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In Vitro Metabolism of the Flame Retardant Triphenyl Phosphate in Chicken Embryonic Hepatocytes and the Importance of the Hydroxylation Pathway

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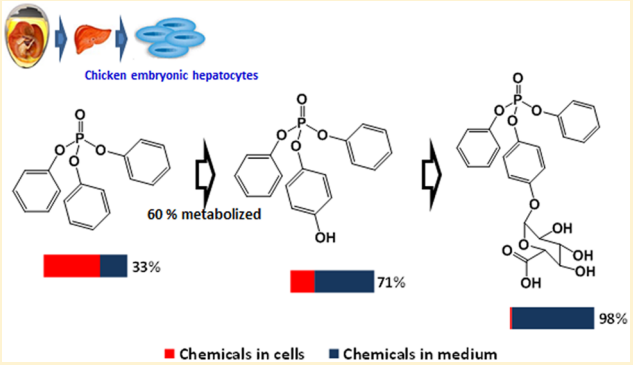
Supporting Information

ABSTRACT: We report for the first time either *in vitro* or *in vivo* the phase I hydroxylation and phase II conjugation metabolic pathways of an organophosphate flame retardant, triphenyl phosphate (TPHP), in addition to diphenyl phosphate (DPHP) metabolite formation. Using a chicken embryonic hepatocyte (CEH) assay, TPHP was phase I metabolized to *p*- and *m*-hydroxy-TPHP metabolites, which were largely present in the assay medium and cells as phase II conjugates with glucuronic acid. After treatment with *β*-glucuronidase, deconjugated *p*-OH-TPHP was present in both the medium and cells at increasing concentrations of 0.073 ± 0.003, 1.95 ± 0.03, and 2.10 ± 0.09 nmol/well at CEH incubation time points of 0, 12, and 36 h, respectively. Similarly, after *β*-glucuronidase treatment, there were increasing *m*-OH-TPHP concentrations of 0.0050 ± 0.0005, 0.18 ± 0.01, and 0.18 ± 0.01 nmol/well. *p*-OH-TPHP at 36 h accounted for 60% of the initial TPHP treatment concentration, which was 3.5- or 12-fold greater than that of the DPHP or *m*-OH-TPHP metabolites, respectively. Overall, in TPHP-exposed organisms, this study demonstrates the importance of phase I and II metabolic processes in the biological fate of TPHP.



INTRODUCTION

Flame retardant (FR) chemicals continue to be in high demand in the global marketplace because of strict fire safety standards worldwide. As a result, FRs are added to various manufactured materials such as plastics, foam, textiles, furniture, and many others to inhibit, suppress, or delay the production of flames and prevent the spread of fire.[1,2] The global phase-out of the commercial penta- and octa-bromodiphenyl ether FR formulations (Penta- and Octa-BDE, respectively)[3] has resulted in an increased demand for FR alternatives, including organophosphate (OP) triester FRs such as triphenyl phosphate (TPHP). Of note is the FR formulation Firemaster 550 (FM550) that is composed of approximately 40% of a mixture of bis(2-ethylhexyl) tetrabromophthalate (TBPH) and tetrabromobenzoate (TBB), and the remaining 60% is a mixture of triaryl phosphates, including TPHP and several isomers of mono-, di-, and tri-isopropylated triaryl phosphates (ITPs).[4,5]

TPHP is an additive FR that is not chemically bonded to polymer products and is therefore likely to be released into the environment over the lifetime of these products.[6] For instance, Stapleton et al.[7] detected TPHP at concentrations of ≤1.8 mg/g in 98% of house dust samples collected from homes in the area of Boston, MA, USA, between 2002 and 2007. TPHP was also detected at concentrations ranging from 42 ± 9 to 200 ± 27 pg/m^3 in particle phase samples collected at five sites in the North American Great Lakes basin from March 2012 to December 2012.[8] However, we recently reported that TPHP, and several other OP triester FRs, could not be detected in any body or egg compartments derived from female herring gulls (*Larus argentatus*) from the Laurentian Great Lakes of North America.[9] Similarly, TPHP concentrations in whole body homogenates of lake trout (*Salvelinus namaycush*) or walleye (*Sander vitreus*) collected from 16 Canadian lakes were not quantifiable, with the exception of one individual lake trout from Great Bear Lake (Northwest Territories, Canada).[10]

The low concentrations of TPHP (log K_{ow} = 4.70) being reported in biotic environmental samples strongly suggest that

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TPHP undergoes rapid metabolism in exposed organisms. Although several *in vitro* studies[11−14] have examined the metabolism of TPHP and several other OP triester FRs, the key metabolic pathways and metabolites formed are still not well understood. For example, two studies investigated TPHP metabolism using adult male Wistar Han rats and human liver microsomes.[11,12] It was suggested that the only metabolism of TPHP resulted from the cleavage of an ester bond between the phosphate group and benzene ring, leading to the formation of diphenyl phosphate (DPHP). However, we recently reported that DPHP was nondetectable in the plasma of herring gulls (*L. argentatus*) collected in 2010 from Chantry Island in eastern Lake Huron.[15] Two recent studies implied a more diverse *in vitro* metabolic pathway profile for TPHP upon its incubation with human liver microsomes, human liver S9 fractions, or chicken embryonic hepatocyte (CEH) samples.[13,14] Specifically, incubation of TPHP with human liver microsomes resulted in the formation of structurally unidentified monohydroxylated TPHP, di-OH-TPHP, and OH-phenyl phosphate metabolites. Incubating TPHP with human S9 liver fraction resulted in several glucuronide and sulfate conjugates of the OH-TPHP metabolites, where the latter were formed as a result of *in vitro* incubation with human liver microsomes. However, the chemical structures of all the reported metabolites were not fully identified on the basis of mass spectral data.[14] A previous CEH *in vitro* TPHP metabolism study from our laboratory found that the resulting concentration of DPHP accounted for only 17% of the initial TPHP dosing concentration.[13] We also screened an ion at m/z 343.0730 using an Agilent 6520A Q-ToF-MS system and found that it shared exactly the same theoretical molecular mass with OH-TPHP and accounted for 20% of the initial administered TPHP concentration.[13] The identities of these OH-TPHP metabolites were not confirmed and could not be quantitatively determined because of a lack of analytical standards.

The objectives of this study were (1) to confirm the identity of the OH-TPHPs formed in TPHP-exposed CEH samples, including the specific substitution positions of the OH group of OH-TPHP, and (2) to quantify the *p*-/*m*-OH-TPHP formed in TPHP-exposed CEH samples, and in comparison to a former confirmed TPHP metabolite, DPHP.

EXPERIMENTAL SECTION

Chemicals and Reagents. To the best of our knowledge, pure standards for any OH-TPHP isomer are not yet commercially available. The mono-*p*- and *m*-OH-TPHP isomers used in this study were synthesized at Duke University in the Duke Small Molecule Syntheiss Facility and prepared by D. Gooden. Details of the OH-TPHP synthesis and chemical structures are provided in the Supporting Information (Figure S1 and Supporting Material I).

For derivatization by methylation of the OH-TPHPs, diazomethane was prepared starting with a small amount of *N*-nitroso-*N*-methylurea (Sigma-Aldrich, St. Louis, MO), which was added into a 50 mL glass bottle containing 20 mL of hexane and 20 mL of a 50% (w/w) NaOH solution (Sigma-Aldrich). The diazomethane that was formed was dissolved into the upper hexane layer and available fresh for OH-TPHP methylation.

***In Vitro* Metabolism of TPHP and *p*-OH-TPHP.** Two independent CEH cultures were prepared for the following chemical exposures: (1) TPHP and (2) *p*-OH-TPHP. The preparation of CEH cultures followed detailed procedures that are provided in our previous publications.[16−19] Briefly, fertilized, unincubated white leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON) and incubated for 19 days (37.5 °C, 60% relative humidity). On incubation day 19, the embryos were euthanized by decapitation, and livers were removed, pooled, and treated with Percoll (GE Healthcare, Little Chalfont, U.K.) and DNase I (Roche Applied Science, Penzberg, Germany). The resulting cell pellet was suspended in 32 mL of Medium 199 (Life Technologies, Burlington, ON) supplemented with 1 μg/mL insulin and 1 μg/mL thyroxine (Sigma-Aldrich). Twenty-five microliters of the cell suspension was added to 500 μL of fresh supplemented medium in 48-well plates. The plates were incubated (37.5 °C and 5% CO_2) for 24 h prior to chemical administration, and then CEH cultures were treated with the DMSO vehicle control (2.5 μL/well) or the target chemicals (2.5 μL/well, final concentration of 10 μM) and incubated for different periods of time (0, 12, and 36 h for TPHP; 0, 1, 2, and 4 h for *p*-OH-TPHP; n = 3 replicate wells). For TPHP-exposed CEH cultures, medium samples were collected and transferred into 1.5 mL brown glass vials at each time point. The remaining cell layer was washed out twice with 200 μL of ethanol and transferred into 1.5 mL brown glass vials. The *in vitro* metabolism experiment of *p*-OH-TPHP was designed to investigate the formation of the *p*-OH-TPHP glucuronide conjugate. Subsequently, a shorter exposure time (<4 h) experiment was conducted, and only the medium samples were collected. All samples were stored at −20 °C until any further analysis.

Methylation of the Hydroxyl Group. Given the substantial response of the detected m/z 343.0730 ion by the Agilent 6520A ESI(+)-Q-ToF-MS system described in a previous publication,[13] medium samples collected at 36 h were subjected to OH methylation. An aliquot of 60 μL of the CEH medium samples or 100 μL of an OH-TPHP stock solution (100 ng/mL in methanol) was transferred into a borosilicate glass tube. The medium or standard samples were blown down to dryness under a gentle nitrogen flow, and 2 mL of a diazomethane hexane solution was added to the tube. The methylation reaction proceeded in a dark environment at room temperature for 12 h. The hexane and remaining diazomethane were then blown to dryness, and the samples were reconstituted in 500 μL of methanol. After being filtered through a centrifugal filter (0.2 μm Nylon membrane, 500 μL; VWR, Mississauga, ON), the samples were ready for instrumental analysis.

Deconjugation of OH-TPHP Glucuronides in CEH Samples. Deconjugation of OH-TPHP glucuronides in CEH samples was performed using β-glucuronidase (from Limpets, Lot 65H3884, Sigma-Aldrich). The β-glucuronidase working stock solution was prepared according to the manufacturer's protocol, and an aliquot of 160 μL of the enzyme solution (activity of 4.6 kU/mL) was combined with 40 μL of CEH medium or cell samples in glass tubes. The combined samples were hydrolyzed for 3 h at 60 °C. After 3 h, the samples were diluted with 800 μL of methanol and filtered through a centrifugal filter for further instrumental analysis.

UPLC-ESI(+)-TQ-S/MS Analysis. The OH-TPHP isomers, MeO-TPHP isomers, and OH-TPHP conjugates were analyzed using a Waters (Milford, MA) ACQUITY UPLC I-Class system (UPLC) coupled to Waters Xevo TQ-S mass spectrometer (TQ-S/MS) using electrospray ionization [ESI-(+)] in multiple-reaction monitoring (MRM) mode. LC

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separation was conducted on a Cortecs UPLC C18 column (2.1 mm × 50 mm, 1.6 μm particle size) (Waters, Mississauga, ON). The LC mobile phases were water (A) and methanol (B), and both contained 2 mM ammonium acetate. The mobile phase flow rate was 0.5 mL/min, and the gradient was as follows: 5% B at 0 min, 95% B (linear) from 0 to 5 min, held for 1 min, 5% B (linear) from 6 to 6.1 min, and held for 4.9 min. The capillary voltage was 0.5 kV. The source and desolvation temperatures were 150 and 600 °C, respectively. The desolvation and cone gas flow rates were 800 and 150 L/h, respectively. See Table S1 of the Supporting Information for detailed operation parameters (i.e., transition, collision voltage).

Accurate concentrations of *p*- and *m*-OH-TPHP in medium and cell samples were determined using an external standard calibration method, and a five-point calibration curve (concentration range of 0.08−50 pg/mL) was run with each batch of samples to confirm the linearity of the instrumental response. Data analysis was conducted using MassLynx version 4.1. The method limit of detection (MLOD) and method limit of quantification (MLOQ) were defined as the concentrations of target compounds producing a peak in a chromatogram with signal:noise ratio of 3 and 10, respectively. MLODs and MLOQs for both *p*- and *m*-OH-TPHPs were 0.03 and 0.01 ng/mL (injection concentration), respectively.

RESULTS AND DISCUSSION

From TPHP-exposed CEH, *p*- and *m*-OH-TPHP metabolites were identified and quantified (Figure 1 and Table 1). To the best of our knowledge, this is the first report that has structurally identified and accurately quantified *p*- and *m*-OH-TPHP metabolites formed *in vivo* or *in vitro* in a TPHP metabolism study. To avoid any potential false positives, possible contamination of the two OH-TPHP isomers in the TPHP stock solutions was investigated. The *p*-OH-TPHP was not present as an impurity in stock standard solutions of TPHP. On the other hand, *m*-OH-TPHP was detected as an impurity in the TPHP stock standard solution, but at a negligible concentration (i.e., <0.008% of the TPHP level).

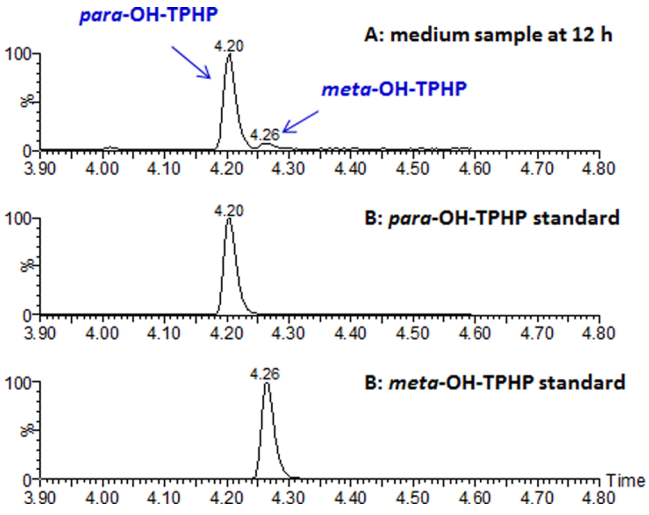

Figure 1. Mass spectral characteristics of detected *p*- and *m*-OH-TPHP in a TPHP-exposed chicken embryonic hepatocyte (CEH) medium sample (A) and their respective standards (B and C) using a Waters ACQUITY UPLC I-Class system coupled to a Waters Xevos TM TQ-S mass spectrometer.

Concentrations of *p*- and *m*-OH-TPHP isomers in medium and hepatocytes were accurately quantified, and both metabolites showed a time-dependent decrease in concentrations over the three time points of 0, 12, and 36 h. Specifically, combined concentrations of *p*-OH-TPHP in medium and cell samples were 0.053 ± 0.004, 0.021 ± 0.005, and 0.0019 ± 0.0003 nmol/well at 0, 12, and 36 h, respectively. This accounted for 1.8, 0.6, and 0.05% of the initial TPHP treatment concentration, respectively (Table 1). Combined concentrations of *m*-OH-TPHP in medium and hepatocytes were 0.0028 ± 0.0003, 0.0020 ± 0.0002, and 0.0009 ± 0.0002 nmol/well at the same time points, respectively, which accounted for 0.08, 0.06, and 0.03%, respectively, of the initial TPHP dose (Table 1). In a recent *in vitro* study of TPHP metabolism in human liver microsomes and S9 fractions, the authors also detected two mono-OH-TPHP metabolites (not structurally identified), and those two peaks were proposed to be *p*- and *m*-OH-TPHPs.[14] However, in another study, *p*-OH-TPHP was identified as the only metabolite of TPHP following *in vivo* metabolism of TPHP in treated houseflies.[20]

Considering that cellular accumulation of an exogenous chemical can be directly related to its specific potency, the cellular uptake of the *p*- and *m*-OH-TPHPs was also investigated in this study. Because the equilibrium of OH-TPHPs between the cells and medium was not achieved at 0 h, and their concentrations at 36 h were very low, the 12 h time point was considered the best point for assessing the accumulation of OH-TPHP in CEH. In fact, 0.006 ± 0.002 nmol of *p*-OH-TPHP/well and 0.0005 ± 0.0002 nmol of *m*-OH-TPHP nmol/well were quantified in the cell samples after the 12 h incubation period and accounted for 29 and 25% of the total *p*-OH-TPHP and *m*-OH-TPHP concentrations, respectively (Table 1). These percentages were lower than those observed for their parent TPHP (46%),[13] suggesting a lower level of cellular accumulation of the two phase I OH-TPHP metabolites compared to the parent TPHP compound.

Although both *p*- and *m*-OH-TPHP were accurately measured in medium and cell samples at the three incubation time points, two questions were raised as a result of these findings. (1) Why did both of these phase I OH-TPHP metabolite concentrations show decreasing trends (Table 1)? (2) Why was there a huge difference in UPLC retention times for the OH-TPHP standards and the detected ESI(+) peaks at *m*/*z* 343.0730 in the TPHP-treated CEH medium samples, even though this ion shared extremely similar ESI(+) daughter ions with *p*-OH-TPHP (Figure S2 of the Supporting Information)? To address these two questions, we conducted diazomethane derivatization of the OH-TPHP isomers in the medium samples from TPHP-treated CEH to investigate the existence of OH functional groups on the chemical structure of the detected *m*/*z* 343.0730 ion. The two OH-TPHP isomers were regarded as positive controls. The results demonstrated that, unlike two OH-TPHP standards, no MeO-TPHP peaks were detected in the medium of the TPHP-treated CEH samples. However, the detected *m*/*z* 343.0730 ion was still detectable in derivatized medium samples but eluted as several UPLC resolved peaks (Figure S3 of the Supporting Information). This finding suggested that the detected *m*/*z* 343.0730 ion might be a fragment ion from a possible OH-TPHP glucuronide conjugate that may contain several hydroxyl groups. If this apparent conjugate could be derivatized via methylation, then different byproducts should be observed depending on the specific positions of different OH functional

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Table 1. Quantified Concentrations of *p*- and *m*-Hydroxyl-Triphenyl Phosphate (OH-TPHP) Isomers in Cell Culture Medium and Cell Samples before and after β-Glucuronidase Treatment (units of nanomoles per well)[a]

chemical[b]		0 h	12 h	36 h
DPHP	% of dosed TPHP	1	5	17
p-OH-TPHP	medium	0.017 ± 0.002	0.015 ± 0.002	0.0013 ± 0.0004
	cells	0.036 ± 0.003	0.006 ± 0.002	0.0005 ± 0.0001
	% of dosed TPHP	1.8	0.6	0.05
p-OH-TPHP (β-glucuronidase-treated)	medium	0.025 ± 0.001	1.89 ± 0.04	2.06 ± 0.11
	cells	0.048 ± 0.002	0.06 ± 0.02	0.04 ± 0.01
	% of dosed TPHP	2.1	55	60
m-OH-TPHP	medium	0.0009 ± 0.0001	0.0015 ± 0.0001	0.0007 ± 0.0002
	cells	0.0019 ± 0.0002	0.0005 ± 0.0002	0.0002 ± 0.0001
	% of dosed TPHP	0.08	0.06	0.03
m-OH-TPHP (β-glucuronidase-treated)	medium	0.0015 ± 0.002	0.17 ± 0.01	0.17 ± 0.01
	cells	0.0035 ± 0.0004	0.007 ± 0.002	0.004 ± 0.001
	% of dosed TPHP	0.14	5	5

[a]These samples were collected at time points of 0, 12, or 36 h following exposure of chicken embryonic hepatocytes. Data are shown as means ± the standard deviation (SD) of three replicates. MLOQs of OH-TPHPs for medium and cell samples were 0.0004 and 0.0001 nmol/well, respectively. [b]Detailed TPHP and DPHP concentrations in the same wells were provided in a previous publication.[13]

groups. To investigate further, the *in vitro* CEH metabolism of *p*-OH-TPHP was investigated. Following exposure of CEH samples to *p*-OH-TPHP, both *p*-OH-TPHP and the *m/z* 343.0730 ion were monitored in medium samples at 0, 1, 2, and 4 h. The result was an increased ESI(+) response of the *p*-OH-TPHP glucuronide conjugate [RT = 3.39 (Figure S4 of the Supporting Information)] and decreased concentration of *p*-OH-TPHP from 0 to 4 h. In fact, we detected an additional *p*-OH-TPHP conjugate [RT = 3.59 (Figure S4 of the Supporting Information)] that was plausibly a sulfate conjugate of an OH-TPHP. This finding was consistent with a previous report of the *in vitro* metabolism of TPHP by the human liver S9 fraction, where both OH-TPHP glucuronide and sulfate conjugates were predicted, although the OH substitution position was not elucidated.[14] In general, xenobiotic metabolism can be divided into different phases: primary metabolism (e.g., direct hydroxylation or via arene epoxide formation; phase I), conjugation (phase II), and excretion (phase III),[21] which is consistent with observed pathways for TPHP in the study presented here.

After the CEH medium and cell samples were treated with β-glucuronidase, *p*- and *m*-OH-TPHP concentrations were quantitatively determined, and again, both showed increasing concentrations from 0 to 36 h (Table 1). Specifically, combined concentrations of *p*-OH-TPHP in medium and cell samples were 0.073 ± 0.003, 1.95 ± 0.03, and 2.10 ± 0.09 nmol/well at 0, 12, and 36 h, respectively, and *m*-OH-TPHP concentrations were 0.0050 ± 0.0005, 0.18 ± 0.01, and 0.18 ± 0.01 nmol/well, respectively. At 36 h, the *p*- and *m*-OH-TPHP concentrations in cells were 0.04 ± 0.01 and 0.004 ± 0.001 nmol/well, respectively, which accounted for 2% for both of the total *p*-OH-TPHP and *m*-OH-TPHP concentrations. This suggested that the resulting OH-TPHP conjugates formed were much less prone to accumulating in CEH cultures than parent TPHP or OH-TPHP compounds but were comparable in that regard to another TPHP metabolite, DPHP. The time−trend concentration profiles and cellular uptake patterns also clearly elucidated a metabolic pathway of TPHP: (1) uptake of TPHP into CEH cultures, (2) phase I hydroxylation to OH-TPHP, (3) rapid OH-TPHP conjugation to form glucuronide-O-TPHP, and (4) excretion of the glucuronide-O-TPHP from the CEH cultures into the surrounding medium.

In previous studies, DPHP was generally regarded as the primary biomarker of TPHP in TPHP-exposed humans and has frequently been detected in human urine samples collected from various countries.[22−24] However, DPHP is not a specific metabolite for TPHP because it can be derived from multiple parent compounds such as ethylhexyl diphenyl phosphate (EHDPP).[25] To the best of our knowledge, we report for the first time via an *in vitro* CEH assay model the identification and quantification of two specific TPHP metabolites, *p*- and *m*-OH-TPHPs in addition to DPHP.[13] Furthermore, the *p*-OH-TPHP metabolite is primarily conjugated with glucuronic acid and excreted from the cells into the surrounding medium. On the basis of these quantitative results, the detected *p*-OH-TPHP (after β-glucuronidase treatment) accounted for 60% of the initial TPHP dosing at 36 h, which was 3.5-fold higher than that detected for DPHP (17%)[13] and 12-fold higher than that detected for *m*-OH-TPHP (5%) in the same wells. This suggested that the glucuronic acid conjugate of *p*-OH-TPHP might serve as another biomarker for the TPHP-exposed biota. However, before such a conclusion can be drawn, species-specific differences in metabolism and differences between *in vivo* and *in vitro* experimental conditions should be investigated. A recent study, which screened potential biomarkers of TPHP in human urine, did not detect diphenyl hydroxyphenyl phosphate or diphenyl sulfophenyl phosphate,[25] each of which was predicted to be a major metabolite in their previous *in vitro* study.[14] In fact, these two metabolites were not predicted as major metabolites in the present TPHP-exposed CEH because OH-TPHP would be conjugated with glucuronic acid very quickly once formed.[25]

■ ASSOCIATED CONTENT

Supporting Information

A description of the synthesis of *m*- and *p*-OH-TPHP, instrumental parameters for OH-TPHP and MeO-TPHP analysis, chemical structures of TPHP and its metabolites, and mass spectra characteristic of *m*- and *p*-OH-TPHP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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