup>J<sub>CC</sub> = 9.04 Hz, <sup>13</sup>CNO<sub>2</sub>), 161.2 (d, <sup>3</sup>J<sub>CC</sub> = 9.04 Hz, <sup>13</sup>COH). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>4</sub>NO<sub>3</sub> [M-H]<sup>-</sup> 140.0258; found: 140.0254

**2.2.4. 1,4-(<sup>13</sup>C)*p*-Nitroaniline 6.** A suspension of **5** (500 mg, 3.54 mmol, 1 eq.), 2-chloroacetamide (596 mg, 5.31 mmol, 1.5 eq.), potassium carbonate (1.47 g, 10.6 mmol, 3 eq.) and potassium iodide (147 mg, 0.89 mmol, 0.25 eq.) in DMF (10 mL) was heated at 90 °C for 2 hours (until the starting material is completely consumed), then heated to reflux for 2.5-3 h. The solvent was evaporated to give a crude product which was purified by flash chromatography on silica gel, eluent (dichloromethane/methanol: 99/1) to afford the pure 1,4-(<sup>13</sup>C)*p*-nitroaniline **6** (410 mg, 2.9 mmol, 82% yield) as a yellow solid. Mp 112-113.5 °C (lit unlabeled 113-114 °C)<sup>34</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 4.37 (br s, 2H, NH<sub>2</sub>), 6.62 (dd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.06 Hz, <sup>2</sup>J<sub>CH</sub> = 9.17 Hz, CH), 8.07 ((ddd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.06 Hz, <sup>2</sup>J<sub>CH</sub> = 8.87 Hz, <sup>3</sup>J<sub>CH</sub> = 3.68 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 113.5 (d, <sup>1</sup>J<sub>CC</sub> = 65.67 Hz, 2 x CH), 126.5 (d, <sup>1</sup>J<sub>CC</sub> = 67.93 Hz, 2 x CH), 139.3 (d, <sup>3</sup>J<sub>CC</sub> = 8.42 Hz, <sup>13</sup>CNO<sub>2</sub>), 152.6 (d, <sup>3</sup>J<sub>CC</sub> = 8.55 Hz, <sup>13</sup>CNH<sub>2</sub>). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>7</sub>N<sub>2</sub>O<sub>2</sub> [M+H]<sup>+</sup> 141.0575; found: 141.0582.

**2.2.5. 1,4-(<sup>13</sup>C)*p*-Phenylenediamine 1.** To a suspension of **6** (200 mg, 1.43 mmol, 1 eq.) and PdCl<sub>2</sub> (25 mg, 0.14 mmol, 0.1 eq.) under argon in dry EtOH (5 mL) was added triethylsilane (663 mg, 5.72 mmol, 4 eq.). The mixture was allowed to stir under argon at room temperature for 1 h. Flash chromatography on silica gel, eluent: AcOEt, gave the desired pure 1,4-(<sup>13</sup>C)*p*-phenylenediamine **1** as a white solid (142 mg, 90% yield). Mp 145-146 °C (lit unlabeled 143-145 °C)<sup>34</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 4.37 (br s, 4H, NH<sub>2</sub>), 6.57 (d, 4H, <sup>2</sup>J<sub>CH</sub> = 7 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 116.8 (dd, <sup>1</sup>J<sub>CC</sub> = 64.31 Hz, <sup>2</sup>J<sub>CC</sub> = 1.93 Hz, 4 x CH), 138.7 (2 x <sup>13</sup>CNH<sub>2</sub>). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>9</sub>N<sub>2</sub> [M+H]<sup>+</sup> 111.0832; found: 111.0823.

### **2.3. Synthesis of 2,5-(<sup>13</sup>C)*p*-phenylenediamine 2**

**2.3.1. 1,4-(<sup>13</sup>C)-1-Chloro-4-nitrobenzene 7.** To a two necked flask containing phosphorous pentachloride (3.9 g, 18.7 mmol, 1 eq.) under argon, was added phenol (5.28 g, 56.2 mmol, 3 eq.). The mixture was heated at 100 °C for 4 h. Then, **5** (2.63 g, 18.7 mmol, 1 eq.) was added to the cooled product which was heated again for 4 h at 100 °C. The crude tetraaryloxyphosphorous monochloride was then decomposed by heating at 200 °C for 20 minutes. Water was added and the product was extracted with diethylether (4 x 30 mL), washed with water (30 mL) and then with brine (30 mL). The combined organic layers were dried over MgSO<sub>4</sub>. Solvents were removed at reduced pressure to give the crude product which was purified by flash chromatography on silica gel, eluent: (pentane:EtOAc /95:5) gave pure 1,4-(<sup>13</sup>C)-1-chloro-4-nitrobenzene **7** as an off-white solid (2.14 g, 13.4 mmol, 72% yield). Mp 81-82 °C (lit unlabeled 79-81 °C)<sup>35</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.52 (ddd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.12 Hz, <sup>2</sup>J<sub>CH</sub> = 9.25 Hz, <sup>3</sup>J<sub>CH</sub> = 3.30 Hz, CH), 8.18 (ddd, 2H, <sup>3</sup>J<sub>HH</sub> = 9.12 Hz, <sup>2</sup>J<sub>CH</sub> = 9.98 Hz, <sup>3</sup>J<sub>CH</sub> = 3.90 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 125.1 (dd, <sup>1</sup>J<sub>CC</sub> = 68.48 Hz, <sup>2</sup>J<sub>CC</sub> = 0.76 Hz, 2 x CH), 129.7 (d, <sup>1</sup>J<sub>CC</sub> = 65.14 Hz, 2 x CH), 141.5 (d, <sup>3</sup>J<sub>CC</sub> = 9.88 Hz, <sup>13</sup>CNO<sub>2</sub>), 146.6 (d, <sup>3</sup>J<sub>CC</sub> = 9.88 Hz, <sup>13</sup>CCl). HRMS GC-FI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>4</sub>ClNO<sub>2</sub> [M<sup>+</sup>] 158.9997; found: 159.0003.

**2.3.2. 1,4-(<sup>13</sup>C)-1,4-Dichlorobenzene 8.** Chlorine gas was bubbled into a solution of dichlorophenylphosphine (4 g, 21.6 mmol, 1.9 eq.) in phenylphosphonic dichloride (24 mL) during 30 min while maintaining the temperature below 30 °C. The initially colorless solution turned pale yellow but remained clear. To this mixture was added **7** (1.8 g, 11.3 mmol, 1 eq.) and the reaction was heated at 150 °C overnight. The cooled reaction was then poured onto crushed ice/water (170 mL) and neutralized with 50% aqueous sodium hydroxide. After extraction with ether (170 mL), the ether extract was washed with brine and dried over MgSO<sub>4</sub>.

Flash chromatography on silica gel, eluent: pentane, gave the pure desired 1,4-(<sup>13</sup>C)-1,4-dichlorobenzene **8** (907 mg, 6.1 mmol, 54% yield). Mp 48-51 °C (lit unlabeled 47-50 °C)<sup>36</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.27 (d, 4H, <sup>2</sup>J<sub>CH</sub> = 7 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 130.0 (dd, <sup>1</sup>J<sub>CC</sub> = 67.84 Hz, <sup>2</sup>J<sub>CC</sub> = 2.49 Hz, 4 x CH), 132.7 (s, 2 x <sup>13</sup>CCl). HRMS GC-FI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>4</sub>Cl<sub>2</sub> [M<sup>+</sup>] 147.9757; found: 147.9747.

**2.3.3. 1,4-(<sup>13</sup>C)-1,4-Dichloro-2,5-diiodobenzene 9.** To a solution of **8** (170 mg, 1.1 mmol, 1 eq.) and potassium iodate (171 mg, 0.8 mmol, 0.8 eq.) in 2 mL of concentrated sulfuric acid (96%) and 5 mL of glacial acetic acid, was added elemental iodine (405 mg, 1.6 mmol, 1.6 eq.). The resulting purple mixture was stirred at 60 °C for 16 h during which the sublimed iodine was intermittently washed back into the reaction mixture by shaking the flask. The resulting mixture was cooled to room temperature and poured into ice water (20 mL), filtered and washed with an aqueous solution of sodium hydroxide (1 M) then with water. The white solid obtained was further dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and washed twice with an aqueous solution of sodium hydroxide (1M) (50 mL) and dried over MgSO<sub>4</sub>. The crude product was recrystallized in a mixture of CH<sub>2</sub>Cl<sub>2</sub>/MeOH to give the pure desired product 1,4-(<sup>13</sup>C)-1,4-dichloro-2,5-diiodobenzene **9** as a white solid (434 mg, 1.08 mmol, 95% yield). Mp 118-119 °C (unlabeled 118-120 °C); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.88 (d, 2H, <sup>2</sup>J<sub>CH</sub> = 6.82 Hz, CH). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 97.9 (d, <sup>1</sup>J<sub>CC</sub> = 70.47 Hz, 2 x CI), 137.8 (2 x <sup>13</sup>CCl), 139.2 (d, <sup>1</sup>J<sub>CC</sub> = 65.75 Hz, 2 x CH). HRMS GC-FI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>2</sub>Cl<sub>2</sub>I<sub>2</sub> [M<sup>+</sup>] 399.7690; found: 399.7690.

**2.3.4. 2,5-(<sup>13</sup>C)-2,5-Dichlorobenzene-1,4-diamine 10.** To a Schlenk tube containing CuI (240 mg, 1.26 mmol, 27 eq.), Fe<sub>2</sub>O<sub>3</sub> (200 mg, 1.25 mmol, 27 eq.) and **9** (900 mg, 2.24 mmol, 1 eq.) under argon, were added 25 mL of EtOH followed by NH<sub>3</sub>·H<sub>2</sub>O (75 mmol, 28% in water, 16 eq.) and

NaOH (800 mg, 20 mmol, 4.5 eq.). The tube was sealed and the reaction mixture was heated for 10 h at 90 °C. Then, the resulting red suspension was cooled to room temperature, extracted with diethyl ether (3 x 50 ml). The combined extracts were washed with brine (50 mL) to give the crude product which was purified by flash chromatography on silica gel, eluent: (pentane:AcOEt/9:1) followed by recrystallization in EtOH to afford the pure desired product 2,5-(<sup>13</sup>C)-2,5-dichlorobenzene-1,4-diamine **10** as a pinkish white powder (220 mg, 1.2 mmol, 55% yield). Mp 162°C (lit unlabeled 164 °C)<sup>37</sup>; <sup>1</sup>H NMR (400 MHz, DMSO): δ 6.75 (m, 2H). <sup>13</sup>C NMR (100 MHz, DMSO): δ 115.7 (d, <sup>1</sup>J<sub>CC</sub> = 73.69 Hz, <sup>2</sup>J<sub>CC</sub> = 3.91 Hz 2 x CH), 117.3 (2 x <sup>13</sup>CCl), 135.9 (d, <sup>1</sup>J<sub>CC</sub> = 70.47 Hz, <sup>2</sup>J<sub>CC</sub> = 1.67 Hz, 2 x CNH<sub>2</sub>). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>7</sub>Cl<sub>2</sub>N<sub>2</sub> [M+H]<sup>+</sup> 179.0053; found: 179.0054.

**2.3.5. 2,5-(<sup>13</sup>C)-*p*-Phenylenediamine 2.** To a mixture of **10** (170 mg, 0.95 mmol, 1 eq.) and Pd/C (10%) (48 mg) in MeOH (6 mL) was added NH<sub>2</sub>NH<sub>2</sub>·H<sub>2</sub>O (0.6 mL, 12 mmol, 12.6 eq.). The reaction mixture vessel was sealed and heated at 120 °C by Discover SP microwave synthesizer for 15 min. After cooling, the reaction mixture was filtered and the solvent was evaporated. Purification by flash chromatography on silica gel (eluent: AcOEt) gave the desired product 2,5-(<sup>13</sup>C)-*p*-phenylenediamine **2** (97 mg, 0.9 mmol, 93% yield). Mp 145-147°C (lit unlabeled 143-145 °C)<sup>34</sup>; <sup>1</sup>H NMR (500 MHz, DMSO): δ 4.16 (s, 4H), 6.34 (m, 2H), 6.34 (dm, <sup>1</sup>J<sub>CH</sub> = 154.9 Hz, 2H). <sup>13</sup>C NMR (125 MHz, DMSO): δ 115.4 (2 x <sup>13</sup>CH), δ 115.4 (dd, <sup>1</sup>J<sub>CC</sub> = 62.64 Hz, <sup>2</sup>J<sub>CC</sub> = 6.30 Hz, 2 x CH), 138.9 (dd, <sup>1</sup>J<sub>CC</sub> = 62.65 Hz, <sup>2</sup>J<sub>CC</sub> = 1.42 Hz, 2 x CNH<sub>2</sub>). HRMS ESI (*m/z*): Calculated for <sup>12</sup>C<sub>4</sub><sup>13</sup>C<sub>2</sub>H<sub>9</sub>N<sub>2</sub> [M+H]<sup>+</sup> 111.0833; found: 111.0837.

#### 2.4. Reaction of **1** and **2** with nucleophilic amino acids and GSH in solution

To a solution of *N*-acetylated amino acid or GSH (60  $\mu\text{mol}$ , 10 eq.) in phosphate buffer (450  $\mu\text{L}$ , 0.1 M, pH 7.4) or in ammonium acetate buffer (450  $\mu\text{L}$ , 0.1 M, pH 10.2) was added a solution of **1** or **2** (6  $\mu\text{mol}$ , 1 eq.) dissolved in 150  $\mu\text{L}$  of  $\text{CD}_3\text{CN}$  at 25  $^\circ\text{C}$ . The reaction mixture was filtered into an NMR tube and the reaction was followed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR on a Bruker 500 MHz spectrometer. Control tests were carried out in the same conditions but in the absence of the amino acid in order to monitor the stability of ( $^{13}\text{C}$ )PPD in solution.

#### 2.5. Reconstructed Human Epidermis (RHE)

*Cell Culture:* The Episkin<sup>®</sup> RHE, large model (thirteen days old, 1.07  $\text{cm}^2$ ), were obtained from Skinethic, Lyon, France. These models are differentiated from human keratinocytes isolated from healthy donors and include a human collagen (Type I) matrix, coated with a layer of Type IV human collagen, and a fully stratified and differentiated epidermis covered with a *stratum corneum*. The Episkin<sup>®</sup> cultures are shipped onto a nutritive gel for transportation to maintain viability. Upon reception, RHE were kept under sterile conditions in a 12-well culture dishes containing 2 mL/well of supplied maintenance medium (DMEM/Ham's F12) pre-warmed at 37 $^\circ\text{C}$ . The medium was changed (2 mL) each 24h.

*Treatment protocol:* The RHE samples were treated topically with a fixed volume (30  $\mu\text{L}$ ) of freshly prepared solutions of **1** or **2** (0.8 M in acetone) or of acetone for the control samples. Different incubation times (1 h, 8 h, 24 h and 48 h) were tested in order to kinetically follow the reactions. Once the desired incubation time was reached, the epidermis was separated from its collagen support using sterilized forceps, placed in an Eppendorf tube and frozen at -80 $^\circ\text{C}$ .

Freezing the samples makes it possible to stop all metabolic activity within the epidermis and keeps them stable until their analysis by HRMAS NMR.

## **2.6. Rotor preparation and data acquisition by HRMAS NMR**

Each sample was prepared at  $-20\text{ }^{\circ}\text{C}$  by introducing 15 to 20 mg of frozen RHE into a disposable 30  $\mu\text{L}$  Kelf insert. To provide a lock frequency for the NMR spectrometer, 10  $\mu\text{L}$  of  $\text{D}_2\text{O}$  were also added to the insert. The insert was then sealed tightly with a conical plug and stored at  $-80\text{ }^{\circ}\text{C}$  until the HRMAS analysis. Shortly before the HRMAS analysis, the insert was placed into a standard 4 mm  $\text{ZrO}_2$  rotor and closed with a cap. The whole set was then inserted into a HRMAS probe precooled at  $4\text{ }^{\circ}\text{C}$ . All HRMAS experiments were performed at  $4\text{ }^{\circ}\text{C}$  and were started immediately after the temperature inside the probe had reached equilibrium conditions (5 min). Upon completion of the HRMAS analysis, the insert was taken out of the rotor and stored back at  $-80\text{ }^{\circ}\text{C}$ . Therefore, complementary NMR analysis can be performed at a later stage on any of the stored samples inserts.

HRMAS spectra were recorded on a Bruker™ Avance spectrometer (University of Strasbourg, CNRS UMR-7177) operating at a proton frequency of 500.13 MHz and equipped with a 4 mm triple resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ ) gradient HRMAS probe or on a Bruker™ Avance HD Narrow Bore 500 spectrometer (University of Strasbourg, CNRS FR-2010, France) operating at a proton frequency of 500.13 MHz and equipped with a Bruker™ 4 mm double resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ) gradient HRMAS iProbe. A Bruker™ Cooling Unit (BCU) was used to regulate the temperature at  $4^{\circ}\text{C}$  by cooling down the bearing air flowing into the probe, in order to reduce the effects of tissue degradation during the spectra acquisition. All NMR experiments were conducted on samples spinning at 3501 Hz in order to keep the rotation sidebands out of the spectral region of interest. For each sample,

1D  $^1\text{H}$  experiments using standard one-pulse and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences coupled with water presaturation, and 1D  $^{13}\text{C}$  experiment with proton decoupling were acquired. All spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm in  $^1\text{H}$  and to 22.7 ppm in  $^{13}\text{C}$ .

## **2.7. Quantification procedure**

In order to quantify the different adducts formed we decided to use the first increment of a 2D  $^1\text{H}$ - $^{13}\text{C}$  g-HSQC (gradient Heteronuclear Single Quantum Coherence). This type of acquisition is the best compromise between the sensitivity of the HSQC experiment for characterizing the  $^{13}\text{C}$  labelled adducts and a reasonable period of time for quantifying these products. The adducts were quantified using the PULCON method, which is a very accurate quantification method (with a very limited percentage of error).<sup>38</sup> Adducts quantification was performed using an external reference standard of  $^{13}\text{C}$ -PPD (7 mM), scanned under the same analytical conditions as the RHE samples. Spectra were normalized according to sample weight. Peaks of interest were automatically defined by an in-house program using MATLAB 7.0 (MathWorks, Natick, MA, USA). Peak integration was then compared to the one obtained with the PPD reference and was corrected according to the number of protons. Quantification results were expressed as nanomoles per milligram of tissue.

## **3. Results and discussion**

### **3.1. Synthesis of 1,4-( $^{13}\text{C}$ )*p*-phenylenediamine 1 and 2,5-( $^{13}\text{C}$ )*p*-phenylenediamine 2**

The site specific carbon-13 substitution of sensitizing compounds in conjunction with NMR technique has been shown to be very helpful in monitoring their reactivity towards isolated

nucleophilic amino acids in solution or with epidermal proteins in a complex tissue like epidermis.<sup>23,39,40</sup> On the one hand, within epidermis the enhanced signal offered by <sup>13</sup>C substitution will allow shorter acquisition time of NMR experiments avoiding an important evolution of biological systems and maintaining high quality spectra.<sup>28</sup> On the other hand, this methodology will ensure an easier interpretation of spectra by minimizing any overlap with signals of <sup>13</sup>C natural abundant atom of metabolites or other species present in the epidermis. The observation of signals arising only from the remaining test chemical, its metabolites or more interestingly its interaction with epidermal proteins will be possible. The best strategy to benefit of <sup>13</sup>C substitution is to directly substitute the carbon atoms at the reactive positions. Thus, we decided to directly incorporate <sup>13</sup>C atoms into the aromatic ring rather than adding them as extra substituents. Taking into account the reactive sites of PPD or its oxidation derivatives and based on previously studied PPD related compound 2,5-(<sup>13</sup>CH<sub>3</sub>)-*p*-benzoquinonediimine<sup>23</sup> reactivity in solution, substitution can be performed in two fashions each on two positions [(2,5-(<sup>13</sup>C) and 1,4-(<sup>13</sup>C)] while maintaining the C<sub>2h</sub> symmetry of the molecule. Keeping the symmetry of the molecule would be very beneficial for the analysis of the spectra because a potential nucleophilic attack on either side of the molecule would give amplified identical NMR signals. Bearing in mind that <sup>13</sup>C disubstituted benzene derivatives are not commercially available, this challenging synthesis required the construction of the aromatic ring using acyclic <sup>13</sup>C substituted precursors adapted for a selective substitution at the desired reactive positions. To the best of our knowledge, the synthesis of <sup>13</sup>C disubstituted arenes with <sup>13</sup>C atoms being in *para*-position to each other is not reported. In this context, we developed two synthetic routes giving access

to the two isotopomers of PPD **1** and **2** (Chart 1). An advantage of this synthetic strategy is that the first three steps leading to the common intermediates 1,4-(<sup>13</sup>C)nitrophenol **5** could be performed in large scale providing materials for both syntheses.

*3.1.1 Synthesis of 1,4-(<sup>13</sup>C)p-phenylenediamine 1.* As shown in Scheme 2 the first key synthetic step involved a modified reaction developed by Mock between boron trifluoride diethyl etherate and triethyl orthoformate to give an intermediate carbenium ion, which was directly reacted with the enolate of 2-(<sup>13</sup>C)acetone in the presence of the Hünig's base to give the <sup>13</sup>C substituted bis-acetal **3** in 80% yield. Following hydrolysis and subsequent cyclisation of the bis-acetal under acidic conditions, the <sup>13</sup>C substituted pyran-4-one **4** was isolated from the aqueous phase as described by Marshal et al.<sup>41</sup> Next, the second key step was the incorporation of the second <sup>13</sup>C atom using the commercially available (<sup>13</sup>C)nitromethane as a precursor followed by aromatization under acidic conditions during the workup to yield nitrophenol **5** <sup>13</sup>C substituted at positions 1 and 4 in 66% yield. This method which combines pyranone and pre-nucleophiles was originally reported by Steglich in 1998<sup>42</sup> for the synthesis of mono <sup>13</sup>C ring labeled phenolic compounds and further optimized by Marshall et al.<sup>33</sup> The structure of compound **5** and the positions of <sup>13</sup>C atoms were confirmed by proton NMR where the spectrum shows a pair of doublet of doublet of doublet at 6.92 and 8.18 ppm corresponding to the aromatic protons each coupled with both <sup>13</sup>C atoms of the aromatic ring (<sup>2</sup>J<sub>CH</sub> and <sup>3</sup>J<sub>CH</sub>) and with the adjacent proton (<sup>3</sup>J<sub>HH</sub> = 9.14 Hz). Noteworthy, the proton of the phenolic OH is coupled through the oxygen to the <sup>13</sup>C atom of the ring and manifested by a doublet (<sup>2</sup>J<sub>CH</sub> = 2.35 Hz) at 5.72 ppm. The <sup>13</sup>C spectrum shows that <sup>13</sup>C atoms are coupled with each other (<sup>3</sup>J<sub>CC</sub> = 9.04 Hz) and illustrated by two doublets at 115 and 126 ppm. The one-pot amination (o-alkylation/Smiles

rearrangement sequences)<sup>43</sup> of **5** using 2-chloroacetamide in the presence of K<sub>2</sub>CO<sub>3</sub> as base gave 1,4-(<sup>13</sup>C)*p*-nitroaniline **6** in 82% yield. Further reduction<sup>44</sup> of **6** using triethylsilane in ethanol in the presence of catalytic amounts of PdCl<sub>2</sub> afforded the desired double substituted final product **1** in excellent yield (90%). This catalytic system was chosen among others because it provides the mildest conditions (room temperature, short time) avoiding any polymerization of the readily formed PPD in the medium. The <sup>13</sup>C spectrum shows one intense singlet corresponding to one type of <sup>13</sup>C atom confirming the symmetry of the molecule.

**3.2.2 Synthesis of 2,5-(<sup>13</sup>C)*p*-phenylenediamine **2**.** Starting from 1,4-(<sup>13</sup>C)*p*-nitrophenol **5** previously obtained in three steps, the phenolic OH was substituted by chlorine using the general method of Coe et al.<sup>45</sup> (Scheme 3). The reaction of **5** with triphenyloxyphosphorus dichloride prepared *in situ* by mixing phosphorus pentachloride with phenol afforded tetraphenyloxyphosphorus monochloride which was further decomposed at high temperature to give **7**. The chlorodenitration of **7** following an optimized procedure reported by Bay et al.<sup>46</sup> was possible using phenyltetrachlorophosphorane prepared *in situ* by the reaction of chlorine gas with dichlorophenyl phosphine. However, it was necessary to lower the originally reported temperature from 170 °C to 150 °C and keep the reaction for a longer time in order to minimize any potential evaporation of the formed product (bp = 174 °C). The <sup>13</sup>C spectrum of the obtained compound **8** shows one singlet at 132.66 ppm corresponding to both <sup>13</sup>C atoms confirming that the molecule is symmetric having two chlorine atoms. The regioselective iodination of **8** at positions C2 and C5 was carried out using a combination of iodine and potassium iodate in concentrated sulfuric acid to give **9** in a good yield (95%) following recrystallization. By keeping the reaction temperature moderately low (60°C), tri-iodinated

derivative formation was avoided, and after 16 h of reaction almost no mono-iodinated derivative remained in the reaction mixture. The iodination at positions C2 and C5 maintained the symmetry of the molecule which was confirmed by the presence of one singlet signal corresponding to both  $^{13}\text{C}$  atoms in  $^{13}\text{C}$  spectrum. Further, the challenging double selective amination<sup>47</sup> of **9** was performed in the presence of aqueous ammonia and  $\text{CuI}/\text{Fe}_2\text{O}_3$  as catalysts in a sealed tube. However, conditions were optimized to give selectively 2,5-( $^{13}\text{C}$ )-2,5-dichloro-*p*-phenylenediamine **10** in 55% yield. Finally, the double dechlorination<sup>48</sup> of **10** using hydrazine and palladium on carbon under microwave irradiation afforded in a quick, clean and convenient work-up procedure the expected 2,5-( $^{13}\text{C}$ )PPD **2** in excellent yield (93%). The synthesized  $^{13}\text{C}$  substituted PPD compounds remained stable under argon at low temperature (-30 °C) for several months.

### **3.2. Behaviour of **1** and **2** in the presence of *N*-Ac-lysine and *N*-Ac-cysteine.**

*3.2.1 Behaviour of **1** in the presence of *N*-Ac-lysine and *N*-Ac-cysteine at pH 7.4.* The behaviour of **1** (10 mM in a 3:1 PBS (pH 7.4)/acetonitrile mixture) was initially studied in solution at 25°C. Compound **1** characterized in  $^{13}\text{C}$  NMR by a singlet at 138.1 ppm was stable over 24 h with the progressive formation of three new signals, corresponding to three magnetically different  $^{13}\text{C}$  atoms (Figure 1). A doublet at 147.9 ppm ( $^3J_{\text{CC}} = 7.17$  Hz), a second doublet at 143 ppm ( $^3J_{\text{CC}} = 8.93$  Hz) and a doublet of doublet at 141.1 ppm ( $^3J_{\text{CC}} = 7.17$  and 8.93 Hz) could be assigned to the Bandrowski's base (BB) using  $^1\text{H}$ - $^{13}\text{C}$  heteronuclear HMBC experiments. The oxidation of PPD to BB and other oligomeric and polymeric products under different conditions in aqueous solution was previously reported in the literature.<sup>21,27,49</sup> PPD was quite stable under our conditions in a closed NMR tube and about 50% of the parent compound was consumed within

one week. This percentage of conversion was obtained within 8 h in oxygen saturated solution as reported by Aeby et al.<sup>27</sup> <sup>1</sup>H NMR spectrum<sup>50</sup> showed two multiplets at 6.72 and 5.76 ppm corresponding to the eight protons of the two benzene rings and the two protons present on the central **BQDI** ring, respectively (Supplementary Figure S1). The HMBC experiment indicated that carbons at 141.1 and 143 ppm were correlated via long range coupling with protons at 6.72 ppm, and carbons at 147.9 ppm are correlated with the protons at 5.76 ppm.

In an attempt to detect adducts between nucleophilic amino acids and PPD in solution, **1** was incubated separately with *N*-Ac-lysine and *N*-Ac-cysteine. New NMR signals could be further used as references for the identification of adducts with lysine and cysteine formed in RHE. Compound **1** and *N*-Ac-lysine were mixed together in a molar ratio of 1/10 to favor the coupling reaction over the polymerization process of **1** when present alone in solution. But even in the presence of a large excess of amino acid, BB was the only product observed in NMR after 7 days (Supplementary Figure S2). This can be explained by the fact that the  $\epsilon$ -amino group of *N*-Ac-lysine is mostly protonated at pH 7.4 making the latter less reactive and allowing self-conjugation of **1**. When compound **1** was incubated under the same conditions with *N*-acetyl cysteine no adducts were detected neither the formation of BB. Similar observations were reported by Coulter<sup>51</sup> and Jenkinson<sup>19</sup> regarding the inhibitory role of cysteine or glutathione on the formation of BB. This is probably due to the reducing character of the thiol group of cysteine and this hypothesis being confirmed by the observation of *N*-Ac-cystine, the dimerization product of *N*-Ac-cysteine, in the NMR spectrum (Supplementary Figure S3). Additionally, the ion  $[M+H]^+$  at  $m/z = 325.0527$  (calculated 325.0523) corresponding to *N*-Ac-cystine was detected in the mass spectrum of this sample.

### 3.2.2. Behaviour of **1** in the presence of *N*-Ac-cysteine, glutathione (GSH) and *N*-Ac-lysine at pH

10.2. In order to enhance the reactivity of the thiol group, cysteine and glutathione were separately incubated with **1** under the conditions described above but at pH 10.2 (100 mM ammonium acetate buffer). Additionally, the formation of the oxidation product of PPD has been shown to be faster in alkaline conditions.<sup>49</sup> The reaction was followed by <sup>13</sup>C NMR and the complete consumption of the parent compound (peak at 138.1 ppm) to give a product with two different <sup>13</sup>C signals (two doublets at 127.2 and 147.2 ppm having the same coupling constant <sup>3</sup>J<sub>CC</sub> of 9.6 Hz) was observed after only 5 days (Figure 2). No further evolution of the spectrum was observed over time. 2D HMBC experiments showed the correlation of each of the new <sup>13</sup>C quaternary carbons via <sup>2</sup>J and <sup>3</sup>J<sub>CH</sub> with the same protons at 6.98 and 6.76 ppm indicating that these signals can be attributed to the same product. The mass spectrum analysis revealed the presence of a new ion [M+1]<sup>+</sup> at *m/z* 272.0983 (calculated 272.0980) (Supplementary Figure S4) corresponding to a covalent adduct of **1** with one residue of *N*-Ac-cysteine. Mechanistically, this suggests the reaction of the thiol group of *N*-Ac-cysteine with the nitrogen atom of the BQDI intermediate to give **11**. BQDI is widely reported as the first oxidation derivative of PPD and can be generated *in situ* following autooxidation of PPD with air oxygen present in solution or with thiyl radicals of *N*-Ac-cysteine. The possible attack of the thiol group on one of the four carbons (CH) of the BQDI ring was excluded because in that case it would afford a product with three different proton signals instead of two. This conclusion is in complete agreement with the results previously obtained by Eilstein et al.<sup>23</sup> where a similar product was identified as the major adduct formed following the incubation of *N*-Ac-cysteine and 2,5-dimethyl-1,4-benzoquinonediimine. At pH 10.2, most of the thiol groups of cysteine (pKa 9.5) or glutathione

(pKa 9.2) are deprotonated to form thiolate ions which are more reactive towards electrophiles. However, Roberts and co-workers showed that the free radical binding mechanism<sup>52</sup> is the privileged one where the thiyl radical of *N*-Ac-cysteine will attack the nitrogen of BQDI or the radical cation of PPD “Würster type radical” formed by the loss of one electron. It is worth to notice that in the case of incubation with *N*-Ac-lysine, spectra were not conclusive. The major product was BB along other unidentified derivatives, and no obvious NMR signals could be assigned to any possible adduct. The formation of BB having six <sup>13</sup>C atoms was confirmed by the presence of the ion [M+H]<sup>+</sup> at *m/z* = 325.1872 (calculated 325.1872) (Supplementary Figure S5). Remarkably at this high pH, the formation of BB was slow (two weeks) which is in agreement with the observations of Abey et al.<sup>27</sup> who demonstrated that the oxidation of PPD to BB is pH dependent reaching its optimum at a pH between 5 and 7 and becoming slow at alkaline pH. In order to confirm the involvement of the thiol group of cysteine in this reaction, the same experiment was conducted replacing *N*-Ac-cysteine with GSH. Spectra clearly showed that no parent compound PPD was remaining after 5 days of incubation and a new species appeared having the same set of <sup>13</sup>C signals as the one observed with cysteine (two doublets at 123 and 147.2 ppm with the same coupling constant <sup>3</sup>J<sub>CC</sub> of 9.6 Hz) (Supplementary Figure S6). This result confirmed that the adduct obtained in both cases was formed following the reaction of the thiol group, the only common nucleophilic group between cysteine and GSH, with the nitrogen atom of BQDI. Notably, BQDI was not detected under our experimental conditions due to its high reactivity and instability.

**3.2.3. Reactivity of 2 in solution with *N*-Ac-cysteine and GSH at pH 10.2.** To have a complete picture and further confirm the results obtained with **1**, the behaviour of **2** in the presence of *N*-

Ac-cysteine or GSH was examined under the same basic conditions (pH 10.2). Superimposed spectra at  $t = 0$  and  $t = 5$  days of the reaction of **2** with *N*-Ac-cysteine are shown in Supplementary Figure S7. At 5 days, complete consumption of PPD (disappearance of the peak at 117 ppm) and the formation of the cysteine adduct **12**, the isotopomer of **11** characterized by two doublets at 116 and 126.1 ppm having the same coupling constant  $^3J_{CC}$  of 5.7 Hz was observed. Compound **2** was also incubated with GSH and the spectra (Supplementary Figure S8) revealed the same set of signals as those obtained with cysteine pointing to the same previous conclusion.

### 3.3. Reactivity of **1** and **2** with RHE

Episkin RHE samples were treated with a fixed dose of **1** or **2** (30  $\mu$ L, 0.8 M in acetone) and then incubated for different times (1 h, 8 h, 24 h and 48 h). Superimposed 1D  $^{13}\text{C}$  spectra of these RHE are shown in Supplementary Figure S9. Compared to the control sample, spectra clearly showed the presence of **1** or **2** characterized by a peak at 138 ppm or 117 ppm, respectively. These signals gradually decreased with time to disappear after 48 h of incubation. Our experimental conditions/protocol did not permit the detection of any adduct between **1** or **2** and epidermal proteins. The absence of reactivity could be explained either by the diffusion of PPD through the epidermis without reacting with epidermal proteins or by a too low level of modification for an HRMAS NMR detection especially with the less sensitive quaternary carbons. A study done by Van Gele et al.<sup>53</sup> showed that RHE Episkin was more permeable than human skin due to the difference in their lipid organization. Moreover, drug transdermal penetration and its bioavailability in reconstructed human epidermis is vehicle dependent and

generally higher than the one of native skin.<sup>54,55</sup> Another hypothesis suggests the elimination of **1** from the epidermis via a detoxication process. To confirm this, the culture media of the control and of the two samples which were incubated in the presence of **1** for 1 h and 24 h, respectively, were analyzed by liquid NMR (Figure 3). On the one hand, the control sample spectrum in blue (culture medium of RHE treated only with acetone and incubated for 24h) shows no <sup>13</sup>C signals. On the other hand, the detoxication process was assessed by the presence in the culture media of the incubated samples with **1** of both unsymmetrical mono-acetylated-PPD (**MAPPD**) (38% of <sup>13</sup>C signals), illustrated by a doublet at 138.1 ppm and the symmetrical diacetylated-PPD (**DAPPD**) (44% of <sup>13</sup>C signals) that has a singlet at 138.3 ppm. This process was more pronounced after 24 h (spectrum in green). Also, the unmodified parent compound **1** was detected at 138 ppm (18% of the <sup>13</sup>C signals present in the culture medium). It should be noted that only traces of BB were observed in the culture medium of RHE even after 24 h of incubation. However, there is no evidence that BB was formed in the RHE and then diffused into the culture medium or the polymerization took place in the culture medium. Hence, the biotransformation of **1** takes place slowly in the epidermis. LC-MS analysis of the culture medium revealed the presence of the acetylated compounds. Therefore, the acetylation reaction of **1** catalyzed by *N*-acetyltransferase (NAT) enzymes appeared to be the main process in RHE.

### **3.7. Reactivity of PPD with RHE under oxidative conditions**

Based on the fact that 2,5-dimethyl-*p*-benzoquinonediimine, the first oxidation derivative of 2,5-dimethyl-*p*-phenylenediamine, was shown to bind covalently to nucleophilic amino acids and

proteins by Eilstein et al.<sup>23-25</sup>, our second hypothesis suggests that PPD was not sufficiently auto-oxidized by air oxygen into BQDI on the RHE surface. Interestingly, a similar conclusion was reported by Abbey et al.<sup>27</sup> indicating that prior oxidation of PPD by ferrocyanide was important to generate strong dendritic cell activation compared to the non-oxidized PPD. This is possibly due to its rapid penetration and further elimination by the detoxication pathway taking place in the RHE epidermis. With the aim of increasing its reactivity, PPD was treated with H<sub>2</sub>O<sub>2</sub> as an oxidant like in hair dyeing conditions prior to its application to RHE. The solution of oxidized PPD was prepared immediately before application to RHE by mixing PPD (0.8 M in acetone) with 0.5 M of 30 % H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> was used as limiting reagent to ensure its complete consumption by PPD or its oxidized derivatives prior to the application of the mixture to RHE and to avoid its potential side reaction with acetone. RHE treated with oxidized **1** were then incubated for different times and <sup>13</sup>C spectra are shown in Supplementary Figure S10. As expected, the amount of remaining **1** having a peak at 138 ppm (maximum at 1 h) decreases with time to reach a minimum at 24 h following its metabolization or clearance to the culture medium. Under these conditions, two new small peaks at 126 and 149 ppm having close chemical shifts to those of the cysteine adduct **11** (123 and 127 ppm) previously observed in solution were detected. These signals were assigned to a possible adduct resulting from the reaction of cysteine residues on epidermal proteins in RHE with PPD. Noteworthy, after 24 h of incubation, these new signals were no longer detected due to a possible evolution or clearance process. In parallel, experiments with **2** were also conducted under the same oxidative conditions. In this case, 2D <sup>1</sup>H-<sup>13</sup>C heteronuclear single quantum correlation (g-HSQC) HRMAS NMR experiments could be carried out taking advantage of the direct <sup>1</sup>H-<sup>13</sup>C spin coupling to monitor the reactivity

of **2** in RHE. The superimposed spectra of the RHE sample treated with **2** incubated for 8 h and the control sample are shown in Figure 4. A simple visual comparison between spectra shows the appearance of a new intense red signal (red colored) at 6.8(<sup>1</sup>H)/117(<sup>13</sup>C) ppm (aromatic CH) arising from the remaining parent compound **2**. Notably, two small signals at 7(<sup>1</sup>H)/115(<sup>13</sup>C) ppm and 7.2(<sup>1</sup>H)/124(<sup>13</sup>C) ppm were detected. These chemical shifts were very close to those of adduct **12** (<sup>13</sup>C signals at 116 and 126.1 ppm) and can be associated to the binding of **2** to the cysteine residues within the epidermis. Apart from these two signals, no other signals resulting from potential covalent binding of PPD to other nucleophilic residues could be observed. The absolute concentrations of remaining PPD and the detected cysteine adduct in RHE were calculated using 1D HSQC spectra. After 8 h of incubation, the concentration of PPD was assessed at 1.3 nmol/mg while the cysteine adduct concentration was assessed at 0.2 nmol/mg reflecting a very low covalent binding of PPD to the epidermal proteins in RHE.

Interestingly, no BB or covalently bound BB to epidermal proteins or other polymeric derivatives of PPD could be detected in RHE under our oxidative and non-oxidative conditions despite the assumption that polymerization is chemically the only fate of PPD in the absence of other competing reaction like those occurring with couplers during the hair dyeing mechanism. The picture in solution is somewhat different and strong peptide reactivity<sup>16,17</sup> of BB has been reported using direct peptide reactivity assay (DPRA). Furthermore, Jahn et al. used electrochemical oxidation coupled with LC/MS approach to study the reactivity of PPD oxidation derivatives toward proteins. The authors demonstrated that, not only BQDI but also BB and other oligomeric derivatives of PPD were able to covalently bind to protein models in solution, primarily with free thiol groups.<sup>21</sup>

#### 4. Conclusion

In the present study, we have reported the challenging synthesis of two  $^{13}\text{C}$ -disubstituted PPD isotopomers **1** and **2**. Unprecedentedly, these molecules allowed us to investigate the reactivity of PPD in a 3D human like epidermis using the HRMAS NMR technique. Our investigations using RHE under non-oxidative conditions demonstrate that upon topical exposure to PPD like in hair dyeing or temporary henna, the surface of RHE allowed a fast permeation of the chemical avoiding its activation/oxidation due to insufficient contact time with air oxygen. This is a considerable pitfall for the use of RHE because these models don't afford a complete barrier function like native human skin. In all experiments conducted with RHE, very "clean" spectra were observed showing only the presence of PPD without any other signals that could arise from possible adducts. Quantification of PPD in RHE with time indicated a rapid clearance of the chemical. Analysis of the culture medium showed the presence of PPD, MAPPD and DAPPD already after 1 h and only traces of BB after 24 h. The mono- and di-acetylated PPD are negative in the LLNA<sup>27</sup> and not able to induce T-cell responses<sup>56</sup> suggesting that *N*-acetylation is a major detoxication pathway that competes with the formation of electrophilic immunogenic PPD derivatives within the epidermis. Notably, these observations are in complete agreement with the study conducted by L'Oréal (France) in 2005 that investigated the biotransformation of ( $^{14}\text{C}$ )PPD.<sup>57</sup> Authors reported that Episkin RHE converted PPD mainly into MAPPD and DAPPD and that the percentage of PPD covalently bound to epidermal proteins<sup>57</sup> ranged from 0.5 to 2.3% depending on the applied dose. In our study as evidenced by NMR, the reactivity of ( $^{13}\text{C}$ )PPD with epidermal proteins under non-oxidative

conditions is too low to be detected by HRMAS NMR. The selectivity of the readily oxidized (<sup>14</sup>C)PPD derivatives binding towards sufficiently activated and available thiol group within proteins<sup>19,20</sup> has been previously reported by Jenkinson *et al.* In our case, the absence of reactivity of RHE under non-oxidative conditions toward PPD suggests the latter was not sufficiently oxidized like in solution, as RHE doesn't lack free cysteine residues and it can exhibit reactivity through these residues with other allergens as isothiazolinones.<sup>30</sup> While the auto-oxidation of PPD in solution is possible leading to the formation of the reactive BQDI, the mechanism with RHE is somehow different. The use of an oxidant as H<sub>2</sub>O<sub>2</sub> seems to be necessary to oxidize PPD prior to its application on RHE. Under such oxidative conditions, we were able to detect and quantify cysteine adducts in RHE (maximum of 0.2 nmol/mg of RHE at 8 h of incubation) while no reaction with other nucleophilic amino acid residues could be observed. Our observations on PPD reactivity towards cysteine residues in solution and RHE suggest a radical intermediates pathway rather than a classical nucleophilic/electrophile mechanism. Free radicals of protein thiol groups are well known in the literature and can be generated in response to oxidative stress by different simulating factors including endogenous and exogenous sources<sup>52</sup> or by BQDI itself.<sup>58</sup> Recently, the increased levels of ROS in human skin have been linked to the skin sensitization potential of PPD<sup>2</sup> while PPD alone has been shown to increase the intracellular ROS in HaCat keratinocytes.<sup>59</sup>

As a conclusion, we suggest that the reaction of PPD with epidermal proteins is a highly complex process primarily associated to the reactivity of thiol moieties where all convenient conditions should be ensured starting from the activation of the two partners of the reaction. Firstly, the oxidation of PPD by air oxygen (like in solution with peptide and HSA) or by employing an

oxidizing agent (like in the case of hair dyeing and our oxidative conditions) to produce the highly reactive BQDI should be carried out. Secondly, the cysteine residues need to be activated into thiyl radicals either by using an oxidizing agent or by the produced oxidation derivatives of PPD in the epidermis.

## ASSOCIATED CONTENT

### Supplementary information

Supplementary Figure S1:  $^1\text{H}$  NMR spectra of 1,4- $(^{13}\text{C})$ PPD **1** in PBS/acetonitrile 3:1 at pH 7.4 showing the formation of Bandrowski's Base **BB** with time.

Supplementary Figure S2:  $^1\text{H}$  NMR spectra of 1,4- $(^{13}\text{C})$ PPD **1** in PBS/acetonitrile 3:1 at pH 7.4 in the presence of *N*-Ac-Lys (10 eq.) showing the formation of Bandrowski's Base **BB** with time

Supplementary Figure S3:  $^1\text{H}$  NMR spectra of 1,4- $(^{13}\text{C})$ PPD **1** in PBS/acetonitrile 3:1 at pH 7.4 in the presence of *N*-Ac-Cys (10 eq.) showing the stability of **1** and the formation of cystine.

Supplementary Figure S4: ESI-MS spectrum of the cysteine adduct **11**.

Supplementary Figure S5: ESI-MS spectrum of the  $(^{13}\text{C})$ hexasubstituted BB formed during incubation of **1** with *N*-Ac-Lys at pH 10.2.

Supplementary Figure S6: Behavior of 1,4- $(^{13}\text{C})$ PPD **1** in the presence of GSH at pH 10.2 followed by  $^{13}\text{C}$  NMR in solution.

Supplementary Figure S7: Behavior of 2,5- $(^{13}\text{C})$ PPD **2** in the presence of *N*-Ac-cys at pH 10.2 followed by  $^{13}\text{C}$  NMR in solution.

Supplementary Figure S8: Behavior of 2,5-<sup>13</sup>C)PPD **2** in the presence of GSH at pH 10.2 followed by <sup>13</sup>C NMR in solution.

Supplementary Figure S9: Superimposed <sup>13</sup>C spectra of RHE treated with (a) 1,4-(<sup>13</sup>C)PPD **1** and (b) 2,5-(<sup>13</sup>C)PPD **2** after different incubation times.

Supplementary Figure S10: Superimposed <sup>13</sup>C spectra of RHE samples treated with **1** mixed with 0.5 molar equiv. of H<sub>2</sub>O<sub>2</sub> after different incubation times.

## **AUTHOR INFORMATION**

### **Corresponding author**

E-mail: [jlepoit@unistra.fr](mailto:jlepoit@unistra.fr)

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### **Notes**

The authors declare no competing financial interest.

## **ABBREVIATIONS**

BB, Bandrowski's base; BQDI, benzoquinonediimine; DPRA, direct peptide reactivity assay; HRMAS, high resolution magic angle spinning; g-HSQC, gradient heteronuclear single quantum coherence; LLNA, local lymph node assay; NAT, *N*-acetyltransferase; PPD, *p*-phenylenediamine; RHE, Reconstructed Human Epidermis.



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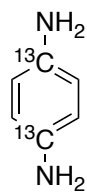
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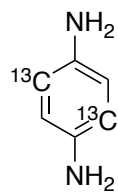
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**Chart 1:** Structures of PPD isotopomers **1** and **2**



**1**



**2**

Figure 1

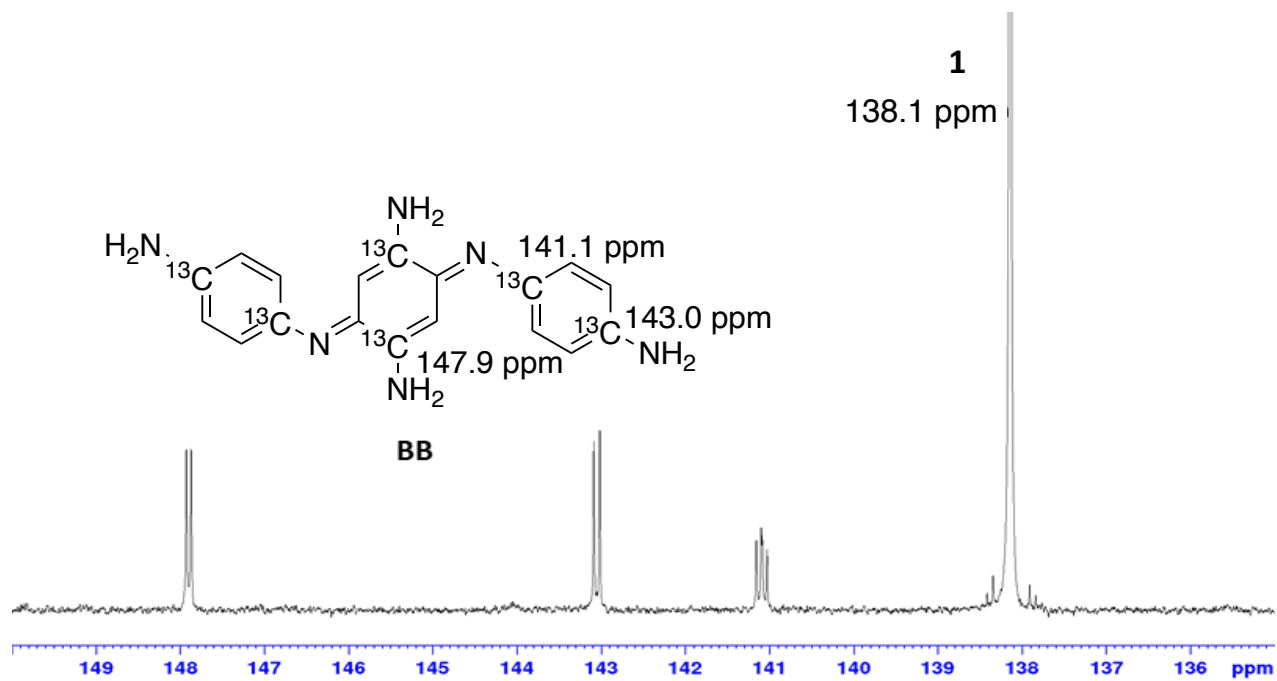


Figure 2

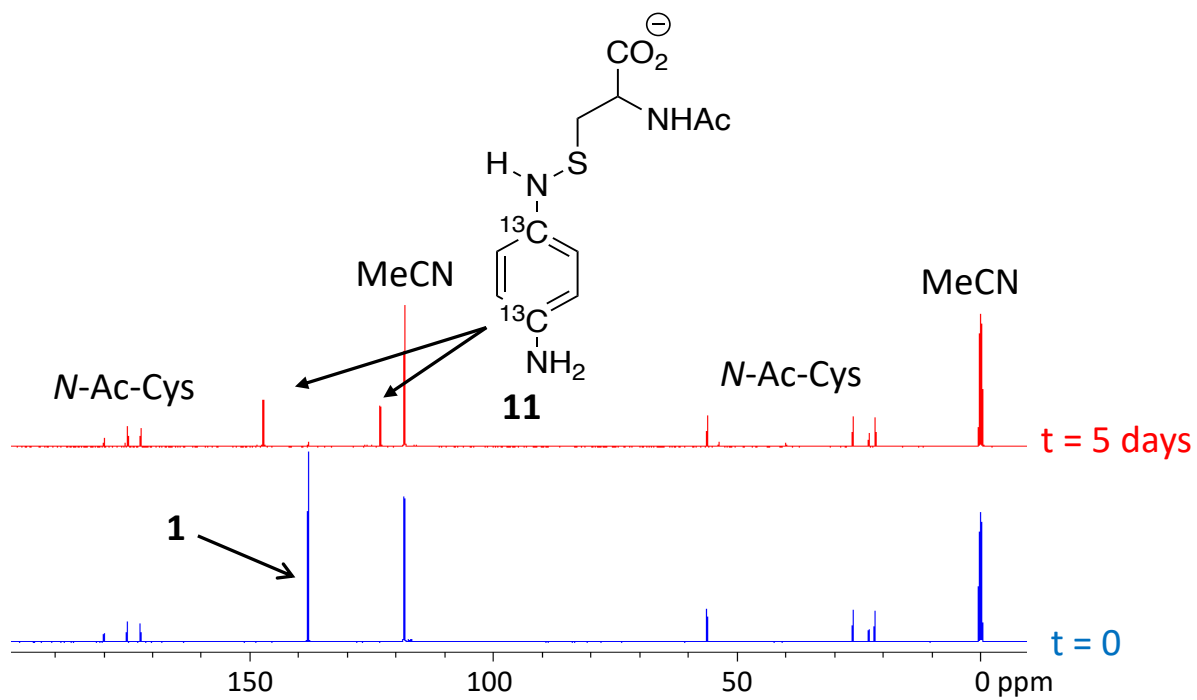


Figure 3

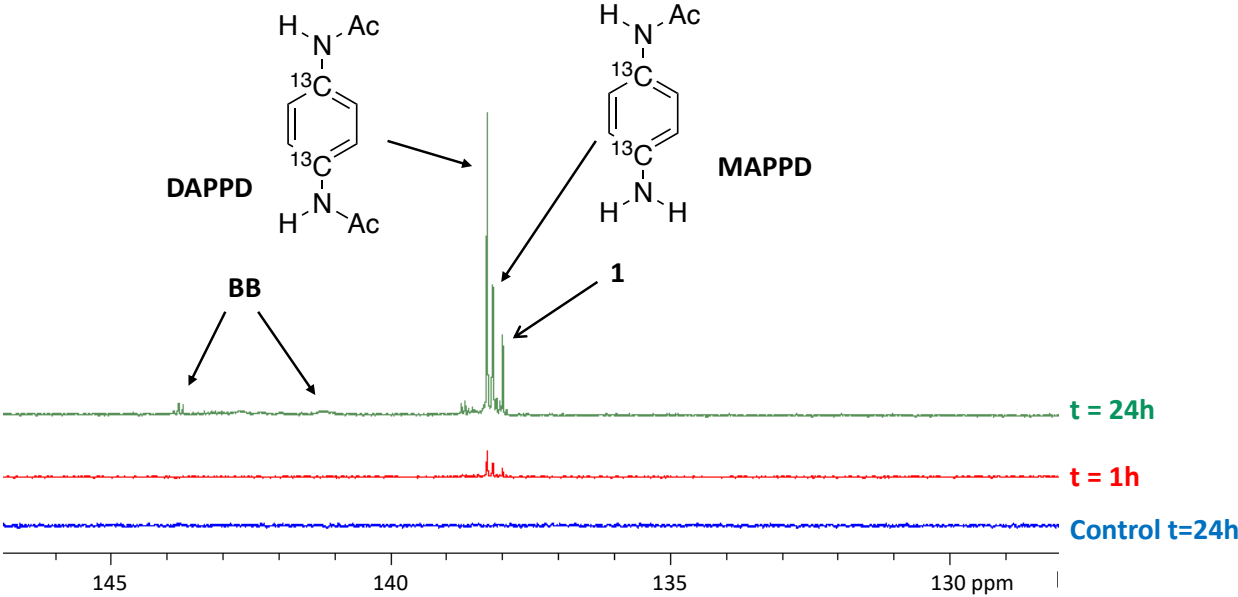
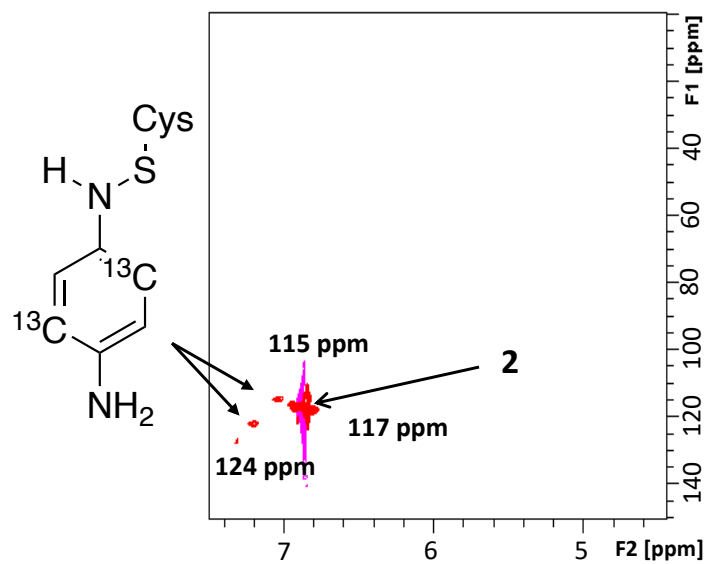


Figure 4



## Figure captions

### Figure 1

$^{13}\text{C}$  NMR spectrum of **1** after 1 week in PBS/acetonitrile 3:1 at pH 7.4

### Figure 2

Reaction of **1** with *N*-Ac-cysteine at pH 10.2 affording adduct **11**.

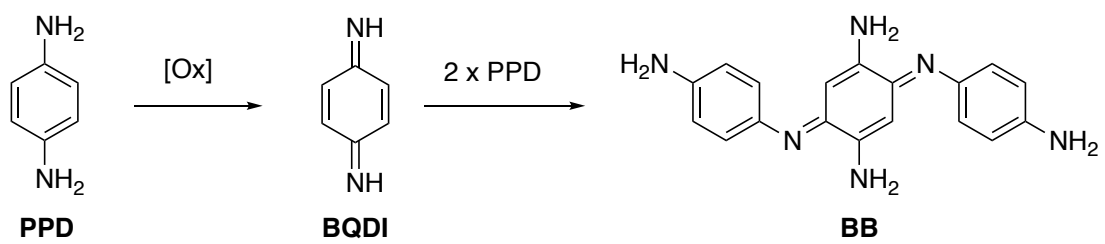
### Figure 3

$^{13}\text{C}$  spectra of the culture media of RHE treated with acetone (control in blue) and RHE treated with **1** and incubated for 1 h and 24 h (red and green, respectively)

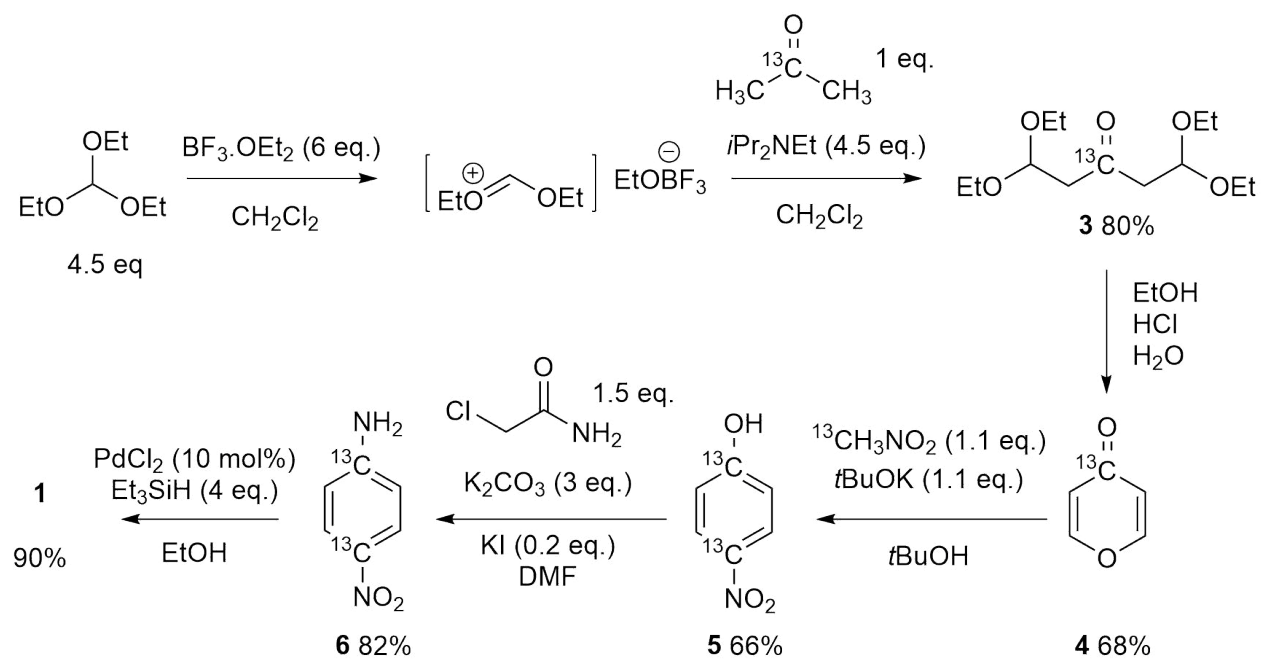
### Figure 4

Superimposed 2D  $^1\text{H}$ - $^{13}\text{C}$  g-HSQC spectra of RHE sample treated with **2** mixed with 0.5 molar equiv. of  $\text{H}_2\text{O}_2$  and incubated for 8 h (red) and control sample treated with acetone (pink)

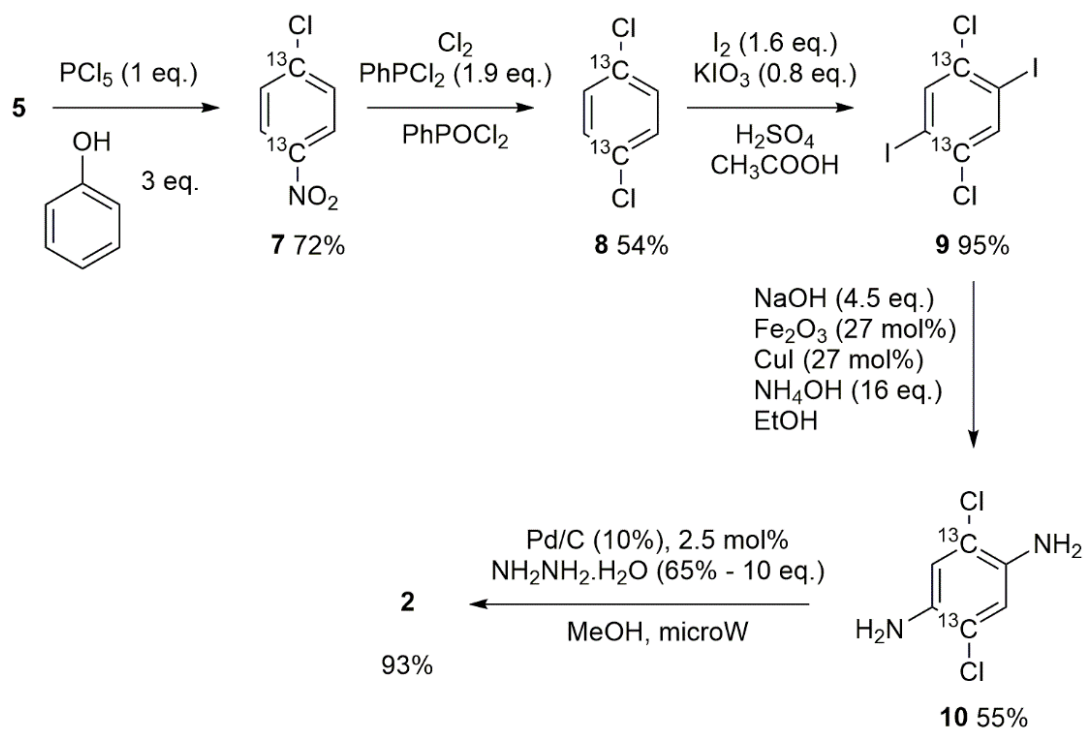
### Scheme 1



Scheme 2



**Scheme 3**



## **Scheme captions**

### **Scheme 1**

Air-oxidation of PPD in solution to form benzoquinonediimine (BQDI) and Brandrowski's base (BB)

### **Scheme 2**

Synthetic pathway giving access to 1,4-<sup>13</sup>C)PPD **1**

### **Scheme 3**

Synthetic pathway giving access to 2,5-<sup>13</sup>C)PPD **2**