



# Formation of environmentally relevant polyhalogenated carbazoles from chloroperoxidase-catalyzed halogenation of carbazole<sup>☆</sup>



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## ABSTRACT

Polyhalogenated carbazoles (PHCs) are a class of emerging organic contaminants that have received increasing concern due to their widespread distribution and dioxin-like toxicity. Although previous studies have suggested possible natural sources of PHCs in the environment, the formation pathways are poorly understood. Here we explored the production of PHCs from halogenation of carbazole in the presence of Br<sup>-</sup> and/or Cl<sup>-</sup> under the catalysis of chloroperoxidase (CPO) isolated from the marine fungus *Caldariomyces fumago*. Overall, a total of 25 congeners including mono- to tetra-substituted chlorinated, brominated, and mixed halogenated carbazoles (with substitution patterns of -BrCl, -BrCl<sub>2</sub>, -BrCl<sub>3</sub>, -Br<sub>2</sub>Cl, -Br<sub>2</sub>Cl<sub>2</sub>, and -Br<sub>3</sub>Cl) were produced from the reactions under various conditions. The PHC product profiles were apparently dependent on the halide concentrations. In the CPO-mediated chlorination of carbazole, 3-mono- and 3,6-dichlorocarbazoles predominated in the formation products. In addition to the less abundant mixed halogenated carbazoles (-Br<sub>2</sub>Cl), 1,3,6-tri- and 1,3,6,8-tetrabromocarbazoles were the dominant products in reactions containing both Br<sup>-</sup> and Cl<sup>-</sup>. The CPO-catalyzed halogenation of carbazole could take place in pH 3–7, but the formation products were pH dependent. Results of this study suggest that CPO-catalyzed halogenation of carbazole may play an important role in the natural formation of PHCs.

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## 1. Introduction

Polyhalogenated carbazoles (PHCs), with a complex chemical structure similar to polychlorinated dibenzofurans, are a class of emerging organic contaminants that have received increasing concern due to their widespread distribution and potential toxicity. The first chlorinated carbazole in the environment was discovered in the early 1980s by Kuehl et al. (1984), which identified  $1.2 \times 10^{-3} - 5.2 \times 10^{-2} \mu\text{mol/kg}$  of 1,3,6,8-tetrachlorocarbazole in sediment samples from the Buffalo River, New York. More than 20 years later, Zhu and Hites (2005) for the first time revealed the occurrence of

brominated carbazoles including 1,3,6,8-tetrabromocarbazole and possible congeners of tri- and pentabromocarbazoles in sediment cores from Lake Michigan. In addition, up to  $\mu\text{mol/kg}$  of 3-mono- and 3,6-dichlorocarbazoles were found in soils and sediments from Germany and Greece (Kronimus et al., 2004; Reischl et al., 2005; Grigoriadou and Schwarzbauer, 2011; Tröbs et al., 2011; Mumbo et al., 2015). Using two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC × GC-TOF MS) analysis, Pena-Abaurrea et al. (2014) discovered the first mixed halogenated carbazole 1,8-dibromo-3,6-dichlorocarbazole in sediments from Southern Ontario, Canada. In another study, a total of 17 PHC congeners with substituted patterns of Br<sub>2</sub>-, Br<sub>3</sub>-, Br<sub>5</sub>-, ClBr<sub>2</sub>-, ClBr<sub>3</sub>-, ClBr<sub>4</sub>-, ClBr<sub>3</sub>I-, Br<sub>4</sub>I-, and Br<sub>3</sub>I<sub>2</sub>-, were found in Lake Michigan's sediments (Guo et al., 2014). More recently, Wu et al. (2016) found carbazole and 11 PHCs including four chlorinated carbazoles, five brominated carbazoles, and two mixed halogenated carbazoles, in

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sediment samples from the Saginaw River and Saginaw Bay Lake Huron, Michigan.

Recent studies have demonstrated that PHCs exhibit dioxin-like toxicity and are persistent and bioaccumulative. For example, Riddell et al. (2015) revealed that mono- to tetra-halogenated carbazoles act through aryl hydrocarbon (Ah) receptor in the induction of cytochrome P450 1A1 (CYP1A1) and CYP1B1 gene expression in Ah-responsive MDA-MB-468 breast cancer cells assay. Compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the relative effect potency (REP) values for 1,3,6,8-tetrahalogenated carbazoles ranged from 0.00031 to 0.00066 in CYP1A1 induction and from 0.0058 to 0.0097 in CYP1B1 induction, which lie in between the toxic equivalency factor (TEF) for higher chlorinated dibenzofurans, dibenzo-*p*-dioxins, and some coplanar polychlorinated biphenyls (Riddell et al., 2015). Mumbo et al. (2015) revealed that 3,6-dibromocarbazole and 3-chlorocarbazole strongly induced ethoxresorufin-*O*-deethylase activity in H4IIA rat hepatoma cell line bioassay. Laboratory incubation studies indicated that 3-chlorocarbazole and 3,6-dichlorocarbazole were persistent to degradation in soil (Tröbs et al., 2011; Mumbo et al., 2015). Based on the Online Chemical Modeling Environment database (Online Chemical Database Website), log transformed octanol-water partition coefficients ( $\log K_{ow}$ ) were estimated to be more than 5.0 for di-, tri- and tetra-substituted brominated and chlorinated carbazoles, indicating that these congeners are bioaccumulative in the food chain (Mumbo et al., 2015).

The widespread occurrence of PHCs in soils and sediments has sparked considerable interest in their origin. Both anthropogenic and natural sources have been suggested by previous studies. Parette et al. (2015) proposed that halogenated indigo dyes are a likely source of 1,3,6,8-tetrabromocarbazole and some other halogenated carbazoles in the environment. However, Peverly and Hites (2016) did not observe the presence of PHCs in a sample of 5,5',7,7'-tetrabromoindigo from Sigma Aldrich. Interestingly, Parette et al. (2016) found the impurities of 1,3,6,8-tetrabromocarbazole, 1,3,6,8-tetrachlorocarbazole, and 1,8-dibromo-3,6-dichlorocarbazole in a historical synthetic 7,7'-dibromo-5,5'-dichloroindigo produced by BASF. Although Parette et al.'s (2016) hypothesis can partially explain the occurrence of some PHCs in the environment, natural origins cannot be entirely excluded. Enzymatic synthesis of PHCs may be a possible natural source. In fact, enzyme-mediated bromination and chlorination have long been recognized as important formation pathways for naturally occurring organohalogen compounds (Gribble, 2010). For example, chloroperoxidase (CPO), secreted by the marine fungus *Caldariomyces fumago*, is a versatile enzyme with the capacity to catalyze the incorporation of halogen atoms into organic molecules in the presence of peroxides such as  $H_2O_2$  (Neidleman and Geigert, 1986; Hofmann et al., 1998). Generally, CPO-mediated reaction involves the formation of halogenium ions ( $X^+$ ,  $X = Cl$  or  $Br$ ) or hypohalous acid (HOX) intermediates, leading to the halogenation of organic substrates via electrophilic substitution (Van et al., 1994; Wagenknecht and Woggon, 1997). Mumbo et al. (2013) reported the formation of mono-, di-, tri-, and tetra-substituted chloro- and bromocarbazoles from CPO-catalyzed halogenation of carbazole in the presence of  $Cl^-$  and  $Br^-$ , respectively. However, possible formation of mixed halogenated carbazoles from the reaction was not investigated in previous studies.

The objective of this study was to investigate the production of environmentally relevant PHCs from CPO-catalyzed halogenation of carbazole in the presence of  $Cl^-$  and  $Br^-$  as a potential mechanism of their generation in the environment. The analysis of PHCs is complicated because there are 210 congeners for chlorinated or brominated carbazoles and even much more for mixed chlorinated/brominated carbazoles. To enhance the chromatographic

separation of possible reaction products, a GC  $\times$  GC-TOF MS was employed to identify and quantify the formation congeners. The effects of halide concentrations and solution pH on PHC production were also evaluated.

## 2. Materials and methods

### 2.1. Chemicals

CPO isolated from the marine fungus *Caldariomyces fumago* (lyophilized powder), NaCl (>99.5%, with <0.005% of bromide), and standards of carbazole (>95%), 3,6-dichlorocarbazole, 3-bromocarbazole, and 3,6-dibromocarbazole were purchased from Sigma-Aldrich (Shanghai, China). Reference standards of 3-chlorocarbazole, 3-bromocarbazole, 1,3,6-tribromocarbazole, 1-bromo-3,6-dichlorocarbazole, 1,3,6,8-tetrachlorocarbazole, and 1,8-dibromo-3,6-dichlorocarbazole were purchased from Wellington Laboratories (Guelph, ON, Canada). The standard of 1,3,6,8-tetrabromocarbazole was purchased from AccuStandard (New Haven, CT). Phosphate buffers with pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 (0.1 mol/L) were prepared from 0.1 mol/L of phosphoric acid and appropriate amount of NaOH.

### 2.2. Incubation experiments

Incubation experimental procedures were modified from those of Mumbo et al. (2013). All experiments were carried out in 2-mL amber glass vials at  $25 \pm 1$  °C. Briefly, 10  $\mu$ L of 50  $\mu$ mol/L of carbazole (in methanol) was added to the vial and the solvent was allowed to evaporate, followed by the addition of 425  $\mu$ L of 0.1 mol/L phosphate buffer solution containing certain amount of NaCl and NaBr and 50  $\mu$ L of  $1.0 \times 10^3$   $\mu$ mol/L of  $H_2O_2$ . Subsequently, 25  $\mu$ L of 2.0 units/mL of CPO were added to the solutions to initiate the reaction. The treated samples were incubated in darkness and the reaction time was measured from the addition of enzyme. At designated time intervals (1, 4, 16, 60, 240, and 960 min), triplicate separately treated samples (one vial for each sample) were taken out and immediately subjected to liquid-liquid extraction. Unless otherwise noted, reactions were carried out in pH 3.0 phosphate buffer solutions containing 1.0  $\mu$ mol/L of carbazole,  $1.0 \times 10^2$   $\mu$ mol/L of  $H_2O_2$ , and 0.1 units/mL of CPO. A CPO-free control treatment containing 1.0  $\mu$ mol/L of carbazole,  $1.0 \times 10^2$   $\mu$ mol/L of  $H_2O_2$ ,  $5.0 \times 10^5$   $\mu$ mol/L of  $Cl^-$ , and  $1.0 \times 10^3$   $\mu$ mol/L of  $Br^-$  was also included to check any possible non-enzymatic formation process in the reaction.

The CPO-catalyzed chlorination of carbazole was carried out in solutions by varying  $Cl^-$  concentrations at  $1.0 \times 10^2$ ,  $1.0 \times 10^3$ , and  $5.0 \times 10^5$   $\mu$ mol/L. The CPO-catalyzed bromination of carbazole was investigated by changing  $Br^-$  at 1.0, 10, and  $1.0 \times 10^3$   $\mu$ mol/L. A suite of experiments was carried out in solutions in the presence of both  $Cl^-$  and  $Br^-$ . To mimic the halide ions in seawater (Kuehl et al., 1984; Flury and Papritz, 1993),  $Cl^-$  and  $Br^-$  concentrations were set at  $5.0 \times 10^5$   $\mu$ mol/L and  $1.0 \times 10^3$   $\mu$ mol/L, respectively. To mimic moderately saline water with trace levels of  $Br^-$ ,  $Cl^-$  and  $Br^-$  concentrations were set at  $1.0 \times 10^5$   $\mu$ mol/L and 10  $\mu$ mol/L, respectively. The pH effect was evaluated in solutions with pH ranging from 3.0 to 8.0 and contained  $5.0 \times 10^5$   $\mu$ mol/L of  $Cl^-$  and  $1.0 \times 10^3$   $\mu$ mol/L of  $Br^-$ .

### 2.3. Enzymatic assay

The CPO activities were determined based on the conversion of monochlorodimedon to dichlorodimedon in the presence of NaCl and  $H_2O_2$  (Morris and Hager, 1966). Briefly, the reaction mixture contained 300  $\mu$ mol of phosphate buffer (pH 3.0), 60  $\mu$ mol of NaCl,

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