77PD-Quinone: Synthesis, Coho Salmon Toxicity Assessment, and Comparison with the Commercial Antidegradant 77PD

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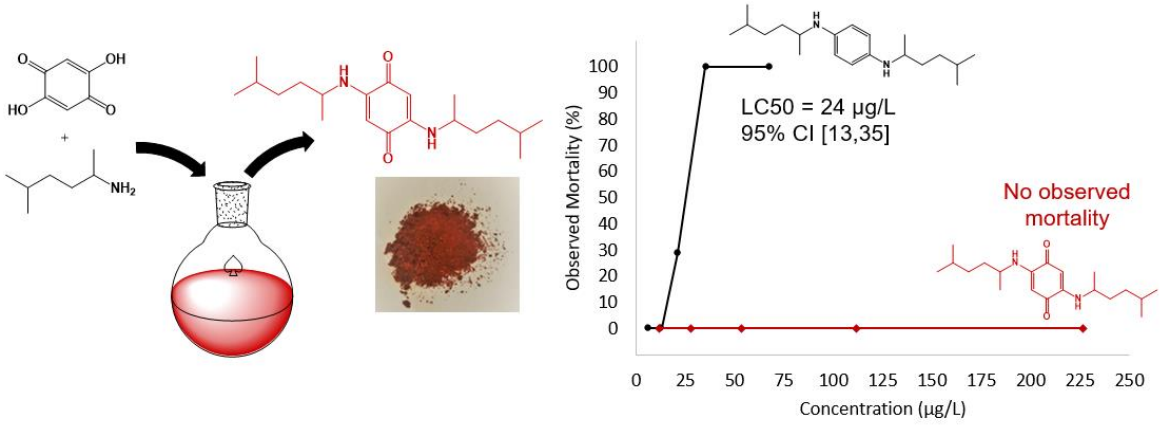
KEYWORDS

Antidegradant; 77PD; 77PD-quinone; 6PPD; 6PPD-quinone; Coho salmon; aquatic toxicity; LC_{50}, No Observed Effect Concentration (NOEC); Limit of Quantitation (LOQ); Limit of detection (LOD).



ABSTRACT

There is a growing interest in assessing the toxicity of para-phenylenediamine-based rubber additives and their quinone transformation products. A technique for the multi-gram scale synthesis of 2,5-diaminoquinones was developed to prepare 2,5-bis((5-methylhexan-2-yl)amino)cyclohexa-2,5-diene-1,4-dione (77PD-quinone) for toxicity assessment. The toxicities of N,N'-bis(5-methyl-2-hexanyl)-1,4-benzenediamine (77PD) and 77PD-quinone were evaluated in coho salmon. Juvenile coho salmon were exposed to a geometric series of five test concentrations of 77PD or 77PD-quinone, a negative control (dilution water), and a solvent control (100 µL/L dimethylformamide) for 96 hours under flow-through conditions. 77PD was found to be toxic to coho salmon with a 96-hour LC_{50} value of 24 µg/L active ingredient (a.i.), and the NOEC was 13 µg a.i./L. No mortality was observed for 77PD-quinone at the highest attainable dose level, 226 µg a.i./L, at which signs of test water saturation were observed.

INTRODUCTION

Since the 1960's, compounds based on para-phenylenediamine chemistry have maintained leading positions as antidegradants of choice for rubber and rubber containing compounds.[1-4] N-(4-methyl-2-pentanyl)-N'-phenyl-1,4-benzenediamine (6PPD) is widely used in tires and is currently produced in volumes exceeding 225,000 tons annually to meet the global market demand.[5-8] The California Department of Toxic Substances Control has listed motor vehicle tires containing 6PPD as Priority Products.[9] Consequently, there is a need to find a replacement for 6PPD in rubber applications. Numerous chemicals including N,N'-bis(5-methyl-2-hexanyl)-1,4-benzenediamine (77PD) are being evaluated. In 2022, the Washington State Department of Ecology conducted a GreenScreen® based evaluation of 6PPD alternatives which included 77PD.[10] The outcome of the evaluation was that 77PD was given a BM-2 rating (i.e. Use but Search for Safer Substitutes) owing in part to its known acute aquatic toxicity, while the other five para-phenylenediamine based antidegradants evaluated were rated BM-1 (i.e. Avoid: Chemical of High Concern). The tire industry estimated that 77PD would not provide the same long-lasting tire protection as 6PPD.[11] Being part of the para-phenylenediamine class of antidegradants, 77PD, has the potential to form a 2,5-diaminobenzoquinone.[12] Indeed, 2,5-bis((5-methylhexan-2-yl)amino)cyclohexa-2,5-diene-1,4-dione (77PD-quinone) has been detected in particulate matter collected from the Chinese cities of Taiyuan and Guangzhou.[13] Consequently, the toxicities of 77PD and that of 77PD-quinone to coho salmon are of substantial interest to the scientific community. This article describes a robust preparation of 77PD-quinone, as well as the first evaluation of 77PD and 77PD-quinone toxicities in coho salmon.

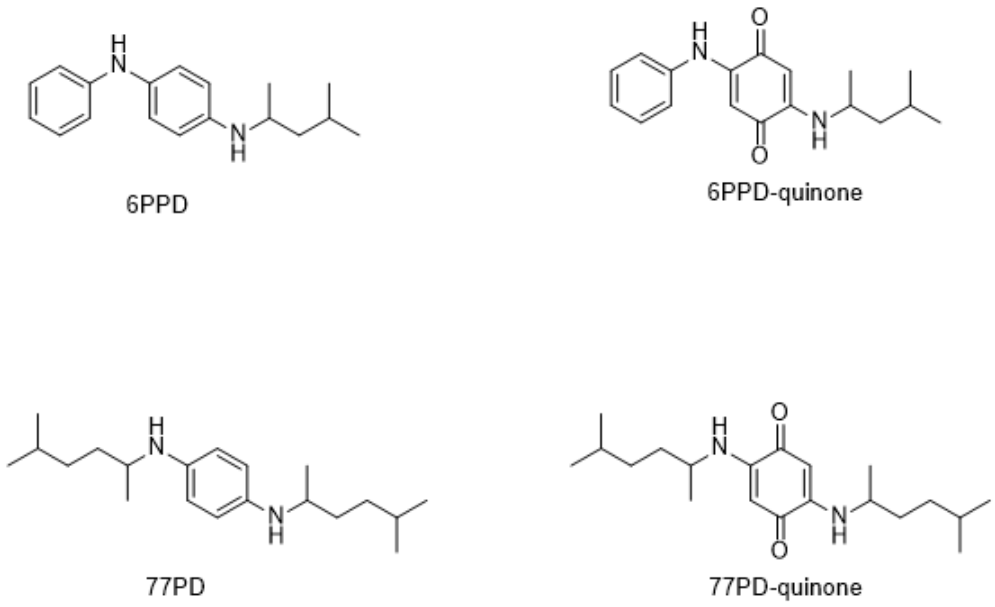


Figure 1. Structures of 6PPD, 6PPD-quinone, 77PD, and 77PD-quinone

Materials and Methods

Chemicals and Reagents. All solvents and reagents were purchased from suppliers and utilized without further purification. See Supporting Information for details. 77PD was obtained from Flexsys. 77PD-quinone was prepared as described in the Supporting Information.

Fish Source. Coho salmon used in the tests were received as juveniles from Aquatic Research Organisms of Hampton, New Hampshire. Identification of the species was verified by the supplier. All fish used in the tests were juveniles from the same source and year class, and the length of the longest fish measured was no more than twice the length of the shortest. For the studies, daily during the holding period, except during periods of fasting prior to testing, the fish were fed a commercially-prepared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania. Fish use

in the study was based on procedures in the OECD Guidelines for Testing of Chemicals, Guideline 203: Fish, Acute Toxicity Test;[14] the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OCSPP 850.1075: Freshwater and Saltwater Fish Acute Toxicity Test;[15] and ASTM Standard E 729 96: Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians.[16]

Exposure Experiments. The acute effects of 77PD and 77PD-quinone were tested on coho salmon (*Oncorhynchus kisutch*) for 96-hour exposures under flow-through test conditions. Coho salmon were exposed to a geometric series of five test concentrations, a negative control (dilution water) and a solvent control (100 µL/L dimethylformamide). A single test chamber was maintained in each treatment and control group, with seven fish in each test chamber. Nominal test concentrations were selected based on exploratory range-finding toxicity data. For 77PD, nominal test concentrations selected were 15, 24, 39, 63, and 100 µg active ingredient (a.i.)/L. For 77PD-quinone, nominal test concentrations selected were 25, 50, 100, 200, and 400 µg a.i./L. Test concentrations were measured in samples of test water collected from each treatment and control group scheduled at 2, 24, and 96 hours after initiation, and were used to calculate mean measured test concentrations. Observations of mortality and other signs of toxicity were made approximately 3, 6, 9, and 12 hours following test initiation, and at 24 hours (±1 hour). Observations on days two to four of the test were performed twice, with one set of observations performed at 48, 72 and 96 hours (±1 hour of test initiation). Cumulative percent mortality observed in the treatment groups was used to determine the median lethal concentration (LC_{50}) values at 24, 48, 72 and 96-hour intervals. The no-observed-effect concentration (NOEC) was determined by statistical analysis of the mortality data. The mortality data were analyzed using the computer program of C. E. Stephan.[17] Based on the mortality pattern in this study, nonlinear interpolation was used to

calculate the 24, 48, 72, and 96-hour LC_{50} values and binominal probability was used to calculate the 95% confidence intervals. The NOEC based on mortality was analyzed using Fisher's Exact test ($p \leq 0.05$).[18]

Individual stock solutions were prepared for each of the five concentrations tested. For 77PD, solutions were prepared daily during the test. For 77PD-quinone, solutions were prepared every two days after flows were initiated in the diluter. All test solution concentrations were adjusted to 100% active ingredient during preparation, based on the test substance purity. For 77PD, a primary stock solution was prepared by mixing a calculated amount of test substance into dimethylformamide (DMF) at a nominal concentration of 1.0 mg a.i./mL. For 77PD-quinone, a primary stock solution was prepared by mixing a calculated amount of test substance into DMF at a nominal concentration of 4.0 mg a.i./mL. For each, the primary stock solution was partially brought to volume, sonicated for 15 minutes, and stirred for 15 minutes. The stock solutions contained no visible precipitates.

For 77PD, four secondary stock solutions were prepared in DMF at nominal concentrations of 150, 240, 390, and 630 µg a.i./mL by proportional dilution of the primary stock. For 77PD-quinone, four secondary stock solutions were prepared in DMF at nominal concentrations of 0.25, 0.50, 1.0, and 2.0 mg a.i./mL by proportional dilution of the primary stock. For each, the secondary stock solutions were mixed on a magnetic stir plate until fully dissolved with no visible precipitates.

The toxicity test was conducted using a continuous-flow diluter system to provide each concentration of the test substance, a negative control (dilution water only) and a solvent control (100 µL DMF/L) to test chambers. Syringe pumps were used to deliver volumes of test substance stock solutions to mixing chambers impartially assigned to each treatment group. DMF was

delivered to a separate mixing chamber assigned to the solvent control. The stock solutions or solvent were mixed with well water in the mixing chambers in order to prepare the test solutions at the appropriate nominal concentrations prior to delivery to the test chambers. Well water alone was delivered to a mixing chamber for the negative control. The flow of dilution water into each mixing chamber was controlled using rotameters and was initially adjusted to provide approximately 10 volume additions of test solution during the test in each test chamber per day. The approximate number of volume additions was increased to 24 volume additions three days prior to initiating the test. After mixing, the test water in each mixing chamber was delivered to the appropriate test chamber.

Syringes with each stock were placed on the delivery system pumps daily during the test. For each test substance, during the exposure period, the stock solutions were pumped into the diluter mixing chambers assigned to the treatment groups at a target rate of 25.0 µL/minute and were mixed with dilution water in the mixing chambers, delivered at a target rate of 250 mL/minute to achieve the desired nominal test concentrations (see Supporting Information for details). The negative control received dilution water only. The solvent control was prepared by delivering DMF to the mixing chamber for the solvent control at the same rate as the test substance stock solutions. The concentration of DMF in the solvent control and all treatment groups was 100 µL/L. All coho salmon in the negative and solvent control groups appeared normal throughout the test with 77PD and 77PD-quinone.

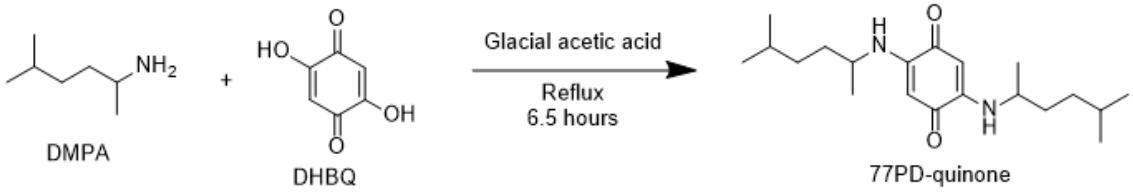
Analytical Chemistry. ^{1}H and ^{13}C NMR spectra were acquired on a 600 MHz Bruker Avance IIIHD NMR spectrometer. HPLC-UV/MS chromatograms used to confirm 77PD-quinone structure and purity were obtained using an Agilent 1260 Infinity II series HPLC system (see Supporting Information for details). Concentrations of 77PD in sample extracts over the exposure

periods were determined using an Agilent model 7890A gas chromatograph equipped with an Agilent Model 5975C mass selective detector (GC-MS) operated in the selective ion monitoring (SIM) mode. Separations were performed using Agilent DB-5MS column (30 m x 0.250 mm, 0.25 µm film thickness). The limit of quantitation (LOQ) for these analyses was set at 5.00 µg a.i./L, defined as the lowest nominal concentration in a matrix fortification sample with a mean recovery between 70-120% of nominal and a relative standard deviation ≤ 20%. The limit of detection (LOD) for these analyses was set at 1.33 µg a.i./L, defined as the dilution factor of the LOQ times the nominal concentration of the lowest calibration standard. Concentrations of 77PD-quinone in the samples over the exposure periods were determined using an Applied Biosystems/MDS Sciex API 4000 Mass Spectrometer coupled with an Agilent Infinity 1200 Series HPLC system. Chromatographic separations were achieved using a Thermo Betasil C-18 analytical column (50 mm x 2.1 mm, 3 µm particle size) and a Thermo Betasil C-18 guard column (10 x 2.1 mm). The limit of quantitation (LOQ) for these analyses was set at 2.50 µg a.i./L, defined as the lowest nominal concentration in a matrix fortification sample with a mean recovery between 70-120% of nominal and a relative standard deviation ≤ 20%. The limit of detection (LOD) for these analyses was set at 0.750 µg a.i./L, defined as the dilution factor of the matrix blank times the nominal concentration of the lowest calibration standard.

Results and Discussion

The preparation of symmetrical 2,5-dialkylamino-1,4-benzoquinones by reaction of alkyl-amines with hydroquinone or 1,4-benzoquinone is widely described in the literature including the synthesis of 77PD-quinone reported by Wang et al.[13, 19-22] However, when these approaches were attempted in our laboratories starting from 2-aminoheptane or 1,4-dimethylpentylamine, only poor yields of desired products (5-25% of the theoretical yields) were obtained. The limitation of these

approaches likely originates from excessive reactivity of 1,4-benzoquinone due to the electrophilicity of all four of its methine carbons. This leads to polymerization side-reactions, particularly in the presence of bases such as amines.[23] A similar problem arises when starting from hydroquinone due to *in situ* formation of benzoquinone by oxidation with elemental oxygen or air.[24,25] The less reactive (i.e. more chemoselective) substituted quinone 2,5-dihydroxy-1,4-benzoquinone (DHBQ) is a commercially available substance that is commonly used for reaction with certain nucleophiles. Schweinfurth et al. have showed the reaction of primary amines with DHBQ can afford 2,5-diaminobenzoquinones.[26] We employed a similar approach to synthesize 77PD-quinone by reaction of DHBQ with racemic 1,4-dimethylpentylamine (DMPA) in refluxing glacial acetic acid (Scheme 1). This efficient one-step approach was successful in consistently producing 77PD-quinone in moderate yields (see Supporting Information for details).



Scheme 1. Reaction of DHBQ with DMPA to form 77PD-quinone

The structure of the material synthesized was authenticated by ^{1}H and ^{13}C NMR spectroscopy. Diastereomers were detected by chromatographic and spectroscopic analyses. TLC (silica gel; 20% ethyl acetate in hexanes) displays two dots of similar intensity (UV light at 254-365 nm) at Rf = 0.5 & 0.6, and HPLC-UV/MS exhibits two co-eluting peaks at 14.62 min and 14.70 min with comparable ultraviolet and mass spectra. The mass spectra show mass to charge ratios of the parent ions at 335 amu (M+1) that align with the calculated molecular weight of 77PD-quinone (334.5

g/mol). It should be noted that 77PD-quinone as its diastereomeric mixture was used for the *in vivo* testing described herein, and any possible differences in toxicity across the stereoisomers were not examined.

Acute Toxicity of 77PD in Coho Salmon. 77PD exhibited low stability in the test water. Measured concentrations of the test water samples ranged from approximately 26 to 68% of nominal. The maximum flow rate of 250 mL/min allowable while maintaining appropriate environmental conditions for the test animals was therefore chosen to provide the greatest amount of volume turnovers within the test chambers in an effort to maintain the most consistent exposure concentration. When measured concentrations of the samples collected during the exposure test were averaged, the mean measured test concentrations for this study were 6.3, 13, 21, 35, and 68 µg a.i./L, representing 42, 55, 53, 56, and 68% of nominal concentrations, respectively. The results of the study were based on mean measured concentrations.

All fish in the 6.3 and 13 µg a.i./L treatment groups also appeared normal throughout the test, with no mortalities or overt signs of toxicity observed. Percent mortality in the 21, 35, and 68 µg a.i./L treatment groups at test termination was 29, 100, and 100%, respectively (Figure 2). Signs of toxicity observed among the surviving fish in the 21 µg a.i./L treatment group at test termination included surfacing and loss of equilibrium. While not statistically significant, a treatment-related effect in the 21 µg a.i./L treatment group could not be precluded, since there was a 29% decrease in survival in comparison to the pooled control. Therefore, in this study, the 96-hour LC_{50} value was 24 µg a.i./L, with a 95% confidence interval of 13 to 35 µg a.i./L and the NOEC for mortality was 13 µg a.i./L.

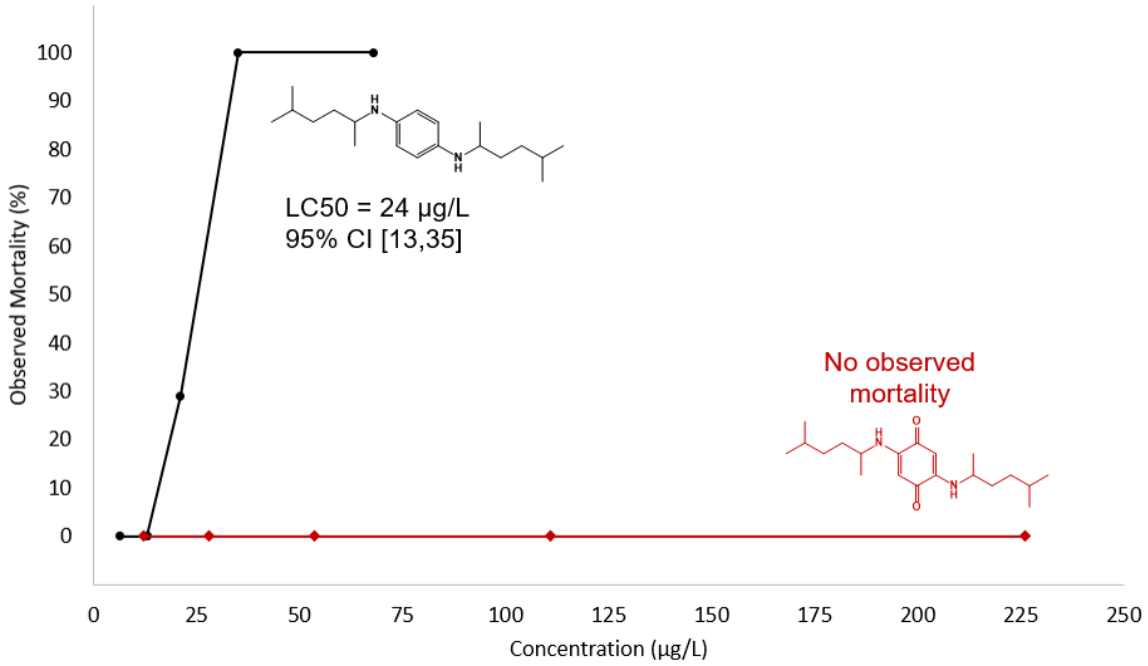


Figure 2. Concentration-response curve for 96-hour coho salmon exposure using 77PD.

In this study 77PD was observed to be more toxic to coho salmon compared to the reported 96-hour LC_{50} value of 60 µg/L for 77PD in fathead minnow,[27] and similar in toxicity to the reported 96-hour LC_{50} value of 28 µg/L for 6PPD in fathead minnow.[28] Tian et. al observed a 24-hour LC_{50} of 251 µg/L for 6PPD in coho salmon, whereas in this study no toxicity or mortality was observed at the highest (68 µg/L) 77PD dose level within that exposure time.[29] While a direct comparison of toxicity towards coho salmon cannot be made between 6PPD and 77PD in the two 24-hour periods, the available data suggests that the two compounds would exhibit similarly high 96-hour toxicities. Additionally, the 77PD 96-hour LC_{50} value of 24 µg/L in coho salmon is below the 0.1 mg/L acute toxicity limit proposed by the Washington State Department of Ecology in the 6PPD Alternatives Assessment Hazard Criteria, and thus would not meet the minimum requirements to be considered a potential replacement for 6PPD.[30]

Acute Toxicity of 77PD-quinone in Coho Salmon. 77PD-quinone was also found to be unstable in the test water, and a flow rate of 250 mL/min was employed. Measured concentrations of the samples ranged from approximately 45 to 61% of nominal, and the mean measured test concentrations for this study were 12, 28, 54, 111, and 226 µg a.i./L, representing 49, 56, 54, 56, and 57% of nominal concentrations, respectively. The results of the study were based on mean measured concentrations.

There were no coho salmon mortalities in any treatment group or control. One fish in the 12 µg a.i./L treatment group exhibited loss of equilibrium at 72 hours through termination. All other fish appeared normal, with no signs of toxicity. The absence of mortality in any of the treatment groups during the test (Figure 2) precluded the statistical calculation of LC_{50} values at 24, 48, 72 and 96 hours. Therefore, the LC_{50} values were estimated to be greater than the highest concentration tested. The NOEC was empirically estimated from the mortality data. The 96-hour LC_{50} value for mortality was >226 µg a.i./L, the highest concentration tested. The NOEC was 226 µg a.i./L. Attempts to test at higher levels were prevented by the low solubility of 77PD-quinone in the test water. Tyndall scattering was observed in the tank at the highest dose level, confirming that the dose concentration had reached saturation with no observed mortality.

The absence of mortality observed in the 96-hour exposure test for 77PD-quinone in coho salmon is in stark contrast with the reported toxicity of 6PPD-quinone in coho salmon.[29] However, 77PD-quinone exhibits significantly less stability in water, and considering these results it is unsurprising that 77PD-quinone has not been detected in aqueous environments despite its reported detection in roadside dust and fine particulate matter.[13] It is important to note that the toxicity of the products of 77PD-quinone hydrolysis have not been rigorously tested in this study,

and that toxicity towards other species of fish for either 77PD-quinone or its aqueous degradation products is not known.

A facile, multi-gram, one-step preparation of 77PD-quinone is reported herein. This allowed for enough material to be synthesized to conduct a flow-through aquatic toxicity study. This synthetic approach also enables researchers to produce quinone compounds for future environmental investigations. The first acute aquatic toxicity studies of 77PD and 77PD-quinone in coho salmon reveal that while 77PD is highly toxic (LC_{50} = 24 µg a.i./L), its quinone counterpart surprisingly exhibits no effects at the highest attainable dose level (226 µg a.i./L). While this investigation focuses on 77PD and 77PD-quinone, a profile of the transformation products that form upon ozonation of 77PD and rubber products containing 77PD is still needed.

Notes

Flexsys is a manufacturer of 77PD.

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