PERSISTENT BROMO-AND CHLOROCARBAZOLES: ENZYMATIC SYNTHESIS AND ELUCIDATION OF THEIR STRUCTURES BY MOLECULAR MODELING

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1. Introduction

Recent studies have shown the presence of halogenated carbazoles in sediments, soils and water [1, 2, 3]. Halogenated carbazoles belong to the group of heterocyclic aromatic hydrocarbons some of which are known hazardous environmental pollutants [4]. Bromo- and chlorocarbazoles are rarely reported environmental contaminants. The frequency of detection in environmental samples creates the need to understand their origin and occurrence given their potential for persistence and toxicity. It has been suggested that they could be naturally derived [1, 2]. Chlorination of carbazole most likely proceeds by a type of electrophilic chlorination of the aromatic moity that is an enzymatically controlled oxidation [3]. 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. These are incorporated catalytically by haloperoxidase enzymes into organic molecules in the presence of peroxides such as H_2O_2 [5, 6].

Molecular modelling based density functional theory (DFT), a quantum mechanical calculations method, has been used to calculate the most stable products of the halogenated carbazoles [3]. The determination of substitution position on aromatic compounds based on this method follows the general principle of electrophilic substitution of aromatic systems where the intermediate sigma (σ) complex is the rate and product determining step [7]. The most stable reaction mechanism with the lowest energy of activation determines regioselectivity. It is also the fastest rate determining intermediate yielding the major product [8]. 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. Enzymatic synthesis of bromo- and chlorocarbazoles in the aquatic environment was investigated and the prediction of the preferred stable isomers was done using DFT calculations.

2. Materials and methods

2.1 Chemicals and enzyme

Carbazole, hydrogen peroxide, potassium phosphate buffer salt, dichloromethane, purified chloroperoxidase (CPO) from *Caldariomyces fumago*, potassium chloride and sodium bromide were purchased from Sigma Aldrich (Taufkirchen, Germany) including reference standards for 3,6-dibromocarbazole, 3-chloro- and 3,6-dichlorocarbazoles. All chemicals were of analytical grade while the solvents were of pico grade quality.

2.2 Enzyme assay and water samples and preparation

0.1M potassium dihydrogen phosphate (KH_2PO_4) buffer solution of pH 3.0 at 25°C was prepared in distilled water. 11.96µM carbazole solution was then prepared in 0.1M KH_2PO_4 buffer solution prepared earlier. 11.96µM, 119.6µM and 11.96mM potassium chloride (KCl) solutions were also prepared separately in buffer solution prepared earlier. 5.98µM, 35.88µM and 0.598mM hydrogen peroxide (H_2O_2) solutions were prepared separately by diluting 30% (w/w) H_2O_2 in distilled water. CPO enzymatic assay was also prepared separately by dissolving 0.03ml CPO in 200ml cold buffer solution prepared earlier equivalent to 6.345units/ml.

2.2 Enzymatic halogenation of carbazole by Chloroperoxidase CPO

Carbazole concentration was kept constant against varying enzymes, bromides, chlorides and hydrogen peroxide concentrations. Temperature and pH of the buffer solution were kept constant. NaBr and KCl were used in

bromination and chlorination reactions respectively. In vitro enzymatic reactions of different reactant ratio treatments were carried out. Bromination reaction treatments were *Rx.1 (2:2:1 ratio)*, *Rx.2 (1:10:3 ratio)* and *Rx.3 (1:1000:50 ratio)* while chlorination reaction treatments were *Rx.4 (2:2:1 ratio)*, *Rx.5 (1:10:3 ratio), Rx.6 (1:1000:1)* and *Rx.7 (1:1000:50 ratio)* where *Rx.* describes the reaction mixtures composition of the substrate, halide and H_2O_2. The halides were dissolved in 0.1M KH_2PO_4 buffer solution containing 11.96µM of the substrate followed by addition of CPO at intervals of 5 min for the next 20min. 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 20min. It was then removed from the water bath and stored under -28°C in a refrigerator. Enzyme concentration varied in chlorination reactions but kept constant in all bromination reactions treatments.

2.3 Extraction and clean-up

Dichloromethane, DCM (CH_2Cl_2) was used to extract the synthesized halogenated carbazoles from the reaction mixtures with the use of a separation funnel. 300µL of the extracted solution was then subjected to a clean-up by column chromatography using silica gel, alumina and sodium sulphate 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 1mL. 0.5mL acetonitrile was added to the concentrate and then reduced under a steady flow of gentle nitrogen gas to approximately 0.5mL. The sample was subjected to another clean-up 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 0.2mL 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 three times using hexane under a steady but gentle flow of nitrogen gas to a final volume of 20µL. The amber vials were sealed and then taken for analysis using high resolution GC-MS.

2.4 GC/MS Analysis

Identification of bromo- and chloro-carbazoles was carried out by high resolution gas chromatography coupled to a mass spectrometer (GC-MS). GC Agilent 6890 Series II described elsewhere [9] was used. Two detection modes were employed, full scan mode with a resolution of 1000 and SIM mode with a resolution of >8500. All samples were analysed in single ion monitoring (SIM) mode, whereby the two most intensive masses of the molecular ion cluster was registered. In addition full scan analyses were carried out for identification of halogenated carbazoles by mass spectra. 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. Because of lack of reference standards for all halogenated carbazoles identified, a semi-quantative approach was used for calculating the ratios of amounts, whereby an equal response for all compounds was assumed. The variations between the analysis were normalized by use of the syringe standard.

2.5 DFT calculations

The electronic structure calculations of the intermediate sigma complexes of the halogenated carbazoles were performed using Density Functional Theory, incorporated in Gaussian 03W program package[10]. All geometry optimizations and frequency calculations were performed as *singlet* using the Becke 3-Lee-Yang-Parr (B3LYP) functional method and *opt=gdiis freq=noraman rb3yp/6-31+G(d,p)* basis set to obtain the zero point vibrational energy. Thermal energy corrections were computed and single point energy (SPE) calculations were carried out using *uB3LYP/6-311+G(2d,p)guess=mix* basis set. Stability tests were done using *stable B3LYP/6-311+G(2d,p)* basis set to ensure the wave function computed is stable and that the calculations corresponds to the ground state of the molecule [11].

3. Results and Discussion

3.1 *Enzymatic Bromination and Chlorination Reactions*

Mono-, 3,6-di-, tri- and tetra-bromocarbazoles were synthesized in the enzymatic controlled bromination of carbazole. Mono- and 3,6-di-bromocarbazole 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. 1A). 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. 1A). 3-Mono-, 3,6-di-, tri- and tetra-cholorocarbazoles were synthesized in the enzymatic controlled chlorination reactions of carbazole (Fig. 1A). 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. 1B). 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. The blanks did not show halogenation in both reactions.

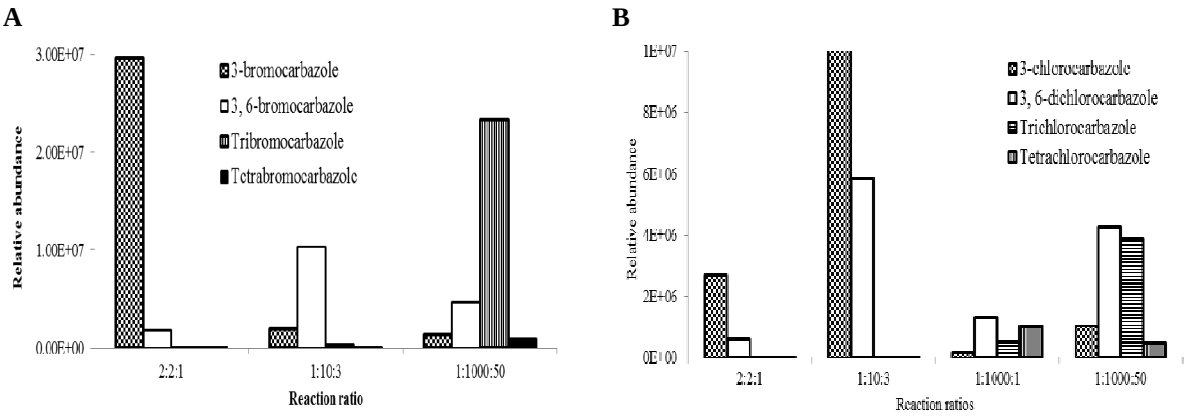

Fig. 1 Normalised relative abundances of bromocarbazoles and chlorocarbazoles formed in different reaction mixtures. *Rx.1*, *Rx.2* and *Rx.3* represent 2:2:1; 1:10:3 and 1:1000:50 reaction ratios for bromocarbazoles (A) while *Rx.4, Rx.5, Rx.6* and *Rx.7* represent 2:2:1, 1:10:3, 1:1000:1 and 1:1000:50 reaction ratios for chlorocarbazoles respectively. Increase in H_2O_2 results in increased concentration of compounds formed in *Rx.7* relative to *Rx.6.*

3-Bromo-, 1,3,6-bromo-, 1,3,6,7-bromocarbazoles were determined by DFT method as the most stable intermediate sigma complexes of the mono-, tri- and tetra- bromoisomers respectively while 1,3,6-trichloro- and 1,3,6,8- tetrachlorocarbazoles were the most stable intermediate sigma complexes of the tri- and tetra-chloro isomers. Di- and tetra-bromocarbazole previously detected as unknown environmental contaminants [2] were synthesized. The presence of bromocarbazoles in seawater samples can therefore be attributed to probable enzymatic catalysed synthesis of these compounds in the sea. Enzyme concentration neither influenced formation nor concentration of chlorocarbazoles formed. Variation in enzyme concentrations (*Rx.4, Rx.5, Rx.6* and *Rx.7* treatments) did not cause significant difference in comparison to bromination reactions where the enzyme concentration was constant.

3.3 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 chloro-carbazoles 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. 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. These were similar to substitutions positions on isomers of compounds isolated from environmental samples [1, 2]. Stereo- selectivity in substitution at 3- and 3,6- positions was consistent with heterocyclic compounds [12]. 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 between the two. 3-mono- and 3,6-di-substituted isomers were the dominant isomers in almost all treatments (Fig. 1) seemingly to explain their

occurrence and wide distribution in some of the reported samplings [2, 3]. Their dominance in environmental samples can be attributed to the high charge density at the *para* positions relative to the *ortho* positions and the effect of the lone pair of electrons in NH_2 moiety that enhances the amount and stability of para-products [7].

3.4 Enzymatic halogenation and toxicity potentials in the environment

Enzymatic synthesis of halogenated carbazoles is highly possible in natural conditions. Environmental concentrations of carbazole in contaminated sites are significant but quite high in oil and creosote [13]. Bromide is high in seawater while chloride levels in rivers, oceans and waste water are equally high. H_2O_2 is available in freshwater and seawater. Its production is higher in polluted [14] and eutrophic waters. 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. Organic compounds have shown enhanced toxicity when transformed into its halogenated products [15]. An increase in K_{ow} is observed in carbazole in relation to its transformation products, bromo- and chlorocarbazoles. 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.

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