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RESEARCH ARTICLE

Enzymatic synthesis of bromo- and chlorocarbazoles and elucidation of their structures by molecular modeling

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Abstract 3-Chlorocarbazole, 3,6-dichlorocarbazole, dibromocarbazole, and 1,3,6,8-tetrabromocarbazole are emerging environmental contaminants which have been detected recently in water, sediment, and soil samples. However, their sources and occurrence have not been explained. Here, we report an enzymatic synthesis of bromo- and chlorocarbazoles by chloroperoxidase from *Caldariomyces fumago* in water. Density functional theory (DFT) method was used to predict the most stable products. Carbazole and chloroperoxidase were assayed in vitro in the presence of hydrogen peroxide, bromide, and chloride ions in different substrate ratio treatments against constant and varying enzyme concentrations. Halogenated carbazoles formed were identified by high-resolution gas chromatography coupled to mass spectrometry. In all treatments, bromination and chlorination took place, but the composition and concentration of compounds formed varied from one treatment to another. Mono-, di-, tri-, and tetra-substituted bromo- and chlorocarbazoles which include the reported environmental contaminants were synthesized. 3-Substituted and 3,6-substituted congeners were relatively higher in concentration. Enzyme concentration did not favor preferential formation of any of the compounds synthesized. However, their synthesis was influenced by halide concentration. Congeners with bromine and chlorine at position of C-3, C-3,6, C-1,3,6, and C-1,3,6,8 were calculated as the stable intermediate sigma complexes by DFT method. Regioselectivity in halogenation is discussed and hypothesis of the likely stable products in the environment explained. This study provides evidence that bromo- and chlorocarbazoles reported previously can be formed enzymatically in the environment, demonstrating the need to consider aromatic pollutants transformation and their potential toxicity enhancements in the management of water pollution and contaminated sites.

Keywords Bromocarbazoles · Chlorocarbazoles · Chloroperoxidase · Density functional theory (DFT) calculations · Enzymatic bromination and chlorination · Regioselectivity in halogenation

This publication is dedicated to Prof. Otto Hutzinger (1933–2012) in recognition of his significant contribution in Ecological Chemistry and Ecotoxicology.

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Introduction

Recent studies have shown the presence of halogenated carbazoles in sediments, soils, and water. 1,3,6,8-Tetrabromocarbazole and congeners of other bromocarbazoles were found for the first time in the environment of the sediment cores of Lake Michigan,

USA (Zhu and Hites 2005). 3-Chlorocarbazole and 3,6-dichlorocarbazoles were isolated in Bavarian soils (Tröbs et al. 2011; Reischl et al. 2005) and the latter in sediments of Lippe River in Germany (Kronimus et al. 2004). Dibromo-, 3-chloro-, and 3,6-dichlorocarbazoles were also detected in sea and soil samples in the Kavala City of Greece (Grigoriadou and Schwarzbauer 2011). Polychlorinated carbazoles were detected in soil samples of sites contaminated by chloralkali processes in Japan and from a historic site of chlorine production in Germany (Takasuga et al. 2009). Halogenated carbazoles belong to the group of heterocyclic aromatic hydrocarbons some of which are known as hazardous environmental pollutants (Seo et al. 2009). Bromo- and chlorocarbazoles are rarely reported as environmental contaminants. The frequency of their detection in environmental samples creates the need to understand their origin and occurrence given their potential for persistence and toxicity in the environment.

3,6-Dichlorocarbazole has dioxin-like toxicological potential (Tröbs et al. 2011). Carbazole itself is carcinogenic and toxic (Tsuda et al. 1982). Its derivatives are toxic (Sverdrup et al. 2002) and mutagenic (Jha and Bharti 2002). They undergo further transformation generating more toxic hydroxynitrocarbazoles (Benedik 1998). 8-Chlorocarbazole-1-acetic acid, a photodegradation intermediate product of diclofenac, was found to cause cell lysis (Encinas et al. 1998). Just like the polycyclic aromatic hydrocarbons (PAHs) (Seo et al. 2009), they have petrogenic, pyrogenic, and biogenic sources. Carbazoles are commonly found in environmental matrices contaminated by coal tar, crude oil, and creosote (Kilbane et al. 2002) and coexist with other aromatic compounds including PAHs. Dibenzocarbazole, a carcinogen, including other heterocycles such as dibenzofurans and dibenzothiophenes is emitted during combustion processes (Junk and Ford 1980). It has been suggested that halogenated carbazoles could also be of natural origin. Grigoriadou and Schwarzbauer (2011) reported that 3-chlorocarbazole and 3,6-dichlorocarbazoles in soils could be the result of xenobiotic formation, while Zhu and Hites (2005) alluded that bromocarbazoles in sediments could be naturally derived.

According to Tröbs et al. (2011), chlorination of carbazole most likely proceeds by a type of electrophilic chlorination of the aromatic moiety that is an enzymatically controlled oxidative chlorination. Bromination and chlorination of natural aromatic structures are enzymatically controlled oxidation processes of the bromide and chloride ions yielding electrophilic bromine (Br^+) and chlorine (Cl^+) as reactive species (Neidleman and Geigert 1986). Haloperoxidases are peroxidase enzymes known to catalyze the incorporation of halogen atoms into organic molecules in the presence of halide ions and peroxides such as H_2O_2 (Neidleman and Geigert 1986; Hofmann et al. 1998). Bromoperoxidase, chloroperoxidase, and iodoperoxidase are the known haloperoxidases. Chloroperoxidase (CPO) has several species of enzymes some of which are the heme CPO of *Caldariomyces fumago* and the vanadium CPO of *Curvularia inaequalis*. The reaction mechanism involves formation of a halogenium ion (X^+) or hypohalous acid (HOX) intermediate by the CPO (van Schijndel et al. 1994; Wagenknecht and Woggon 1997) to effect electrophilic substitution with electron-rich aromatic substrates (Libby et al. 1992; Yamada et al. 1985).

Halogenated metabolites are frequent in nature and produced by many organisms and have been isolated from bacteria, fungi, marine algae, lichens, higher plants, mammals, and insects (Gribble 2003). Bromine-containing metabolites are the most abundant naturally synthesized organohalogens by organisms living in marine environments due to the relatively high bromine concentration in sea water compared to soil (Neidleman and Geigert 1986). In contrast, chlorinated metabolites are preferentially produced by terrestrial organisms (De Jong et al. 1992). The first chlorinating enzyme was isolated from the fungus *C. fumago* (Morris and Hager 1966). Bromoperoxidase has also been isolated from different algae and sea urchin eggs (Deits et al. 1984). It has been reported that haloperoxidase enzymes lack both substrate and regiospecificity in comparison to flavin-dependent halogenases (Pée and Patallo 2006; van Pée 1996). However, *C. fumago* has been able to chlorinate aromatic hydrocarbon including PAHs yielding mono-, di-, and tri-chlorinated compounds (Vázquez-Duhalt et al. 2001; Wannstedt et al. 1990; Yamada et al. 1985; Dembitsky 2003).

Molecular modeling based on density functional theory (DFT), a quantum mechanical calculation method, has been used to calculate the most stable products of the chlorinated carbazoles within the mono- and di-homologue groups (Tröbs et al. 2011). These structures based on this method follow the general theory of electrophilic substitution of aromatic system (Smith and March 2007). According to this theory, the intermediate sigma (σ) complex is the rate- and product-determining step. It is not the stability of the formed products but the stability of the intermediate σ-complex (Smith and March 2007). Carbazole undergoes electrophilic aromatic substitution reaction with various chlorinating agents (Bonesi and Erra-Balsells 1997). Halogenation oxidation via an electrophilic bromine (Br^+), chlorine (Cl^+), or iodine (I^+) is also the more common mechanism for enzymatic halogenation of natural products (Fujimori and Walsh 2007). Therefore, DFT method is more appropriate compared to the explanation of the charge densities based on the ground state of the product (Bonesi and Erra-Balsells 1997). It is consistent with the chemistry of carbazole showing strong activation at *ortho* and *para* positions to nitrogen on the compound (Katritzky and Taylor 1990).

Electrophilic aromatic substitution involves a distinct intermediate. According to Hammond's postulate, the rate-determining step involves the formation of a transition state that closely resembles the intermediate referred to as the σ-complex (Carey and Sundberg 2008). The rate-determining step's activation energy can be estimated according to the

energy of the σ-complex. A stable, low-energy σ-complex results in low activation energy and a high reaction rate, while a high-energy σ-complex represents a low activation energy and a low reaction rate. Regioselectivity is also determined by the most stable mechanism intermediate with the lowest energy of activation, therefore the fastest rate determining intermediate yielding of the major product (Fox and Whitesell 2004).

The enzymatic synthesis of bromo- and chlorocarbazoles was investigated in this study. There are no previous reports of enzymatic or chloroperoxidase type-catalyzed halogenation of carbazole yielding bromo- and chlorocarbazoles compounds. We hypothesize that since the halides, hydrogen peroxide, chloroperoxidase, and carbazole are available in nature, their interaction could lead to the synthesis of the halogenated carbazoles in the environment. Carbazole derivatives were not used as the reactions have been done and explained elsewhere (Bonesi and Erra-Balsells 1997). The results of this study could therefore be used to determine the outcome when derivatives are used. It was therefore our intention to investigate the enzymatic synthesis of bromo- and chlorocarbazoles in the aquatic environment and to predict the preferred stable isomers using DFT calculations.

Materials and methods

Chemicals and enzyme

Carbazole, hydrogen peroxide, potassium phosphate buffer salt, dichloromethane, purified CPO from *C. fumago*, potassium chloride, and sodium bromide were purchased from Sigma Aldrich (Taufkirchen, Germany). Reference standards for 3,6-dibromocarbazole, 3-chloro-, and 3,6-dichlorocarbazoles were also obtained from the same company. All chemicals were of analytical grade while the solvents were of picograde quality.

Enzyme assay and water samples and preparation

A 0.1 M potassium dihydrogen phosphate (KH_2PO_4) buffer solution of pH 3.0 at 25 °C was prepared in distilled water. Carbazole solution of 11.96 μM in concentration was then prepared by dissolving carbazole in 0.1 M KH_2PO_4 buffer solution prepared earlier. To increase the solubility of carbazole in water, 13.5 % ethanol (C_2H_5OH) was used. Potassium chloride (KCl) solutions of 11.96 μM, 119.6 μM, and 11.96 mM in concentration were prepared separately by dissolving KCl in buffer solution prepared earlier while ensuring the pH 3 at 25 °C was maintained. Hydrogen peroxide (H_2O_2) solutions of 5.98 μM, 35.88 μM, and 0.598 mM in concentration were prepared separately by diluting 30 % (*w*/*w*) H_2O_2 in distilled water. To have enough enzyme material for the analysis, 200 ml CPO enzymatic assay was prepared separately by dissolving 0.03 ml CPO, approximately 1,263.6 U, in 200 ml cold buffer solution of 0.1 M potassium phosphate buffer solution of pH 3.0 at 25 °C equivalent to 6.345 U/ml.

Enzymatic halogenation of carbazole by chloroperoxidase

In order to find out factors that influence formation of halogenated carbazoles, the concentration of carbazole was kept constant against varying concentrations of the enzymes, bromides, chlorides, and hydrogen peroxide. Temperature of the buffer solution, pH=3.0, was also kept constant at 25 °C. NaBr and KCl were used in bromination and chlorination reactions, respectively. H_2O_2 was kept low due to its potential inhibitory effect at high concentrations (above 25 mM during biocatalysis) (Nicell and Wright 1997), especially when used as a co-substrate for peroxidase activity.

In vitro enzymatic reactions of different reactant ratio treatments were carried out. The halides were dissolved in 0.1 M KH_2PO_4 buffer solution containing 11.96 μM of the substrate (carbazole) and C_2H_5OH, followed by the addition of CPO at intervals of 5 min for the next 20 min. The reaction was started with the addition of H_2O_2 semi-continuously at every interval during incubation. The reaction mixture was stirred on a water bath at 25 °C and stopped after 20 min. The reaction mixture was then removed from the water bath and stored under −28 °C in a refrigerator. The concentration of enzymes was however kept constant for all bromination reactions treatments.

Bromination reaction mixtures: Rx.1 (2:2:1 ratio), Rx.2 (1:10:3 ratio), and Rx.3 (1:1,000:50 ratio) treatments

CPO concentration was kept constant in all reaction mixtures. However, the reaction mixtures composition described by *Rx.1*, *Rx.2*, and *Rx.3* treatments varied. Rx.1 contained 11.96 μM carbazole, 11.96 μM NaBr, and 5.98 μM H_2O_2 and Rx.2 contained 11.96 μM carbazole, 119.6 μM NaBr, and 35.88 μM H_2O_2, while Rx.3 contained 11.96 μM carbazole, 11.96 mM NaBr, and 0.598 mM H_2O_2, all in separate solutions each of 20 ml 0.1 M potassium phosphate buffer at pH 3.0 and 2.1 % ethanol. CPO was added to each stirring mixture at 25 °C at 5-min intervals in 2 U aliquot during the 20-min incubation period (10 U in total). The blank did not contain CPO enzyme.

Chlorination reaction mixtures: Rx.4 (2:2:1 ratio), Rx.5 (1:10:3 ratio), Rx.6 (1:1,000:1), and Rx.7 (1:1,000:50 ratio) treatments

Under chlorination reaction, CPO concentration was not constant but varied. The reaction mixtures composition described by *Rx.4*, *Rx.5*, *Rx.6*, and *Rx.7* treatments also varied. Rx.4 contained 11.96 μM carbazole, 11.96 μM KCl, 5.98 μM H_2O_2, and 32 U aliquots CPO (6.4 U of CPO added at 5 min intervals), while Rx.5 contained 11.96 μM carbazole, 119.6 μM KCl, 35.88 μM H_2O_2, and 159 U

aliquots CPO (31.8 U of CPO added at 5 min intervals); Rx.6 contained 11.96 μM carbazole, 11.96 mM KCl, 11.96 μM H_2O_2, and 10 U (2 U of CPO added at 5 min intervals); and finally Rx. 7 with 11.96 μM carbazole, 11.96 mM KCl, 0.598 mM H_2O_2, and 317 U aliquots CPO (63.4 U of CPO added at 5 min intervals), all in separate solutions each of 20 ml of 0.1 M potassium phosphate buffer at pH=3.0 and 2.1 % ethanol. CPO was added to the stirring mixture at 25 °C during the 20-min incubation period. The blank did not contain CPO enzyme.

Extraction and cleanup

Dichloromethane, DCM (CH_2Cl_2), was used to extract the synthesized halogenated carbazoles from the reaction mixtures with the use of a separation funnel. Three hundred microliters of the extracted solution was then subjected to a cleanup using column chromatography using silica gel, alumina, and sodium sulfate to remove any material that could cause interference. The extract was eluted using hexane–DCM (1:1). The eluted extract was then concentrated by rotary evaporation to approximately 1 mL. Acetonitrile (0.5 mL) was added to the concentrate and then reduced under a steady flow of gentle nitrogen gas to approximately 0.5 mL. The sample was subjected to another cleanup through C18 column using acetonitrile as the eluent. This was followed by concentration of the eluted extract under a steady flow of gentle nitrogen to approximately 0.2 mL before being transferred to amber vials. Syringe standards (pentachlorotoluene and 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin-$^{13}C_{12}$) were added to the transferred cleaned extract in the vials. The extract and standard were concentrated thrice using hexane under a steady but gentle flow of nitrogen gas to a final volume of 20 μL. The amber vials were sealed and then taken for analysis using high-resolution gas chromatography coupled to mass spectrometry (GC/MS).

GC/MS analysis

Identification of bromo- and chlorocarbazoles was carried out by high-resolution gas chromatography coupled to a mass spectrometer. The specifications and operating conditions are summarized in Table 1. The criteria for the identification of individual compounds involved the use of retention times, mass spectra, and isotope ratio. Pure reference standards of 3-chlorocarbazole, 3,6-dichlorocarbazole, and 3,6-dibromocarbazole were used as standards for their respective compounds, while the others were identified using their mass spectrum. All samples were analyzed in single ion monitoring (SIM) mode, whereby the two most intensive masses of the molecular ion cluster were registered. In addition, full scan analyses were carried out for identification of halogenated carbazoles by mass spectra. Because of lack of reference standards for all halogenated carbazoles identified, a semi-quantitative approach was used for calculating the ratios of amounts, whereby an equal response for all compounds was assumed. The variations between the analyses were normalized by use of the syringe standard.

Computational details

The electronic structure calculations of the intermediate sigma complexes of the halogenated carbazoles were performed on an Intel®Core™ i7-3520M CPU 2.90 GHz 2.90 GHz personal computer using density functional theory, incorporated in Gaussian 03W program package (Frisch et al. 2004). The geometries of halogenated carbazoles in gas phase were carried out using the Becke 3-Lee-Yang-Parr (B3LYP) functional method. All geometry optimizations and frequency calculations of *intermediate sigma complex* were performed as *singlet* using opt = gdiis, freq = noraman, rb3yp/6-31+G(d,p) basis set to obtain the zero-point vibrational energy. This value was multiplied by the scaling factor of 0.9804 in order to compute thermal energy corrections. Single-point energy (SPE) calculations were carried out using the unrestricted functional with mixing orbitals: uB3LYP/6-311+G(2d,p)guess = mix basis set. SPE enables the computation of very accurate energy values and other properties for an optimized geometry at a lower level of theory. Stability tests on all calculated structures were performed using stable B3LYP/6-311+G(2d,p) basis set to ensure that the wave function computed is stable and that the calculations corresponds to the ground state of the molecule (Foresman and Frisch 1996).

Results and discussion

Enzymatic bromination reactions

Mono-, 3,6-di-, tri-, and tetra-bromocarbazoles were synthesized in the enzymatic controlled bromination of carbazole. The presence of these compounds in the assay was confirmed by their retention times using reference standards and their individual mass spectra (Figs. S1A and S2–S5). Mono- and 3,6-dibromocarbazole were the most abundant compounds in Rx.1 and Rx.2 treatments with the mono-isomer being more in Rx.1 compared to 3,6-dibromocarbazole (Fig. 1). With increasing amount of bromide in the reaction mixture, an increased abundance of the higher brominated isomers could be observed. Tribromocarbazole was the most abundant compound in Rx. 3 treatment (Fig. 1).

The most stable isomers in mono-, tri-, and tetra-homologue groups of bromocarbazoles were not identified due to lack of reference materials. However, through DFT calculations, 3-bromo-, 1,3,6-bromo-, and 1,3,6,7-bromocarbazoles were calculated as the most stable intermediate sigma complexes of the

Table 1 GC/MS parameters for the isomer specific detection of bromo- and chlorocarbazoles

GC	Type	Agilent 6890 series II
	Column	Rtx-Dioxin2, 40 m, 0.18 mm ID, 0.18 μm film thickness (Restek)
	Temperature program	60 °C, 1.5 min, 25 °C min^{-1}, 140 °C, 8 °C min^{-1}, 300 °C, 300 °C, 20 min
	Carrier gas	Helium with a constant flow of 1.3 mL^{-1} min^{-1}
	Injector	Cold injection system CIS 4 (Gerstel)
	Temperature program injector	120 °C, 12 °C s^{-1}, 280 °C, 5 min
	Temperature transfer line	300 °C
	Autosampler	A200S (CTC)
	Injection volume	0.5 μL pulsed splitless
MS	Type	MAT 95S (Thermo)
	Ionization mode	EI^+, 50 eV, 260 °C
	Detection	Full scan mode
	Resolution	1,000
	Detection	SIM mode
	Resolution	>8,500

Fullscan and SIM modes were used. Their resolutions are provided depending on which mode was used

mono-, tri-, and tetra-isomers, respectively (Table 2). The blanks did not show bromination. This is almost similar to previous H_2O_2 oxidation of bromine that did not take place at 1 mM concentration (Terrón et al. 1998) though our results were obtained at H_2O_2 concentrations below 1 mM. Enzyme concentration which was kept constant in all treatments against varying halide concentration did not influence the preferential formation of a specific bromocarbazole isomer with respect to another or their concentration. This confirms the role of enzymes in facilitating oxidation of the bromide to effect electrophilic substitution with the electron-rich carbazole (Silverthorn 2004; Stenesh 1998) irrespective of the treatment composition. Terrón et al. (1998) reported bromine oxidation, but this was done at very high H_2O_2 concentration and under long incubation periods. At 1 mM, under similar conditions, no oxidation took place emphasizing the role of enzymes at very low or environmental H_2O_2 levels.

Dibromocarbazole and tetrabromocarbazole previously detected as unknown environmental contaminants by Grigoriadou and Schwarzbauer (2011) were synthesized in this study. The presence of bromocarbazoles in seawater samples can therefore be explained based probable enzymatic catalyzed synthesis of these compounds in the sea. This could be attributed to the fact that the bromide concentration is higher in seawater than in ground and river waters coupled with the fact that there exist sources of bromoperoxidase enzymes in the sea (Deits et al. 1984). Hypobromous acid is also a more reactive halogenating agent towards aromatic species than hypochlorous acid in water (Voudrias and Reinhard 1988). Bromocarbazole concentration in aquatic environment can therefore be concluded to be higher compared to chlorocarbazoles.

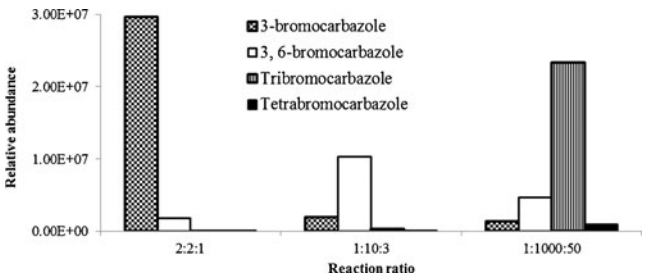
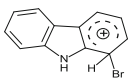
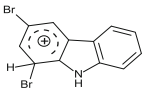
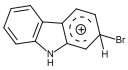
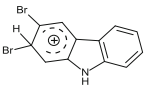
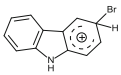
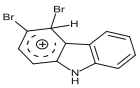
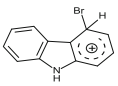
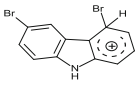
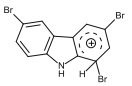
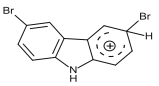
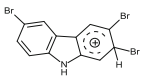
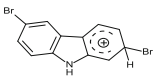
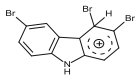
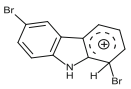


Fig. 1 Normalized relative abundances of bromocarbazoles formed in different reaction mixtures. Rx.1, Rx.2, and Rx.3 are represented here as 2:2:1; 1:10:3, and 1:1,000:50, respectively

Enzymatic chlorination reactions

3-Mono-, 3,6-di-, tri-, and tetra-cholorocarbazoles were synthesized in the enzymatic controlled chlorination reactions of carbazole (Fig. 2 and Fig. S1B). They were confirmed by reference materials and the mass spectra of the individual chlorocarbazoles (Fig. S6 to S8 in Electronic supplementary material (ESM)). 3-Chlorocarbazole and 3,6-dichlorocarbazole were the abundant compounds in Rx.4 and Rx.5 treatments. However, all four compounds, namely 3-mono-, 3,6-di-, tri-, and tetra-chlorocarbazoles, were formed in Rx.6 and Rx.7 treatments (Fig. 2). Similarly, the type of the compound formed was found to be influenced by halide concentration. When the halide concentration was minimal as in Rx.4 and Rx.5 treatments, 3-chlorocarbazole and 3,6-dichlorocarbazole were preferentially formed. With increased halide concentration in Rx.6 and Rx.7 treatments, all four chlorocarbazoles were formed (Fig. 2). The blanks did not show chlorination. Terrón et al. (1998) recorded no oxidation of chlorine even at higher H_2O_2 concentrations without the use catalyst.

Table 2 Summarized results of DFT calculations of the intermediate sigma complexes of bromocarbazoles synthesized including their energies in atomic units and kilocalories per mole

Intermediate Sigma Complex	Br-C	Energy in atomic units (au)	Relative energy (kcal/mol)	Intermediate Sigma Complex	Br-C	Energy in atomic units (au)	Relative energy (kcal/mol)
A				**B**			
 Sigma-1-bromocarbazole	1	-3091.2928	3.89	 3-brominesigma-1-bromocarbazole	1	-5664.8315	5.46
 Sigma-2-bromocarbazole	2	-3091.2913	4.82	 3-brominesigma-2-bromocarbazole	2	-5664.8329	4.58
 Sigma-3-bromocarbazole	3	-3091.2990	0.00	 3-brominesigma-4-bromocarbazole	4	-5664.8301	6.29
 Sigma-4-bromocarbazole	4	-3091.2886	6.53	 3-brominesigma-5-bromocarbazole	5	-5664.8293	6.82
C							
 3,6-brominesigma-1-bromocarbazole	1	-8238.3729	0.00	 3-brominesigma-6-bromocarbazole	6	-5664.8402	0.00
 3,6-brominesigma-2-bromocarbazole	2	-8238.3729	0.01	 3-brominesigma-7-bromocarbazole	7	-5664.8317	5.33
 3,6-brominesigma-4-bromocarbazole	4	-8238.3718	0.67	 3-brominesigma-8-bromocarbazole	8	-5664.8341	3.79

Br-C : position of brominated carbon

A, B, and C indicate intermediate sigma complexes of the respective mono-, di-, and tri- bromocarbazoles

Enzyme concentration neither influenced the formation nor the concentration of chlorocarbazoles. The variation in enzyme concentrations in Rx.4, Rx.5, Rx.6, and Rx.7 treatments did not cause any significant difference in comparison to bromination reactions where the enzyme concentration was kept constant. The results from Rx.4 and Rx.5 treatments (Fig. 2) support the suggestions that 3-monochloro- and 3,6-dichlorocarbazoles detected in most sampling campaigns as the stable forms of chlorocarbazoles in environment (Reischl et al. 2005) assuming the halide concentration does not occur in excess on the sampling site. Increase in hydrogen peroxide was found to cause an increase in concentration of compounds formed as was revealed in Rx.6 and Rx.7. However, this is attributable to enzyme activity as elsewhere (Terrón et al. 1998) recorded no halogenation even at high concentrations of the oxidant in the absence of enzymes or catalysts. 1,3,6-Trichloro- and 1,3,6,8-tetrachlorocarbazoles were calculated as the stable intermediate sigma complexes of the tri- and

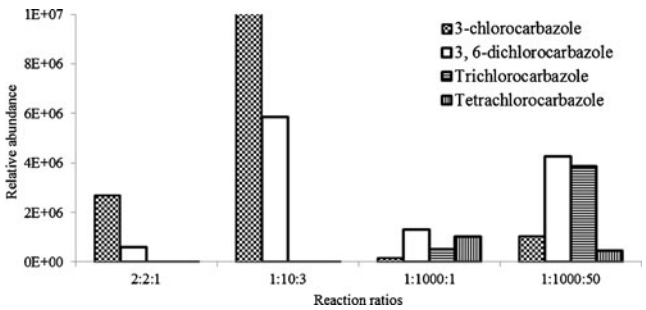
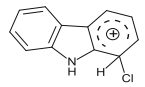
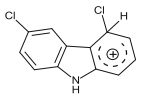
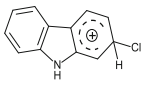
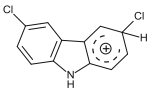
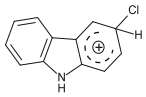
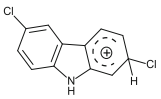
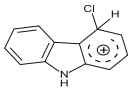
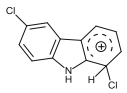
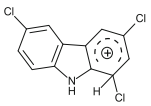
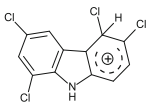
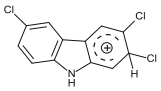
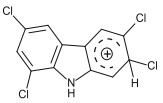
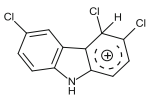
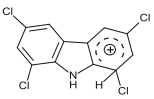


Fig. 2 Chlorocarbazoles synthesized in different reaction mixtures. Rx.4, Rx.5, Rx.6, and Rx.7 are represented as 2:2:1, 1:10:3, 1:1,000:1, and 1:1,000:50. Increasing H_2O_2 concentration results in increased concentration of the compounds in Rx.7 relative to those in Rx.6

Table 3 Summarized results of DFT calculations of the intermediate sigma complexes of chlorocarbazoles synthesized including their energies in atomic units and kilocalories per mole

Intermediate Sigma Complex	Cl-C	Energy atomic units(au)	Relative energy (kcal/mol)	Intermediate Sigma Complex	Cl-C	Energy atomic units(au)	Relative energy (kcal/mol)
A				**B**			
 Sigma-1-chlorocarbazole	1	-977.3747	3.50	 3-chlorinesigma-5-chlorocarbazole	5	-1436.9961	7.23
 Sigma-2-chlorocarbazole	2	-977.3715	5.46	 3-chlorinesigma-6-chlorocarbazole	6	-1437.0076	0.00
 Sigma-3-chlorocarbazole	3	-977.3802	0.00	 3-chlorinesigma-7-chlorocarbazole	7	-1436.9982	5.91
 Sigma-4-chlorocarbazole	4	-977.3695	6.72	 3-chlorinesigma-8-chlorocarbazole	8	-1437.0021	3.44
C				**D**			
 3,6-chlorinesigma-1-chlorocarbazole	1	-1896.6264	0.00	 1,3,6-chlorinesigma-5-chlorocarbazole	5	-2356.2522	1.24
 3,6-chlorinesigma-2-chlorocarbazole	2	-1896.6261	0.19	 1,3,6-chlorinesigma-7-chlorocarbazole	7	-2356.2528	0.84
 3,6-chlorinesigma-4-chlorocarbazole	4	-1896.6249	0.74	 1,3,6-chlorinesigma-8-chlorocarbazole	8	-2356.2542	0

Cl-C : position of chlorinated carbon

A, B, C, and D indicate intermediate sigma complexes of the respective mono-, di-, tri-, and tetra-chlorocarbazoles

tetra-isomers by DFT method (Table 3). The results were similar to those obtained by semiempirical PM3 method based on electron charge density on carbazole at the ground state (Bonesi and Erra-Balsells 1997). They also seem to follow electrophilic aromatic substitution pattern that favors halide substitution at the *ortho* and *para* positions relative to nitrogen on the carbazole (Bonesi and Erra-Balsells, 1997; Katritzky and Taylor 1990), implying that the formation mechanism in

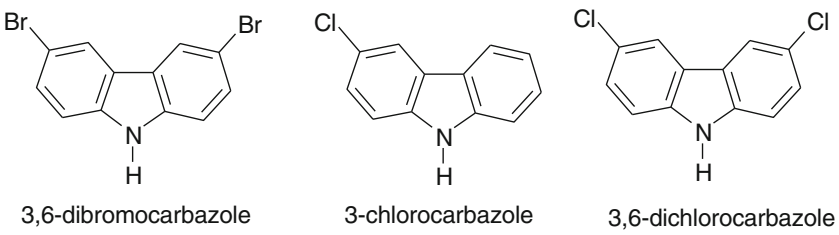


Fig. 3 Substitution positions of bromo- and chlorocarbazoles identified using of reference materials

enzymatic synthesis of chlorocarbazoles proceeds in a similar manner as chemical synthesis of the same compounds.

Role of enzymes and regioselectivity in halogenation reactions

Enzyme concentration was found not to affect either the type of isomer or the concentration of the bromo- and chlorocarbazoles formed. Our hypothesis that enzyme concentration could play a role in determining the type of compound to be synthesized and then proceed to influence its concentration was therefore ruled out. This shows that enzyme activity is generally limited by substrate concentration (Stenesh 1998). Increase in concentration only influences the rate of reaction through increase in the number of the enzyme active binding sites, at optimal temperatures specific to that enzyme (Silverthorn 2004). In the absence of enzymes, reactions may take place slowly or may not at all depending on other environmental variables. The preferred substitution positions obtained in this study were 3-, 3,6-, 1,3,6-, and 1,3,6,8- for the respective mono-, di-, tri-, and tetra-isomers of the two congeners. They were similar to substitutions patterns on isomers of compounds (Fig. 3) isolated from environmental samples (Grigoriadou and Schwarzbauer 2011; Reischl et al. 2005; Zhu and Hites 2005; Kronimus et al. 2004). Stereoselectivity in substitution at 3- and 3,6- positions was consistent with heterocyclic compounds (Sumpter and Miller 1954). This was the same for tri- and tetra-isomers but did not come out strongly due to very close relative energies to reveal the difference (Tables 2 and 3). 3-Mono- and 3,6-disubstituted isomers were the dominant isomers in almost all treatments (Fig. 4), seemingly to explain their occurrence and wide distribution in some of the reported samplings (Grigoriadou and Schwarzbauer 2011; Tröbs et al. 2011). Their dominance in environmental samples can be attributed to the high charge density at the *para* positions relative to the *ortho* positions (Fig. 5) and the effect of the lone pair of electrons in NH_2 moiety that enhances the amount and stability of *para*-products (Effenberger and Maier 2001; Smith and March 2007).

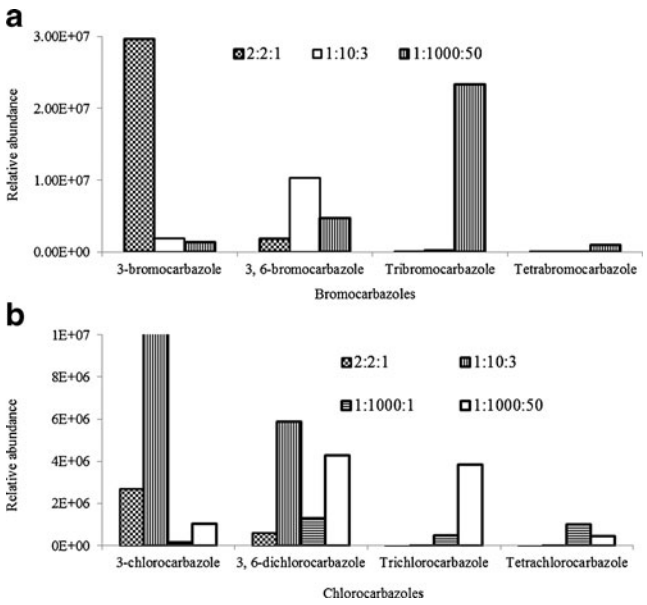


Fig. 4 The formation of bromocarbazoles (**a**) and chlorocarbazoles (**b**) in different reaction treatments. 3-Substituted and 3,6-substituted congeners were synthesized in all treatments and in relatively higher concentration in relation to the others

Enzymatic halogenation and toxicity potentials in the environment

Enzyme catalyzed halogenation is not unique to carbazole. Many organohalogen compounds with bromine and chlorine are produced by living organisms or by natural abiotic processes (Gribble 2003). Enzymatic synthesis which we propose is also not the only pathway to halogenation. Carbazole can react photochemically to form halogenated carbazoles (Metcalf and Eddy 1991). Indirect photolysis and photochemical transformation by bromine and chlorine radicals in aquatic environment are possible (Calza et al. 2008; Kochany and Maguire 1994). However, volatilization and oxidation processes involving carbazole are very slow (Kochany and Maguire 1994) requiring long incubation periods. The formation of only one isomer in the mass spectra within each of the four homologue groups (Fig. S2–S8 in ESM) also shows remarkably that our process is stereoselective making abiotic radical halogenation highly unlikely.

Enzymatic synthesis is highly possible in natural conditions. Environmental concentrations of carbazole in contaminated sites are significant ranging from 0.066 to 1.08 g/L in wastewater effluents and 0.52 to 2.77 mg/L in waste water treatment sludge but quite high in oil and creosote (Table S1 in ESM). Bromide is high in seawater while chloride levels in rivers, oceans, and waste water are equally high (Table S1 in ESM). H_2O_2 is available in freshwater (0.001 to 0.109 mg/L) and seawater (0.001 to 0.0136 mg/L) (Table S1 in ESM). Its production is higher in polluted (Cooper et al. 1988) and eutrophic waters. Chloroperoxidase halogenation has been demonstrated at 1 μM H_2O_2 (Hoekstra et al.1995). Considering that most halogenated carbazoles reported in literature were isolated in sites with carbazole contaminants, our experimental concentrations were in close comparison to environmental levels in polluted sites making this pathway possible in nature.

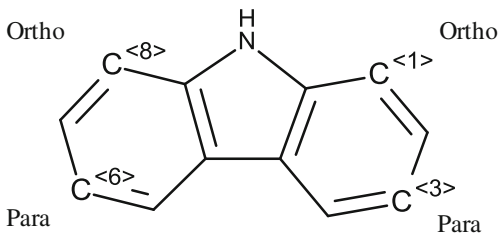


Fig. 5 Ortho and para positions on carbazole

Organic compounds have shown enhanced toxicity when transformed into its halogenated products (Wittsiepe et al. 1999). An increase in K_{ow} is observed in carbazole in relation to its transformation products, bromo- and chlorocarbazoles (Table S2 in ESM). Bioaccumulation, lipophility, and toxicity potentials of the drug gemfibrozil increased after transformation in wastewater to chloro- and bromogemfibrozil, respectively (Bulloch et al. 2012). Given that carbazole is carcinogenic and mutagenic while 3,6-dichlorocarbazole has dioxin-like toxicity, the toxicity of tri- and tetra-congeners could be significantly enhanced.

Conclusions

The role of enzymes in the formation of halogenated compounds of toxic potentials has once again been demonstrated. Environmental contaminants, namely halogenated carbazoles previously suggested to be naturally formed (Tröbs et al. 2011; Grigoriadou and Schwarzbauer 2011; Zhu and Hites 2005), were all synthesized by environmentally abundant enzymes in this study. These results provide evidence that bromocarbazoles and chlorocarbazoles can be formed naturally in the environment. The scope of halogenated carbazoles synthesized could be more to include penta- to octa- forms. This was limited by the extent of the study that focused mainly on compounds reported in literature. Bromocarbazole was found to be preferentially formed in aquatic environment in comparison to chlorinated derivatives. A hypothesis was deduced on the most abundant substituted congeners in environmental samples. These compounds just like 3,6-dichlorocarbazole could have toxic potentials. Further studies are required to understand their occurrence, toxicity, persistence, and environmental fate.

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