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A Chlorine Isotope Effect for Enzyme-Catalyzed Chlorination

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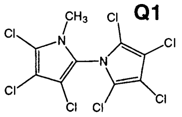
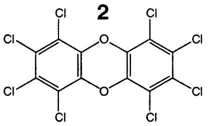
Chlorinated organic compounds (COCs) such as heptachloro-1′-methyl-1,2′-bipyrrole (**Q1**)[1,2] and octachlorodibenzodioxin (**2**)[3,4] have been detected worldwide, sometimes at high levels in soils, sediments, Antarctic air, seabird eggs, the blubber of marine mammals, and most notably, even in human milk.[1−8] To date, it has been difficult to determine whether these compounds are natural products or derived from human synthesis (i.e., anthropogenic input). If these compounds are indeed natural, then they may be useful mimics for understanding how anthropogenic COCs behave and interact in the environment. Alternatively, if these compounds derive from anthropogenic activity, careful consideration would need to be given as to their source and mode of production and whether their emissions can be controlled.

Selected chlorinated compounds found in the environment. Q1: heptachloro-1′-methyl-1,2′-bipyrrole;[1,2] 2: octachlorodibenzodioxin;[3,4] 3: pentachloroanisole;[5] 4: 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-furan;[4] 5: 1,1′-dimethyl-3,3′,4-tribromo-4′,5,5′-trichloro-2,2-bipyrrole;[6] 6: 2-(2′,3′,4′,5′,6′-pentachlorophenoxy)-3,4,5,6-tetrachloroanisole.[7]



Q1



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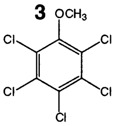

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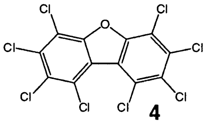

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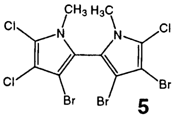

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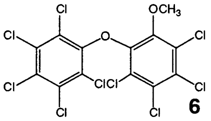

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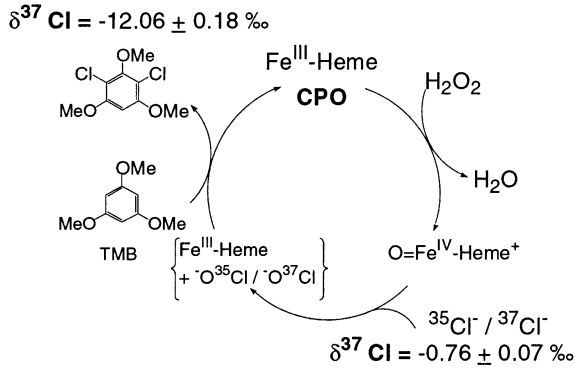
Haloperoxidases[9−13] and halogenases[14] are thought to be the major source of natural halogenated organic compounds in the environment, along with *S*-adenosyl-L-methionine methyl transferase, which produces specifically halomethanes.[15] To determine whether enzyme-catalyzed chlorination produces COCs with distinct stable Cl isotope (^{35}Cl and ^{37}Cl) ratios, we investigated the Cl isotope effect for enzyme-catalyzed chlorination compared to that for uncatalyzed chlorination processes. We report herein that Fe(III)-heme-chloroperoxidase (CPO) isolated from the fungus *Caldariomyces fumago* catalyzes the preferential incorporation of ^{35}Cl into aromatic organic substrates over that of molecular chlorination with hypochlorite. Furthermore, the large isotope effect by CPO suggests that naturally chlorinated COCs can be distinguished from anthropogenic COCs by their Cl isotope ratios.

Scheme 1



Because Cl isotope ratios generally vary by ∼2% or less, their ratios are often expressed in terms of the "δ" notation:

$$\delta^{37}\text{Cl}(‰) = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000 \qquad (1)$$

where R_{sample} is the ^{37}Cl/^{35}Cl of the sample and R_{standard} is the ^{37}Cl/^{35}Cl of standard mean ocean chloride (SMOC).[16] This notation expresses only the remarkable portions of the isotopic abundance while also providing a convenient scale to compare different samples. Bioaccumulating, anthropogenic COCs (such as polychlorinated biphenyls) have δ^{37}Cl values that span from −5.1 to +1.2‰.[20]

Numerous mechanistic studies of CPO reveal that CPO reacts first with H_2O_2, forming the two-electron oxidized species, O═Fe(IV)-heme^{+}, Compound I, which in turn oxidizes chloride in a rate-limiting step (Scheme 1). The oxidized Cl species then chlorinates the organic substrate. While the speciation of the oxidized Cl intermediate is debated, it is formally at the level of "Cl^{+}" (i.e., as in hypochlorite).

CPO (*C. fumago*) was treated with 1,3,5-trimethoxybenzene (TMB) or 3,5-dimethylphenol (DMP), potassium chloride, and hydrogen peroxide in citrate/phosphate buffer pH 3 (Scheme 1).[21] The H_2O_2 was added slowly by syringe pump to limit the side reaction of the CPO-catalyzed disproportionation of H_2O_2.[21] Each experiment was performed in triplicate in solutions of excess KCl with a known chlorine isotopic composition (−0.76 ± 0.07‰, n = 3). Extraction and analysis by gas chromatography−mass spectrometry revealed that the TMB was almost fully dichlorinated (90%) with traces of the mono- and trichlorinated congeners. DMP was trichlorinated (∼75%) and dichlorinated (∼25%). Bulk δ^{37}Cl values of the solvent extracts were −12.06 ± 0.18‰ (n = 3) and −11.08 ± 0.08‰ (n = 3) for the TMB and DMP halogenated products, respectively.[22] The magnitude of the kinetic isotope effect

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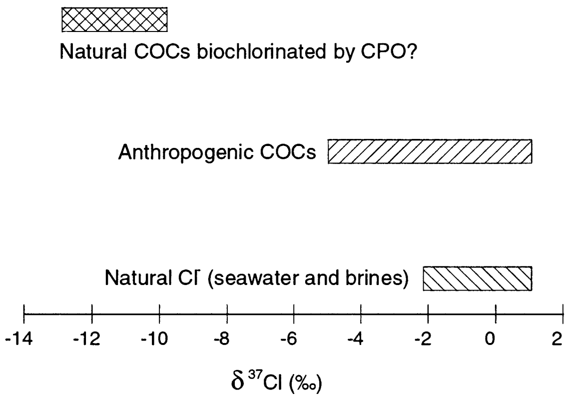

Figure 1. Plot of known δ^{37}Cl values for semi-volatile anthropogenic COCs[20] and chloride salts.[27] Also shown are the possible δ^{37}Cl values for natural COCs chlorinated by CPO.

(KIE), which can be expressed as the ratio of k_{35}/k_{37}, was determined using eq 2:

$$\frac{k_{35}}{k_{37}} = \frac{\log(1-f)}{\log[1-(f \times R_{\text{org}}/R_{\text{Cl}^-})]} \qquad (2)$$

where f is the fraction of Cl^- incorporated into the organic substrate and R_{org} and R_{Cl^-} are the $^{37}\text{Cl}/^{35}\text{Cl}$ ratios for the organic extracts and the Cl^-, respectively.[23] (Mean values of f were 0.044 for TMB and 0.055 for DMP). The KIEs were 1.012 for TMB and 1.011 for DMP. Hence, $^{35}\text{Cl}^-$ was incorporated into the substrate at a rate ~1% faster than $^{37}\text{Cl}^-$ and is the first such finding for any natural chlorination process. To test whether abiotic chlorination of these same substrates causes a similar isotope effect, TMP and DMP were treated with sodium hypochlorite (0.17 ± 0.11‰; $n = 3$).[24] The dichlorinated and trichlorinated products of each substrate had δ^{37}Cl values that were -3.47 ± 0.34‰ ($n = 3$) and -3.62 ± 0.38‰ ($n = 3$) for the TMB ($k_{35}/k_{37} = 1.0037$) and DMP ($k_{35}/k_{37} = 1.0039$), respectively. Therefore, the magnitude of this effect is much smaller than the enzymatic process. In addition, the δ^{37}Cl values observed for the OCl^- experiment are within the range observed for anthropogenic COCs (Figure 1). Further experiments are in progress to determine at what point in the mechanism of CPO isotopic fractionation occurs, as it may be due to one or more processes, such as desolvation of the Cl^-.[25]

In summary, the CPO-catalyzed reactions produce COCs with distinct δ^{37}Cl values that could be used to identify different sources of these compounds. For example, the δ^{37}Cl of natural chlorides ranges from -2 to 1‰ (Figure 1) and should always be in excess for these types of reactions in the environment. Hence natural COCs synthesized by this enzymatic pathway may have δ^{37}Cl values that are approximately -14 to -10‰ and be easily distinguished from anthropogenic COCs. Efforts are underway to isolate from environmental samples COCs with unknown origins for Cl isotope analysis.

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(16) While a consensus on the absolute value for the $^{37}\text{Cl}/^{35}\text{Cl}$ of SMOC has not been reached,[17] a ratio of 0.318988 is used in this study.[18]

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(21) Fe(III)-heme chloroperoxidase (CPO) (*C. fumago*) was used in this study because of its availability and was purchased from Sigma. The reaction conditions consisted of a stirred solution of substrate (ca. 0.4 mM TMB or DMP) in 25 mL of citrate phosphate buffer, pH = 3 containing 20 mM KCl, CPO (21 μg) to which 1 mL of 0.0129 M H_2O_2 was added by syringe pump over a 1.5-h interval. The solution was stirred for 1 h, and then an additional 42 μg of CPO and 1 mL of 0.0129 M H_2O_2 was added by syringe pump over a second 1.5-h interval. Again, after approximately 1 h the procedure was repeated, and the final mixture was stirred overnight before extraction. The total CPO added was 63 μg; the total H_2O_2 was 1.38 mM. For the nonenzymatic chlorination, 0.4 mM TMB or DMP were treated in 50 mL of 0.12 M citrate phosphate buffer, pH 3 with 3 mL of 0.353 M NaOCl added by syringe pump over a 100-min interval. The total added NaOCl was 20 mM. The solution was stirred overnight before extraction. Each experiment was performed in triplicate. In the CPO-catalyzed reactions, the KCl was the reagent in excess, and in the noncatalyzed reaction, the NaOCl was the reagent in excess. The chlorinated compounds were extracted into pentane and analyzed by GC−MS.

(22) The Cl isotope analysis of COCs is discussed in detail elsewhere.[20] Briefly, the organic extracts (in pentane) were transferred to precombusted 12-mm (i.d.) × 22-cm Pyrex tubes. The pentane was removed under a stream of nitrogen and precombusted copper oxide (1 to 2 g) was added to each tube. The tubes were then sealed under vacuum and combusted at 550 °C for 1 h. The resulting copper chloride was treated with CH_3I to produce CH_3Cl, which was then cryogenically distilled and purified. The purified CH_3Cl was introduced into a dual-inlet isotope ratio mass spectrometer that measured the ratio of $CH_3{}^{37}Cl$ (m/z 52) and $CH_3{}^{35}Cl$ (m/z 50), which was used to calculate the δ^{37}Cl of the sample.

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