Synthesis and Toxicity Evaluation of Tire Rubber-Derived Quinones

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Abstract

N-(1,3-Dimethylbutyl)-N'-phenyl-*p*-phenylenediamine-quinone (6PPD-Q), the tire rubber-derived transformation product of 6PPD, was recently discovered to cause the acute mortality of coho salmon (*Oncorhynchus kisutch*). Aiming to identify a potential nontoxic replacement antioxidant for 6PPD, we herein synthesized seven PPD-quinones with distinct side chains to investigate their structure-related toxicities in rainbow trout (*Oncorhynchus mykiss*). While 6PPD-Q exerted strong toxicity (96 h LC_{50} = 0.64 μg/L), toxicity was not observed for six other PPD-quinones despite their similar structures. The fish tissue concentrations of 6PPD-Q after exposure (0.8 μg/L) were comparable to the other PPD-quinones, which indicated that bioaccumulation levels were not the reason for the selective toxicity of 6PPD-Q. Hydroxylated PPD-quinones were detected as the predominant metabolites in fish tissue. Interestingly, a single major aromatic hydroxylation metabolite was detected for nontoxic PPD-quinones, but two abundant OH-6PPD-Q isomers were detected. MS^2 spectra confirmed that hydroxylation occurred on the alkyl side chain for one isomer. Based on this fact, we suggested a 'dual-action' model wherein OH-6PPD-Q was generated by an enzyme with a high regioselectivity, which further attacks an unknown protein to cause lethality. This study reported the selective toxicity of 6PPD-Q and pinpointed the possibility for other PPDs to be applied as safe replacements of 6PPD.

Keywords: rainbow trout; PPD-Quinones; metabolism; LC-MS; antioxidant

Synopsis: The toxicity of 6PPD-Q is highly structurally selective.

Introduction

A recent milestone study identified N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-Q), the tire rubber-derived transformation product of 6PPD, as the primary toxicant in urban runoff water responsible for the acute mortality of coho salmon (*Oncorhynchus kisutch*).[1] 6PPD-Q exerts extreme aquatic toxicity compared to other quinones, with a median lethal concentration (LC_{50}) of 0.095 µg/L in coho salmon,[2] similar to its concentration range in surface waters.[3] 6PPD-Q also exerts toxicity to several other fish species including rainbow trout (*Oncorhynchus mykiss*) with large interspecies variation,[4-6] but its toxicity mechanism remains unclear.

6PPD-Q is formed through the oxidation of 6PPD by ozone.[7] 6PPD is widely used as an antioxidant in rubber tires to increase their longevity and protect them from oxidative degradation. The annual consumption of 6PPD in the United States ranges from 50 to 100 million pounds.[8] Consequently, there is a pressing need to identify nontoxic alternative antioxidants that can replace 6PPD. Broadly speaking, two possible strategies can be considered: 1) The development of a new generation of nontoxic antioxidants (*e.g.,* natural polyphenols)[9] that are structurally unrelated to phenylenediamines,[10] or 2) the selection of a new PPD compound that retains the antioxidant effectiveness while reducing the toxicity of its ozonation-derived quinone product. Notably, the former approach would be quite challenging as alkene rubber compounds are susceptible to ozonation with a higher reactivity (E_a =14.7 kcal mol^{-1}) than most antioxidants.[8]

While the phenylenediamine core structure of 6PPD is essential for its superior antiozonant function to protect alkene rubber compounds,[8] structural variability of the side chain may be less impactful towards this function (Figure 1a). Indeed, several other PPDs, such as N-phenyl-N'-cyclohexyl-p-phenylenediamine (CPPD), which contains a cyclohexane side chain, are commercially used albeit with smaller production volumes than 6PPD.[11] Inspired by this, we hypothesize that a safer PPD compound could be identified by fine-tuning the side chain structure. To explore this, we synthesized seven PPD-quinones with distinct side chains (see structures in Figure 1b) and investigated their structure-related toxicities towards juvenile rainbow trout.

Materials and Methods

Chemicals and Reagents. All chemical reagents were purchased from commercial vendors and used without further purification, unless otherwise specified. All reactions necessitating oxygen- or moisture-free conditions were conducted using oven-dried glassware under a nitrogen atmosphere. Reagent-grade solvents were used for all chemical reactions. HPLC-grade solvents used for sample extractions and liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis were obtained from EMD Chemicals (Gibbstown, NJ, United States). 6PPD, 6PPD-Q, and d_5-6PPD-Q standards were purchased from the Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions of both purchased standards and synthesized standards used for fish exposure experiments were prepared in HPLC-grade methanol.

Synthesis of PPD-Quinones. In addition to the commercial 6PPD-Q standard purchased, we synthesized 6PPD-Q ourselves to verify the validity of our employed synthetic route. Thus, a total of seven PPD-quinones were synthesized. The procedure for the synthesis of PPD-quinones was adapted from MacGregor *et al.*[12] and Hiki *et al.*[5] Benzoquinone was purified via recrystallization from dichloromethane (DCM). Each of the quinones were prepared via sequential Michael additions followed by oxidation back to the quinone. First, the mono-substituted product 2-anilino-1,4-benzoquinone was prepared at a moderate yield (60%), upon which it was combined with corresponding alkylamines to generate four different PPD-quinones (15 – 26 %) that were purified via column chromatography. The symmetric amines (DPPD-Q and DTPD-Q) were prepared via concurrent Michael additions of benzoquinone with ortho-toluidine under reflux and isolated via filtration. The purity of each chemical standard was confirmed via proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 NMR (^{13}C-NMR) conducted on a Brucker 400/500 MHz instrument with comparison to a purchased internal standard (trimethoxybenzene). Purity was further validated by LC-HRMS analysis.

Fish Source and Culture. Rainbow trout eggs were purchased from Lyndon Hatcheries (New Dundee, ON, Canada). Fish were reared from eggs and cultured under flow-through conditions at 15 ±1 °C for 6 weeks prior to exposure experiments. Fish were monitored daily and fed with a commercial fish feed at a daily rate of 1 % of body

102 weight. Experiments were approved by the Ontario Ministry of the Environment,
103 Conservation and Parks (MECP).

104 **96h Acute Lethality Toxicity Test.** The 96-hour acute toxicity test of eight chemicals
105 including 6PPD, 6PPD-Q, and six other PPD-quinones was conducted using juvenile
106 rainbow trout (size 0.3 – 0.7g), according to the requirements of Environment and
107 Climate Change Canada's 'Biological best method: acute lethality test using rainbow
108 trout' (Report EPS1/RM/9).[13] Moderate loss (~ 50 % in 96 hours) of 6PPD-Q was
109 observed in preliminary experiments, mainly due to adsorption to exposure containers.
110 However, a similar rate of loss was observed for both plastic and glass containers, and
111 thus plastic containers were eventually selected for the study. Before the exposure
112 experiments, successful monthly KCl reference toxicant tests were performed,
113 confirming organism health in the test methodology.

114 Exposures of rainbow trout were performed in 20 L plastic containers lined with
115 food grade polyethylene disposable liners at 15 ± 1 °C for 96 ± 2 hours. Rainbow trout
116 were exposed to nominal concentrations of each individual PPD-quinone at 0.2, 0.8, 3,
117 12, and 50 µg/L (25 µg/L for 6PPD-Q), by spiking ~ 2 mL of methanol stock solution
118 into 20 L of water. The toxicity of DPPD-Q was only tested at 12 and 50 µg/L, as
119 toxicity was not observed for the two highest exposure concentrations. Three replicates
120 were performed for each treatment group, with 10 fish being included in each replicate.
121 Control exposures were dosed with the methanol solvent vehicle at the same level as
122 that of the treatment groups (0.01 %). Methanol was selected as the exposure solvent

123 as it showed the best solubility, and no acute toxicity was observed at 0.01 % methanol.
124 Tests were conducted in static conditions and fish were not fed for at least 16-hours
125 before testing and as well as during the 96 hours of exposure. Mortality and immobility
126 of fish were recorded daily.

127 Over the 96 hours of exposure, 0.5 mL of exposure water samples were taken
128 during the exposure to measure the concentrations of PPD-quinones. The water samples
129 were mixed with 0.5 mL of methanol and stored at -80 °C until LC-HRMS analysis.
130 Dead fish were removed from the exposure containers during the experiments and
131 stored at -80 °C. At the end of the exposure, all surviving fish were euthanized with
132 CO_2-charged water and stored at -80 °C until chemical analysis.

133 **Targeted Chemical Analysis in Water and Fish Samples**. To measure the
134 concentrations of PPD-quinones in exposure water samples, water samples (N = 3)
135 from each treatment group were collected, wherein 0.425 mL of each water sample
136 (with methanol) was spiked with 0.0472 mL of 100 µg/L of d_5-6PPD-Q in methanol.
137 After vortexing for 1 minute, the samples were directly subjected to LC-HRMS for
138 analysis. We did notice that the measured concentrations of DPPD-Q in water samples
139 were significantly lower than nominal concentrations, probably due to their low
140 solubility. Therefore, fish tissue measurements and metabolite identification were not
141 conducted for DPPD-Q. For 6PPD and other PPD-quinones, measured water
142 concentrations were similar to nominal concentrations except for the high
143 concentrations at 50 µg/L which might exceed their water solubilities (Table S1). For

instance, the solubility of 6PPD-Q was measured to be 38 ± 10 µg/L by Hu *et al.*[14]

The method used to extract PPD-quinones from fish samples was adapted from a recent study.[4] In brief, the whole fish body was homogenized (N = 3) and was spiked with 90 ng of d_5-6PPD-Q in a 1.5 mL Eppendorf tube. After equilibrium, 300 mg of NaCl, 300 µL of water, and 510 µL of acetonitrile were added to the tube, followed by vortexing for 1 minute. Each sample was shaken for 20 minutes, sonicated for 20 minutes, and then centrifuged at 5000×g for 5 minutes. The supernatant was then carefully transferred to a new Eppendorf tube. The extraction was repeated with an additional 600 µL of acetonitrile. The supernatants were combined, and then 3.6 mg of C18 and 40 mg of $NaSO_4$ was added. After vortexing for 1 minute, the extract was centrifuged at 5000×g for 5 minutes. The extract was then transferred to a fresh amber glass LC vial for LC-HRMS analysis. The fish tissue samples from the 0.2 µg/L exposure groups were not used for chemical measurements as the concentrations were below method detection limits (MDLs).

LC-HRMS. PPD-quinones and their metabolites were measured using a Vanquish ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific) equipped with a Q Exactive orbitrap mass spectrometer (Thermo Fisher Scientific; HRMS). A Hypersil GOLD™ C18 column (50×2.1 mm, 1.5 µm) was used for chromatographic separations. The injection volume was 2 µL, and the column and sample compartment temperatures were maintained at 40 °C and 10 °C, respectively. Mobile phases consisted of 0.1% formic acid in ultrapure water (A) and 0.1% formic

acid in methanol (B) with an LC method as follows: B was increased from 10% to 100% in 5 min, maintained at 100% for 3.5 min, then decreased to 10% at 9 min and maintained for 2 minutes. The flow rate remained at 0.25 mL/min throughout.

Data were acquired in full scan (*m/z* 100 – 900) with electrospray ionization (ESI) in both positive and negative ion modes. Spectra were recorded at resolution R = 70 000 (at *m/z* 200) with a maximum of 3×10^6 ions collected within 100 ms. Other parameters included 2.7 kV spray voltage, 30 L/h sheath gas flow rate, 6 L/h auxiliary gas flow rate, and 300 °C capillary temperature.

Nontargeted Identification of Metabolites. Nontargeted analysis was performed using the in-house algorithm developed in our previous studies.[15-17] In brief, raw mass spectrometry files were converted to a .mzXML format. Only the fish tissue samples from the highest exposure concentrations (25 µg/L for 6PPD-Q, and 50 µg/L for other PPD-quinones, N = 3) were selected for metabolite identification. This, in total, was 27 raw mass spectrometry files including three control fish samples. Peak features from fish tissue extracts were detected using the 'XCMS' R package at a mass tolerance of 2.5 ppm.[18] Features were matched across different samples using the 'group' function, with a 0.5 minute retention time tolerance window. Missing values of peaks were matched using an in-house script by checking exact mass across a pre-assigned retention time window (± 0.5 min). More than 40,000 LC-HRMS features were detected across the 27 mass spectrometry files.

Only peak features which exhibited a 10 times higher abundance (when compared

186 to control fish) with statistical significance ($p < 0.05$), were considered as putative
187 metabolites. This narrowed down the original > 40,000 LC-MS features to ~ 2,000
188 features. To further exclude the possible minor impurities of synthesized chemicals or
189 solvents, putative metabolites were further shortlisted by comparing their peak
190 abundances to the fish tissues of the OPPD-Q exposure group, which showed limited
191 bioaccumulation in fish due to the limited water solubility of OPPD-Q. This further
192 narrowed down the metabolites to ~ 50 LC-HRMS features.

193 We decided to focus on the abundant metabolites with peak intensities > 10^7, as
194 abundant metabolites were more likely to play a major role in toxicity and
195 bioaccumulation. This further narrowed down the LC-MS features to 1 – 3 for each
196 PPD-quinone. Then, elemental compositions of each feature were predicted with a mass
197 tolerance of 5 ppm. Chemical formulas were constrained with 6 – 30 C, 5 – 50 H, 0 –
198 10 N, 0 – 1 S, and 0 – 10 O. All assigned formulas were required to meet the basic
199 chemical criteria as described in previous studies.[19] Collectively, hydroxylated
200 metabolites were identified as the only significant metabolites for PPD-quinones, while
201 an additional N-dealkylation metabolite was identified for 6PPD. The results confirmed
202 hydroxylation as the predominant metabolism pathway of PPD-quinones.

203 **Quality Assurance and Quality Control**. Procedural blanks without tissue samples
204 were incorporated along with each batch of samples to check for potential background
205 contamination. Standard injections were performed immediately following every ~ 20

206 sample injections to check the stability of the instrument. Methanol was injected after
207 standards to monitor for any potential carryover.
208 Recoveries of 6PPD and seven PPD-quinones were assessed by spiking 20 µL of a
209 PPD-quinone mixture (1 mg/L for each chemical) into control fish tissue samples (N =
210 3). Sufficient recoveries were obtained for d_5-6PPD-Q (101 ± 6.2%), 6PPD (54 ± 10%),
211 6PPD-Q (89 ± 4.8%), (IPPD-Q; 91 ± 2.3%), (CPPD-Q; 93 ± 2.4%), (HPPD-Q; 98 ±
212 2.6%), OPPD-Q (58 ± 8.6%), DPPD-Q (97 ± 4.9%), and (DTPD-Q; 69 ± 8.6%). Strong
213 linearity (R^2 > 0.99) was obtained for the external calibration curves of all PPD-
214 quinones (0.2 – 50 µg/L). Quantification of PPD-quinones were determined using
215 relative response against the surrogate standard d_5-6PPD-Q by internal calibration
216 curves. The MDLs were calculated by a 99 % confidence level of y-intercept divided
217 by the slope of the calibration curve, which ranged from 0.1 – 0.6 ng/g in tissue samples.
218 As PPD-quinones in most tissue samples from the 0.2 µg/L exposure group were below
219 the MDLs, these tissue samples were excluded for subsequent measurements. Due to
220 the lack of authentic standards for hydroxylated metabolites, their concentrations were
221 semi-quantified using their corresponding parent PPD-quinones.
222 **Data Analysis**. Statistical analyses were performed using GraphPad Prism (v7.0.4,
223 GraphPad software Inc, San Diego, CA, USA) or R studio (v1.1.456, RStudio, Inc.,
224 Boston, MA, USA). Bioconcentration factors (BCF) of PPD-quinones were calculated
225 using analyte concentrations in fish and their corresponding media (eq 1) as described
226 in previous studies.[20-23]

227 $$BCF = \frac{C_{fish}}{C_w} \quad (1)$$

228 Where C_{fish} and C_w are the concentrations of PPD-quinones in rainbow trout and
229 exposure media, respectively. Note that the BCF of PPD-quinones might be
230 underestimated as steady-state might not be achieved during 4 days of exposure.

231

232 **Results and Discussion**

233 **Synthesis of PPD-quinones.** Seven PPD-quinones were synthesized by use of the
234 synthetic methods previously reported by MacGregor *et al.*[12] and Hiki *et al*.[5] In addition
235 to 6PPD-Q and four other PPD-quinones with commercially used parent PPDs (*i.e.,*
236 CPPD-Q, IPPD-Q, DPPD-Q, DTPPD-Q), HPPD-Q and OPPD-Q were also synthesized
237 to evaluate the potential toxicological impact of the side chain length (see structures in
238 Figure 1b). The purities and identities of all synthesized PPD-quinones were rigorously
239 characterized using a combination of ^{1}H-NMR, ^{13}C-NMR, heteronuclear multiple bond
240 correlation NMR (HMBC-NMR), and LC-HRMS (see Figures S1-S16).

241 The quantification accuracy of synthesized PPD-quinones was crucial for
242 quantitative structure-related toxicity testing. We confirmed this through three
243 independent pieces of evidence: 1) similar ^{1}H-NMR signals were detected for all PPD-
244 quinone stock solutions at the same concentration (within a factor of 1.2), and the
245 internal standard trimethoxybenzene was further used to adjust the concentrations of
246 stock solutions based on total NMR signals; 2) all PPD-quinones showed similar LC-
247 HRMS peak intensities within a factor of 3 (Figure S17); 3) the LC-HRMS peak

intensity of synthesized 6PPD-Q was similar to commercially purchased 6PPD-Q (within a factor of 2). Thus, we concluded that our synthesized standards were accurate and valid for subsequent toxicity testing.

Selective toxicity of 6PPD-Q in rainbow trout. Together with 6PPD and the PPD-quinones, a total of eight chemicals were tested for their toxicity at five concentrations (0.2, 0.8, 3, 12, and 50 µg/L), resulting in a total of ~ 120 individual exposures. To reduce the chemical consumption, we used 2-month old juvenile rainbow trout (0.3 – 0.7 g) for toxicity testing, rather than 1-year old rainbow trout which were previously used by Brinkman *et al*.[6] We first tested 6PPD-Q to investigate whether the smaller rainbow trout could replicate the results reported by Brinkman *et al*. 100 % fish mortality was observed at 3, 12, and 25 µg/L of 6PPD-Q during the first day of exposure. All fish exposed to 0.8 µg/L of 6PPD-Q survived the first day of exposure, but 30 %, 43% and 43% of mortality was observed at Day 2, Day 3, and Day 4, respectively. The 96 h LC_{50} of 6PPD-Q was calculated at 0.64 µg/L, which was slightly more sensitive than 1-year old rainbow trout (96 h LC_{50} = 1.0 µg/L)[6], confirming juvenile rainbow trout as a valid animal model for PPD-quinone toxicity testing.

We then moved forward to test the toxicity of 6PPD and the six other synthesized PPD-quinones. No toxicity effects were observed for any of the other six PPD-quinones up to 50 µg/L. This result indicated the toxicity of 6PPD-Q was at least 62.5-times stronger than other PPD-quinones, even for C_6-side chain derivatives (CPPD-Q and HPPD-Q) with very similar structures to 6PPD-Q. To confirm this finding, multiple

cross-validation experiments were conducted. Firstly, we measured the concentrations of the PPD-quinones in the exposure water samples via LC-HRMS and found that apart from DPPD-Q, which showed low concentrations likely due to limited solubility, the other PPD-quinones exhibited comparable water concentrations (Table S1). This suggested that water solubility should not explain the selective toxicity of 6PPD-Q. Secondly, we compared the toxicities of the synthesized and commercially purchased 6PPD-Q and obtained reproducible toxicity results between the two exposures, which confirmed that our synthesis and purification of the PPD-quinone standards was valid. Lastly, the selective toxicity of 6PPD-Q to rainbow trout was also assessed by the Krogh group (co-author), wherein the toxicity testing of PPD-quinones was conducted entirely independently, under a similar exposure condition (fish size is ~0.3 g). In this separate investigation using commercially purchased standards, an 96h LC_{50} of 0.79 μg/L was observed for 6PPD-Q, while no toxicity was observed for 77PD-Q (5.0 μg/L), CPPD-Q (4.6 μg/L), or IPPD-Q (13 μg/L) even at the highest concentrations tested. Overall, these collective results demonstrate that the selective toxicity of 6PPD-Q to rainbow trout relative to other PPD-quinones should be attributed to intrinsic structure-related properties.

Bioaccumulation cannot explain the selective toxicity of 6PPD-Q. Both bioaccumulation[24] and target protein engagement (*e.g.,* coplanar polychlorinated biphenyls and the aryl hydrocarbon receptor)[25] can lead to the structural selective toxicity of chemical contaminants. To assess the potential contribution of

bioaccumulation, we measured the concentrations of the PPD-quinones in rainbow trout whole-body tissues. A dose-dependent increase of 6PPD-Q concentration (2.34 - 432 ng/g) in fish tissues was observed (Figure 2a). The whole-body bioconcentration factors (BCFs) of 6PPD-Q (2.3 – 432 ng/g) were calculated as 2.9, 19, 25, and 17.2 L/kg at the water concentrations of 0.8, 3, 12, and 25 µg/L, respectively. This was similar to the BCF of 6PPD-Q that was previously reported in *Salvelinus leucomaenis pluvius* (8.6 L/kg in brain, and 24 L/kg in gill).[4] The concentrations of 6PPD ranged from 4.2 – 604 ng/g in fish tissues, which were comparable to those of 6PPD-Q. This demonstrated that bioaccumulation should not be responsible for the selective toxicity of 6PPD-Q compared to 6PPD.

Notably, the tissue concentrations of the six other PPD-quinones (data was not shown for OPPD-Q and DPPD-Q as they were only detected in fish exposed 25 and 50 µg/L) were an order of magnitude lower than those of 6PPD-Q (Figure 2a). The low tissue concentrations of OPPD-Q and DPPD-Q should be primarily attributed to aforementioned low water solubility. However, the low tissue concentrations of HPPD-Q and CPPD-Q were surprising, as their predicted $\log K_{OW}$ values (4.12 and 3.94 for HPPD-Q and CPPD-Q, respectively; EPI Suite) were similar to that of 6PPD-Q (3.98). This demonstrated that the relatively high tissue concentrations of 6PPD-Q compared to other PPD-quinones is unlikely to be attributed to hydrophobicity-driven partitioning. Alternatively, they may be due to a general decrease in the metabolic activity of lethal fish exposed to 6PPD-Q. To test this, we measured the tissue concentrations of PPD-

311 quinones in rainbow trout which survived at an exposure concentration of 0.8 µg/L
312 6PPD-Q. The tissue concentrations of 6PPD-Q in surviving fish (2.9 ± 1.7 ng/g, N =
313 3) were comparable to the other PPD-quinones (0.55 – 2.27 ng/g, N = 3) exposed at the
314 same water concentration of 0.8 µg/L (Figure S18). This demonstrated that the
315 bioaccumulation of 6PPD-Q at a non-lethal exposure concentration was comparable to
316 other PPD-quinones, and thus bioaccumulation may not explain the selective toxicity
317 of 6PPD-Q.

318 **Discovery of regioselective hydroxylated metabolites.** The observed BCF (2.9 – 25
319 L/kg) of 6PPD-Q in rainbow trout was 1 – 2 orders of magnitude lower than that of
320 other well-studied chemical contaminants with similar K_{OW} values such as
321 dichlorobenzene (BCF = 214 L/kg, $\log K_{OW}$ = 3.38) and diphenyl (BCF =437 L/kg,
322 $\log K_{OW}$ = 4.09).[26] This suggested that 6PPD-Q may be rapidly metabolized in rainbow
323 trout, and therefore we employed nontargeted analysis to identify the metabolites of the
324 PPD-quinones in fish tissue. Among the > 40,000 features detected for the 6PPD-Q-
325 exposed trout, hydroxylated 6PPD-Q (OH-6PPD-Q; *m/z* = 315.1690, $C_{18}H_{23}N_2O_3$) was
326 identified as the only metabolite exhibiting significant peak intensity (*i.e.,* > 10^7). This
327 result was consistent with two previous studies that reported the detection of OH-6PPD-
328 Q in fish tissues exposed to 6PPD-Q.[4, 27] Similarly, OH-6PPD (*m/z* = 285.1955,
329 $C_{18}H_{25}N_2O$) was also detected in fish tissues, together with its N-dealkylation product
330 (*m/z* = 186.0907, $C_{12}H_{12}N_2O$). Notably, hydroxylated metabolites were also identified
331 as the predominant metabolites for all six other PPD-quinones (see chromatograms in

Figure 2b). Their identities were clearly supported by their ~ 0.5 min earlier retention times compared to their corresponding parent PPD-quinones. These results confirmed that PPD-quinones were metabolized in rainbow trout via enzymatic hydroxylation.

Due to the lack of authentic standards, we used parent PPD-quinones to semi-quantify the concentrations of their hydroxylated metabolites in fish tissues. The concentrations of OH-HPPD-Q, OH-CPPD-Q, OH-IPPD-Q, and OH-DTPD-Q were comparable or even higher than their parent quinones across all exposure groups (Figure 2a). For surviving fish exposed to 0.8 μg/L of 6PPD-Q, the concentrations of OH-6PPD-Q (6.6 ± 0.52 ng/g) were also ~ 3 times higher than those of 6PPD-Q (2.34 ± 1.70 ng/g) in the same tissue samples. However, the concentrations of OH-6PPD-Q in lethal fish (28.4 – 77.6 ng/g) were generally lower than those of 6PPD-Q (57 – 432 ng/g). This confirmed that the metabolic activity of the lethal fish was impaired, which led to the accumulation of 6PPD-Q and decrease in its metabolite OH-6PPD-Q.

While the generally high concentrations of hydroxylated PPD-quinones could not explain the selective toxicity of 6PPD-Q, upon closer investigation, we noticed that the peak for OH-6PPD-Q was comprised of two abundant isomers (RT=4.39 and 4.75 min, see the peak labeled with asterisk in Figure 2b). This was unique to OH-6PPD-Q as a single predominant hydroxylated metabolite was detected for other PPD-quinones. We proceeded to collect high-resolution MS^2 data to assign the position of the hydroxyl group. We first inspected the MS^2 spectra of OH-HPPD-Q as it was most structurally similar to 6PPD-Q. Three diagnostic fragments at *m/z* 110.0601, 203.0811, and

231.0761 assisted us to unambiguously locate the hydroxyl group as being on the aromatic ring (Figure 3a). Similar results were also obtained for OH-IPPD-Q, OH-DTPD-Q, and OH-CPPD-Q, indicating that the hydroxyl group was located on the aromatic ring for these metabolites as well (Figures S19). Similar aromatic hydroxylation was also observed for the second OH-6PPD-Q isomer eluted later (RT=4.75 min). However, when evaluating the MS^2 spectra of the first OH-6PPD-Q isomer eluted earlier (RT=4.39 min, labeled in Figure 2b), we discovered six diagnostic fragments at *m/z* 94.0651, 99.0809, 187.0862, 215.0811, 241.0971, and 257.1283, which clearly supported that the hydroxylation of the OH-6PPD-Q isomer occurred on the alkyl side chain. Similarly, MS^2 spectra of OH-6PPD also supported its hydroxylation on the alkyl side chain, for both isomers (Figure S20). These results clearly demonstrated the regioselective hydroxylation of 6PPD and 6PPD-Q on the alkyl side chain, in contrast to the aromatic ring hydroxylation of other PPD-quinones (Figure 3b).

Regioselective hydroxylation has been well documented as a strategy for endogenous metabolites to achieve desired biological functions. For instance, docosahexaenoic acid (DHA) can be hydroxylated by lipoxygenases at each double bond to form multiple downstream hydroxylated DHA metabolites that play distinct functions.[28, 29] 7(S)-hydroxylated DHA, but not other hydroxylated DHA isomers, was recently identified as a selective agonist of human peroxisome proliferator-activated receptor alpha (PPARα) by forming a H-bond between the hydroxyl group and Cys-276

374 inside the binding pocket.[30] Inspired by this, we suggested a 'dual-action' model
375 wherein 6PPD-Q is hydroxylated by an enzyme on the side alkyl chain, which
376 subsequently forms a strong H-bond with a presently unknown target protein that
377 mediates lethal effects (Figure 3c). For HPPD-Q and other PPD-quinones, despite their
378 similar chemical structures, the regioselective aromatic hydroxylation precludes the
379 formation of H-bonding with said unknown target protein. The strength of an isolated
380 H bond in proteins is typically 5-6 kcal/mol,[31, 32] corresponding to ~1,000-fold
381 difference in binding affinity ($K_d=e^{\Delta G/RT}$), which can well explain the selective toxicity
382 of 6PPD-Q. Notably, an alternative 'single-action' model mediated by the direct
383 interaction between 6PPD-Q and the protein target might be possible. A 'small'
384 favorable free energy of 0.8 kcal/mol (corresponding to 1.8-fold difference in K_d) was
385 typically observed for the addition of an sp^3 carbon atom at protein-ligand binding.[33, 34]
386 This seems unlikely to explain the toxicity difference of 6PPD-Q, CPPD-Q, HPPD-Q
387 and IPPD-Q, with a similar/same number of sp^3 carbon atom. Thus, 'dual-action' model
388 might be more reasonable, but future studies are warranted to support this by
389 investigating the toxicity of the regioselective OH-6PPD-Q isomers in-depth.
390 **Implications.** We report here the highly selective toxicity of 6PPD-Q to rainbow trout.
391 If these results could be replicated in other fish species, particularly coho salmon, this
392 would pinpoint that several currently commercially used PPDs (*e.g.,* IPPD and CPPD)
393 may be safer replacements for 6PPD. However, many other ozonolysis reaction
394 products of PPDs are formed in addition to PPD-quinones[7], and thus a systematic

toxicity testing of the reaction products of PPDs beyond PPD-quinones is imperative.

The absence of toxicity of other PPD-quinones was surprising considering their similar structures to 6PPD-Q. Such an extreme structural selectivity strongly suggests that protein binding, rather than nonspecific toxicity mechanisms (*e.g.,* reactive oxygen species, DNA damage, or mitochondrial proton uncoupling), should mediate the toxicity. We have proposed a 'dual-action' model based on the results collected from the current study, however, this hypothesis requires further validation studies. Particularly, extensive efforts are needed to further identify the enzyme and the target protein responsible for the reported selective toxicity of 6PPD-Q.

Supporting Information Available

The supporting information provides text and figures addressing: (1) The LC-HRMS chromatograms and NMR spectra of synthesized PPD-quinones; (2) The MS^2 spectra of hydroxylated metabolites of PPD-quinones.

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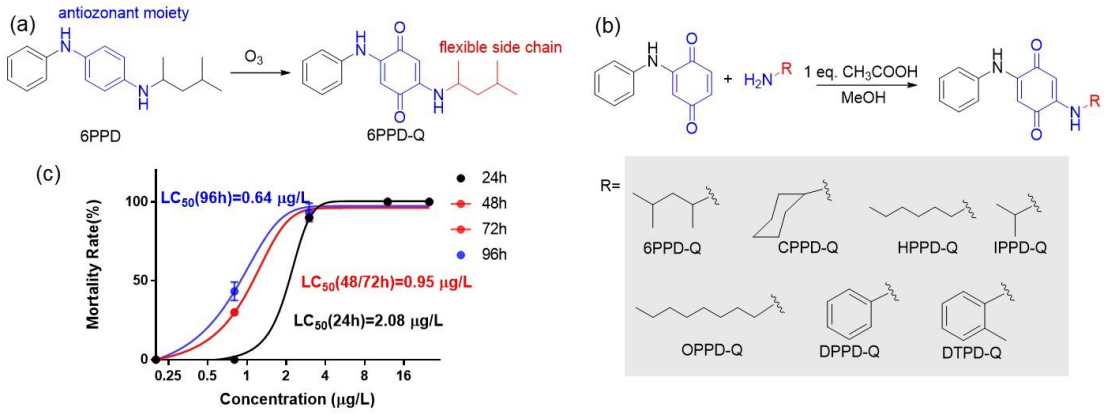

Figure 1. (a) Oxidation of 6PPD to toxic 6PPD-Q by ozone. The essential antiozonant moiety is labeled in blue. The flexible side chain is labeled in red. (b) Synthesis of seven PPD-quinones with distinct side chains. DTPD-Q is symmetric. (c) Acute toxicity of 6PPD-Q to rainbow trout (N=3). Data was not shown for 6PPD and other PPD-quinones, as no toxicity was observed up to 50 μg/L.

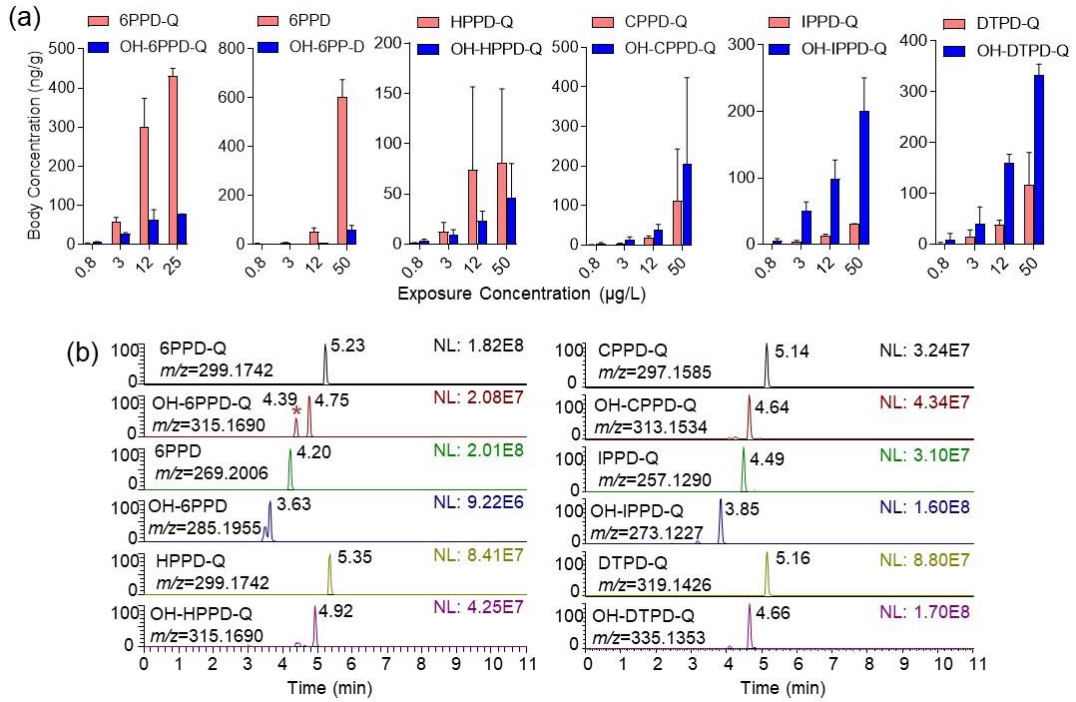

Figure 2. (a) Concentrations of PPD-quinones (red) and hydroxylated PPD-quinones (blue) in whole body rainbow trout (N=3 from each treatment). Rainbow trout exposed to 3, 12 and 25 µg/L of 6PPD-Q were killed at Day 1 and collected, while fish was collected at Day 4 for other treatment groups. Data is not shown for OPPD-Q due to its low fish tissue concentrations. Data is not shown for DPPD-Q due to its low water solubility. (b) Chromatograms of PPD-quinones and corresponding hydroxylated PPD-quinones in fish tissues collected from the highest concentration group. Note that the retention times of hydroxylated PPD-quinones are ~0.5 min earlier than corresponding precursor PPD-quinones. Two abundant isomers of OH-6PPD-Q were detected with retention times of 4.75 and 4.39 min (labeled with asterisk).

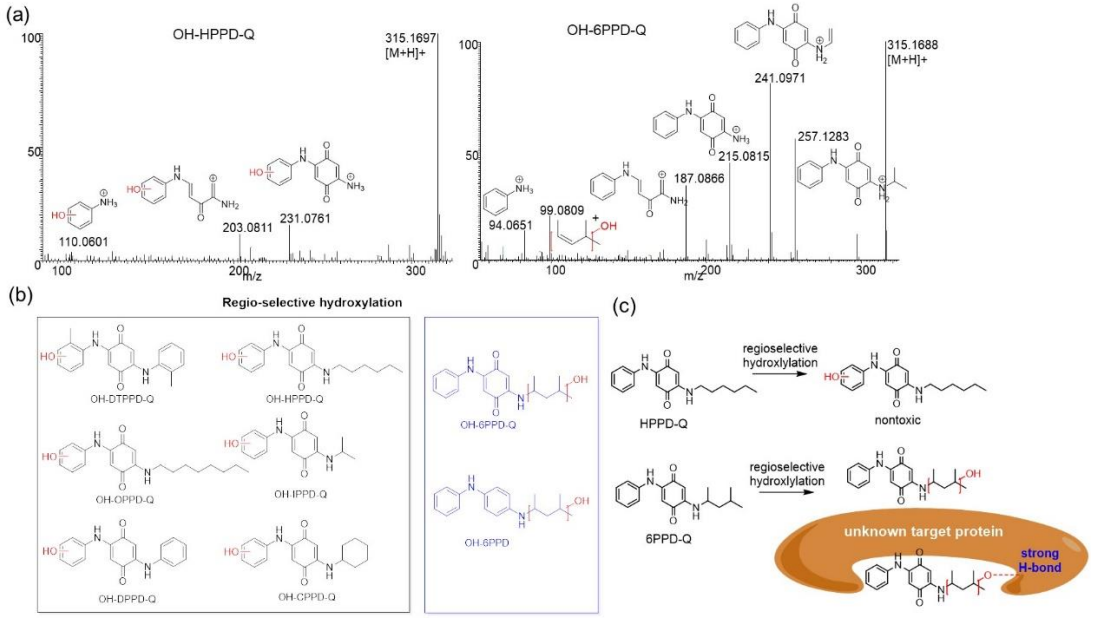

Figure 3. (a) Representative MS^2 spectra of OH-HPPD-Q and OH-6PPD-Q (the isomer eluted at 4.39 min) supporting the location of hydroxyl group. (b) 6PPD-Q and 6PPD are hydroxylated at both the alkyl side chain (blue box) and aromatic ring, while other PPD-quinones are hydroxylated at the aromatic ring (black box). (c) A 'dual-action' model explains the high toxicity selectivity of 6PPD-Q.

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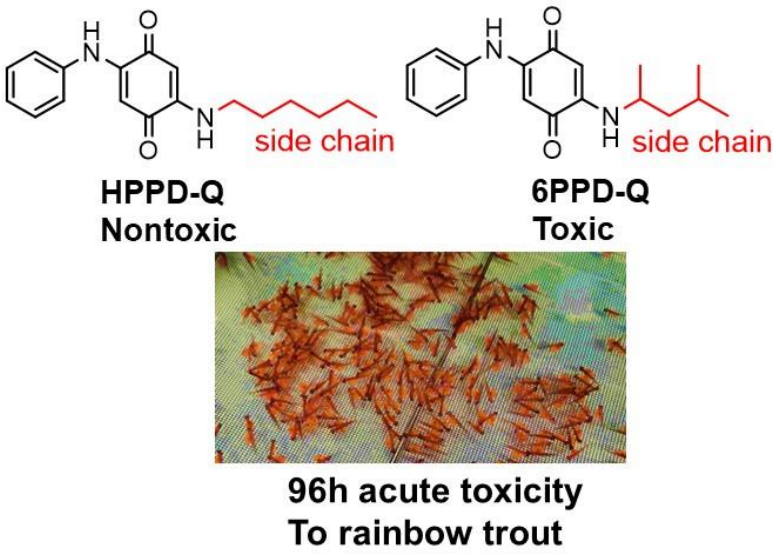

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