New hydroxylated metabolites of 4-monochlorobiphenyl in whole poplar plants

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Abstract
Two new monohydroxy metabolites of 4-monochlorobiphenyl (CB3) were positively identified using three newly synthesized monohydroxy compounds of CB3: 2-hydroxy-4-chlorobiphenyl (2OH-CB3), 3-hydroxy-4-chlorobiphenyl (3OH-CB3) and 4-hydroxy-3-chlorobiphenyl (4OH-CB2). New metabolites of CB3, including 2OH-CB3 and 3OH-CB3, were confirmed in whole poplars (Populus deltoides × nigra, DN34), a model plant in the application of phytoremediation. Furthermore, the concentrations and masses of 2OH-CB3 and 3OH-CB3 formed in various tissues of whole poplar plants and controls were measured. Results showed that 2OH-CB3 was the major product in these two OH-CB3s with chlorine and hydroxyl moieties in the same phenyl ring of CB3. Masses of 2OH-CB3 and 3OH-CB3 in tissues of whole poplar plants were much higher than those in the hydroponic solution, strongly indicating that the poplar plant itself metabolizes CB3 to both 2OH-CB3 and 3OH-CB3. The total yield of 2OH-CB3 and 3OH-CB3, with chlorine and hydroxyl in the same phenyl ring of CB3, was less than that of three previously found OH-CB3s with chlorine and hydroxyl in the opposite phenyl rings of CB3 (2’OH-CB3, 3’OH-CB3, and 4’OH-CB3). Finally, these two newly detected OH-CB3s from CB3 in this work also suggest that the metabolic pathway was via epoxide intermediates. These five OH-CB3s clearly showed the complete metabolism profile from CB3 to monohydroxylated CB3. More importantly, it’s the first report and confirmation of 2OH-CB3 and 3OH-CB3 (new metabolites of CB3) in a living organism.

Background
Polychlorinated biphenyls (PCBs) are still being transported in the environment and exposing humans and biota, even though they have been banned for more than 30 years by many countries. Occasionally their biotransformation products, such as methyl sulfone (MeSO2-) and hydroxylated (OH-) metabolites of polychlorinated biphenyls [1], exhibit greater toxicity than their parent congeners [2-6]. Furthermore, the hydroxylated metabolites of PCBs (OH-PCBs) have been reported in many species and habitats [7-11]. Different taxa, including microorganisms [12], plants [13] and animals [14,15], have been used to elucidate the hydroxylated metabolic pathways of PCBs.

4-Monochlorobiphenyl (CB3), one of the simplest structures of PCBs, is a good congener to study the metabolism of PCBs because it is an important component of commercial PCB products [16], and it is a widespread airborne environmental pollutant which exposes plants, animals and humans [17,18]. Poplar as a model plant with a completely sequenced genome has been widely applied to remediate the pollution of organic compounds [19,20]. Furthermore, three hydroxylated metabolites of CB3 (OH-CB3s), including 2’-hydroxy-4-chlorobiphenyl (2’OH-CB3), 3’-hydroxy-4-chlorobiphenyl (3’OH-CB3) and 4’-hydroxy-4-chlorobiphenyl (4’OH-CB3), have been detected previously [21]. However, two unknown OH-CB3s were not confirmed but were speculated to be 2-hydroxy-4-chlorobiphenyl (2OH-CB3), 3-hydroxy-4-chlorobiphenyl (3OH-CB3) according to their physico-chemical and chromatographic properties - no authentic standards were available at the time [21]. Actually, five OH-CB3s, including 2’OH-CB3, 3’OH-CB3, 4’OH-CB3 and two unknown OH-CB3s also found in rat liver microsomes in vitro and the two unknown OH-CB3s were proposed likely to be 2OH-CB3 and 3OH-CB3 [22]. Therefore, previous studies have neither confirmed the existence of 2OH-CB3 and 3OH-CB3 in the environment nor in whole organisms in vivo.

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In this paper, these two new OH-CB3s were confirmed using three newly synthesized standards. The distribution, concentrations, and masses of these two new OH-CB3s in whole poplar tissues were studied in detail.

**Experimental**

**Reagents and chemicals**

Florisil (60-100 mesh, Acros Organics) was activated at 450°C for 12 h, allowed to cool to ambient temperature in a dessicator and then deactivated with 1% (w/w) water. Anhydrous sodium sulfate, methyl-tert butyl ether (MTBE) (HPLC grade), dichloromethane (HPLC grade), hexane (pesticide grade) and sodium hydroxide (98.6%) were obtained from Fisher Scientific. Methanol (HPLC grade) was purchased from Acros Organics, NJ, USA. The deionized water (18.3MΩ) came from an ultrapure water system (Barnstead International, Dubuque, IA). Other chemicals and reagents were of analytical reagent grade or better in this experiment.

**Synthesis and characterization of OH-CB3 standards**

The 4OH-CB2, 2OH-CB3 and 3OH-CB3 as putative metabolites of CB3 were synthesized via the corresponding methoxylated CB3 derivatives [23]. Briefly, Suzuki-cross coupling of benzene boronic acid with 4-bromo-2-chloro-, 2-bromo-5-chloro- or 5-bromo-2-chloro-anisole yielded 3-chloro-4-methoxybiphenyl, 4-chloro-2-methoxybiphenyl or 4-chloro-3-methoxybiphenyl, respectively. Subsequent demethylation with boron tribromide yielded the desired OH-CB3 with a purity of 98% or better (based on relative peak area as determined by gas chromatography) (figure 1).

### 3-Chloro-4-methoxybiphenyl

Yield: 96% (white solid); mp 87-89°C; 1H NMR (CDCl3, 300 MHz): δ/ppm 3.93 (s, 3H), 6.99 (d, J = 8.5 Hz, 1H), 7.29-7.36 (m, 1H), 7.38-7.46 (m, 3H), 7.48-7.54 (m, 2H), 7.55 (d, J = 2.2 Hz, 1H); 13C NMR (CDCl3, 75 MHz): δ/ppm 116.5, 120.2, 126.7, 127.1, 127.2, 127.5, 128.8, 134.9, 139.5, 150.7; Anal. Calcd for C11H9ClO: C, 70.41; H, 4.44; Found: C, 70.25; H, 4.40; GC-MS (m/z, relative abundance %): 218 (M⁺, 100), 175 (48), 139 (28).

### 4-Chloro-2-methoxybiphenyl

Yield: 96% (white solid); mp 39-42°C; 1H NMR (CDCl3, 300 MHz): δ/ppm 3.76 (s, 3H), 6.94 (d, J = 8.5 Hz, 1H), 7.21 (d, J = 8.1 Hz, 1H), 7.27-7.43 (m, 3H), 7.44-7.50 (m, 2H); 13C NMR (CDCl3, 75 MHz): δ/ppm 55.7, 111.8, 120.8, 127.2, 128.0, 129.1, 129.3, 131.5, 133.8, 137.4, 156.9; GC-MS (m/z, relative abundance %): 218 (M⁺, 94), 204 (12), 168 (100), 152 (14), 139 (46).

### 4-Chloro-3-methoxybiphenyl (crude)

Yield: ~ 100% (colorless oil); 1H NMR (CDCl3, 300 MHz): δ/ppm 3.88 (s, 3H), 7.02-7.08 (m, 2H), 7.28-7.43 (m, 4H), 7.44-7.50 (m, 2H); 13C NMR (CDCl3, 75 MHz): δ/ppm 110.7, 119.8, 121.4, 126.9, 127.5, 140.1, 141.1, 154.9; GC-MS (m/z, relative abundance %): 218 (M⁺, 100), 175 (48), 139 (28).

### 4OH-CB2

Yield: 92% (white solid); mp 73.5-75°C (hexane); 1H NMR (CDCl3, 300 MHz): δ/ppm 7.09 (d, J = 8.5 Hz, 1H), 7.29-7.36 (m, 1H), 7.37-7.46 (m, 3H), 7.48-7.54 (m, 2H); 13C NMR (CDCl3, 75 MHz): δ/ppm 116.5, 120.2, 126.7, 127.1, 127.2, 127.5, 128.8, 134.9, 139.5, 150.7; Anal. Calcd for C11H9ClO: C, 70.41; H, 4.44; Found: C, 70.67; H, 4.32; GC-MS (m/z, relative abundance %): 204 (M⁺, 100), 139 (47).

### 2OH-CB3

Yield: 98% (white solid); mp 73.5-75°C (hexane); 1H NMR (CDCl3, 300 MHz): δ/ppm 6.94-7.02 (m, 2H), 7.17 (d, J = 8.2 Hz, 1H), 7.36-7.52 (m, 5H); 13C NMR (CDCl3, 75 MHz): δ/ppm 116.5, 120.2, 126.7, 127.1, 127.2, 127.5, 128.8, 134.9, 139.5, 153.0; Anal. Calcd for C11H9ClO: C, 70.41; H, 4.44; Found: C, 70.25; H, 4.40; GC-MS (m/z, relative abundance %): 204 (M⁺, 100), 168 (31), 139 (22), 115 (18).

### 3OH-CB3

Yield: 80% (white solid); mp 46.5-47.5°C (hexane); 1H NMR (CDCl3, 300 MHz): δ/ppm 6.94-7.02 (m, 2H), 7.17 (d, J = 8.2 Hz, 1H), 7.36-7.52 (m, 5H); 13C NMR (CDCl3, 75 MHz): δ/ppm 116.1, 121.0, 126.7, 128.2, 128.9, 131.0, 134.2, 136.0, 153.0; Anal. Calcd for C11H9ClO: C, 70.41; H, 4.44; Found: C, 70.25; H, 4.40; GC-MS (m/z, relative abundance %): 204 (M⁺, 100), 168 (31), 139 (22), 115 (18).

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**Figure 1** Synthesis and chemical structure of 2OH-CB3, 3OH-CB3 and 4OH-CB2
127.8, 128.8, 129.1, 139.9, 142.0, 151.6; Anal. Calcd for C₁₁H₉ClO: C, 70.41; H, 4.44; Found: C, 70.50; H, 4.36; GC-MS (m/z, relative abundance %): 204 (M⁺, 100), 168 (12), 139 (66), 115 (21).

Experimental design and OH-CB3 analysis
The poplar cuttings (Populus deltoides × nigra, DN34), the exposure method, extraction and cleanup procedure, and qualitative and quantitative analysis were the same as described previously [21].

Results and discussion
Confirmation of two new hydroxylated metabolites of CB3 in whole poplar plants
Three hydroxylated metabolites, including 2’OH-CB3, 3’OH-CB3 and 4’OH-CB3, have been identified previously [21]. However, there were two unknown mono-OH-CB3s detected in different tissues of whole poplars, which were not identified due to a lack of authentic standards previously [21]. The possible hydroxylated metabolites of CB3 with chlorine and hydroxyl in the same phenyl ring, including 2OH-CB3 and 3OH-CB3, were proposed according to their physicochemical and chromatographic properties. In order to confirm the two new hydroxylated metabolites of CB3 and to elucidate the complete metabolic pathway of CB3 hydroxylation in whole poplar plants, three newly synthesized hydroxylated compounds of CB3, including 2OH-CB3, 3OH-CB3 and 4OH-CB2, were synthesized as shown in figure 1 and used as analytical standards.

2OH-CB3 was easily separated from 3OH-CB3 and 4OH-CB2 using a previously established LC-MS method, which allowed confirmation of the second peak (figure 2) to be 2OH-CB3 based on the retention time. However, with such the similar structures and only the change of chlorine and hydroxyl position, 3OH-CB3 and 4OH-CB2 could not be separated completely. The peaks of 3OH-CB3 and 4OH-CB2 overlapped almost fully with their retention times of 2.336 and 2.313 min, respectively. However, the retention time of the first peak (figure 2) in the root sample of poplar plant was 2.338 min, which allowed accurate confirmation that the first unknown compound was 3OH-CB3. The elution sequence was 3OH-CB3 and 2OH-CB3. As shown in figure 2, the five hydroxylated metabolites of CB3 can be separated perfectly with the elution sequence of 3OH-CB3, 2OH-CB3, 4’OH-CB3, 3’OH-CB3 and 2’OH-CB3. The OH-CB3s with chlorine and hydroxyl in the same phenyl ring eluted first from the chromatographic column because they have a higher polarity than the OH-CB3s with chlorine and hydroxyl in the different phenyl rings. To our knowledge, it is the first report of these new hydroxylated metabolites of CB3 with chlorine and hydroxyl in the same phenyl ring in living organisms.

![Figure 2 Chromatograms of five OH-CB3s in the root sample and standard. A: root sample; B: standards of five OH-CB3s.](image-url)
2OH-CB3 and 3OH-CB3 in the solutions of exposed plants and controls

Due to the possible oxidation of CB3 in aqueous solution [22], the potential for formation of hydroxylated products 2OH-CB3, 3OH-CB3, in deionized water and hydroponic solution (as abiotic controls) was firstly analyzed. In order to avoid microbial degradation and photodegradation, the abiotic controls were autoclaved and wrapped with aluminum foil prior to analysis (deionized water alone, and hydroponic solution alone). It can be seen from figure 3 that the concentrations of 2OH-CB3 and 3OH-CB3 increased following the exposure time during the 10 day experiment with 1.0 mg L⁻¹ of CB3 spiked into the reactors as the starting concentration. Degradates 2OH-CB3 and 3OH-CB3 were not detected at the beginning (day 0), confirming that the CB3 standard and abiotic reactors were not contaminated by these two OH-CB3s. The concentrations of 2OH-CB3 and 3OH-CB3 in the abiotic hydroponic solution controls (Hoagland’s solution) were about 10 times higher than those in deionized water, which likely due to the relatively high concentrations of ions in hydroponic solution accelerating the process of CB3 oxidation. Reactors contained higher concentrations of 2OH-CB3 than 3OH-CB3 in hydroponic solution controls, which meant that 2OH-CB3 was easily produced during the oxidation reaction. However, their yields were very low. The total masses of 2OH-CB3 and 3OH-CB3 at day 10 accounted for only 0.00032% and 0.0029% of the total mass of spiked CB3 in deionized water controls and hydroponic solution controls, respectively.

In treatments containing poplars, 2OH-CB3 and 3OH-CB3 were not detected in the hydroponic solutions of “blank poplar controls”, in which no CB3 was spiked into the reactors. This provides evidence that the reactors and poplars were not contaminated during the course of the experiment. It can be seen from figure 4 that 2OH-CB3 and 3OH-CB3 were detected in the solutions of autoclaved poplars and dead poplars, which were used as “sterile” controls. Furthermore, the concentrations of 2OH-CB3 and 3OH-CB3 were roughly identical in these solutions, which suggests that

![Figure 3 Abiotic Controls of deionized water (A) and hydroponic solution (B). Concentrations of 2OH-CB3 and 3OH-CB3 (n = 3) developed over 10 days following an initial concentration spike of parent compound CB3 at a concentration of 1.0 mg L⁻¹.](image-url)
Microorganisms were present in sufficient number to produce some 2OH-CB3 and 3OH-CB3 from CB3, and the concentrations of 2OH-CB3 and 3OH-CB3 in the solutions of autoclaved poplars and dead poplars were much higher than those in deionized water controls and in hydroponic solutions after 10 days exposure.

In the solutions of washed and whole poplars, the concentrations of 2OH-CB3 and 3OH-CB3 were higher than those in the dead poplars, indicating that the presence of whole poplars metabolized considerably more CB3 into 2OH-CB3 and 3OH-CB3. In addition, the concentration of 2OH-CB3 was 2.9 and 2.5 times as high as that of 3OH-CB3 in the solutions of washed poplars and whole poplars, respectively, demonstrating a higher yield of 2OH-CB3 in poplar.

Production of 2OH-CB3 and 3OH-CB3 in various tissues of whole poplars
As shown in figure 5, except for the leaves, 2OH-CB3 and 3OH-CB3 were detected in all the parts of whole poplar plants. In general, the concentration of 2OH-CB3 was higher than that of 3OH-CB3 in all tissues of the plant investigated, which is consistent with the concentrations observed in hydroponic solutions of the treatment reactors.

The concentrations of 2OH-CB3 and 3OH-CB3 in the bark samples were relatively higher than those in the woody tissue samples, which might be due to the following reasons: First, the bark, especially bottom bark, was in direct contact with CB3 and OH-CB3s in the hydroponic solution and, therefore, could absorb OH-CB3s from the hydroponic solution and diffuse upwards towards the middle bark. The bottom bark had the highest concentration of 2OH-CB3 in all the tissues, reaching 111.8 ± 27.3 ng g⁻¹. At the same time, the middle bark could also absorb additional CB3 from air in the reactor due to the volatility of CB3. The porous bark is a main conduit for transport of solutes due to the “wick effect” and diffusion. Thus, it is also a transport pathway for xenobiotic chemicals like CB3 and OH-CB3s.

It is important to note that the roots did not show the highest concentrations of 2OH-CB3 and 3OH-CB3, even though they were in direct contact with CB3 and took-up CB3 directly from the hydroponic solution. This provides evidence that CB3 and OH-CB3s were easily translocated from the roots to other tissues in whole poplars. Another apparent phenomenon was the different 2OH-CB3 to 3OH-CB3 ratios, suggesting that the transfer abilities or formation rates of 2OH-CB3 and 3OH-CB3 were different in different plant tissues due to different enzymes and/or activities. Overall, the detection of 2OH-CB3 and 3OH-CB3 in different tissues demonstrated that whole poplar can also metabolize CB3 into 2OH-CB3 and 3OH-CB3.
Total Masses of 2OH-CB3 and 3OH-CB3 in various solutions and poplar plants

The total masses of 2OH-CB3 and 3OH-CB3 were compared in different solutions and poplar plants. It can be seen from Table 1 the total masses of 2OH-CB3 and 3OH-CB3 in deionized water controls and hydroponic solution controls were 1.29 ± 0.22 ng and 11.60 ± 1.17 ng, respectively, which was negligible compared to their total masses in the treatment solutions and poplars. Total masses of 2OH-CB3 and 3OH-CB3 in the solutions of dead poplars were slightly higher than those in the solutions of autoclaved poplars, while total masses of 2OH-CB3 and 3OH-CB3 in the dead poplars were lower than those in the autoclaved poplars. However, the sum of 2OH-CB3 and 3OH-CB3 in the dead poplars and their solutions was similar to that in the autoclaved poplars and their solutions, with the values of 134.7 ng and 156.6 ng, respectively. The reasons might be that autoclaved poplars (detrital organic matter) were susceptible to the growth of microorganisms during the experiment, even though there were few microorganisms (very low activity) present at the beginning.

More importantly, the total masses of 2OH-CB3 and 3OH-CB3 in the systems of washed poplars and whole poplars were 702.0 ng and 1092 ng, respectively, much higher than those in the systems of autoclaved poplars and dead poplars. In addition, the total masses of 2OH-CB3 and 3OH-CB3 in washed poplars and whole poplars were much higher than those in their solutions. Therefore, the treatments containing plants (washed poplars and whole poplars) played a major role in the metabolism of CB3 to 2OH-CB3 and 3OH-CB3, whereas microorganisms made only a minor contribution. The total masses of 2OH-CB3 and 3OH-CB3 in washed poplars were lower than those in whole poplars, which suggested that ethanol pretreatment killed a significant fraction of the microorganisms on the roots and also partly damaged the root tissues. Of the two hydroxylated metabolites of CB3, 2OH-CB3 was major product with more than three times more mass than 3OH-CB3 in whole poplars. All in all, whole intact poplars produced the greatest mass of these two new metabolites of CB3 within all the treatments, although the overall yield of 2OH-CB3 and 3OH-CB3 was relatively small (about 0.23% of total CB3 spiked in the solution).

Comparison of OH-CB3s with hydroxyl and chlorine in the same phenyl ring and the different rings

We identified five OH-CB3s in whole poplars in our studies: three OH-CB3s with hydroxyl and chlorine
substituents in different phenyl rings, and two OH-CB3s with hydroxyl and chlorine in the same phenyl ring. This is the maximum number of monohydroxy metabolites of CB3 that are possible in the absence of any chlorine shifts. In general, the total masses of OH-CB3s with hydroxyl and chlorine substituents in the different phenyl rings were much higher than those with hydroxyl and chlorine in the same phenyl ring in all control samples and in whole poplars. Especially in whole poplars, the total masses of OH-CB3s with hydroxyl and chlorine in the different phenyl rings (1.06% of OH-CB3s/CB3) [21] were 4.6 times higher than those with hydroxyl and chlorine in the same phenyl ring (0.23% of OH-CB3s/CB3), clearly indicating OH-CB3s with hydroxyl and chlorine in the different phenyl ring were more easily formed.

Another apparent difference was their distribution in whole poplars. The maximum concentrations of 2OH-CB3 and 3OH-CB3 were in bottom bark (figure 5); while the maximum concentrations of 3’OH-CB3 and 2’OH-CB3 were in bottom bark and 4’OH-CB3 was in middle bark [21]. Furthermore, the roots contained the second largest concentrations of 2OH-CB3 and 3OH-CB3 in whole poplars (figure 5); while the roots contained the second lowest concentrations of 4’OH-CB3, 3’OH-CB3, 2’OH-CB3 and 4’OH-CB3. There were no other monohydroxy metabolites of CB3 with chlorine shift detected in our experiments. Therefore, our results strongly support the pathway of epoxide intermediates and complete metabolic transformation from CB3 to OH-CB3s.

Pathway of hydroxylated metabolites of CB3 in whole poplars

Two possible mechanisms have been used previously to explain the formation of mono OH-PCBs from PCBs in living tissues. One was the enzymatic transformation of OH-PCBs from PCBs via the epoxide intermediates. The epoxide intermediates then rearranged to different mono OH-PCBs [15,24,25]. The other mechanism is the direct insertion of the hydroxyl group into a biphenyl to form OH-PCBs from PCBs, which mainly explains the formation of a single OH-PCB [26]. In research reported here, five monohydroxy metabolites of CB3 were identified in whole poplars: 2OH-CB3, 3OH-CB3, 2’OH-CB3, 3’OH-CB3 and 4’OH-CB3. There were no other monohydroxy metabolites of CB3 with chlorine shift detected in our experiments. Therefore, our results strongly support the pathway of epoxide intermediates and complete metabolic transformation from CB3 to OH-CB3s.

The proposed metabolic pathway from CB3 to OH-CB3s is depicted in figure 6. The parent compound, 4-monochlorobiphenyl (CB3), was likely first metabolized by cytochrome P450 enzymes [26] to the corresponding 2’,3’,4’-epoxide, 3’,4’-epoxide and 2,3-epoxide. These three epoxide intermediates then rearranged to the five possible monohydroxy CB3s. The major products in these five monohydroxy metabolites of CB3 were 4’OH-CB3 and 2OH-CB3.

Conclusions

Plants account for a great amount of biomass on earth so that they have a very important function to metabolize and detoxify organic persistent pollutants, including PCBs, in the environment. Understanding the metabolic processes of OH-CB3s in plants, including poplars, is
important. This is the first report of two new metabolites of CB3 (3OH-CB3 and 2OH-CB3), with chlorine and hydroxyl in the same phenyl ring from CB3 in whole plants. These results also implied 3OH-CB3 and 2OH-CB3 should extensively exist in the environment due to the ubiquitous existence of CB3.

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Authors' contributions
GZ performed the experiments and drafted the manuscript. HL synthesized the standards of OH-PCB3 and revised the manuscript. JS participated in the conception, design of study and revision of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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