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Ozone-Activated Halogenation of Mono- and Dimethylbipyrrole in Seawater

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Abstract

Polyhalogenated *N*-methylbipyrroles of two different structure classes have been detected worldwide in over 100 environmental samples including seawater, bird eggs, fish, dolphin blubber, and in the breast milk of humans that consume seafood. These molecules are concentrated in the fatty tissues in comparable abundance to some of the most important anthropogenic contaminants, such as the halogenated flame-retardants and pesticides. Although the origin of these compounds is still unknown, we present evidence that the production of these materials can involve the direct ozone activated seawater halogenation of *N*-methylbipyrrole precursors. This observation shows that environmental polyhalogenated bipyrroles can be produced via an abiotic process, and implies that the ozone activated halogenation of a variety of natural and anthropogenic seawater organics may be a significant process occurring in surface ocean waters.

Graphical Abstract

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Author Contributions

W.F. conceived of the study, A.K. performed the abiotic halogenations and with M.B. the analyses, W.F., L.A., and A.K. analyzed the data, formulated the figures and composed the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.6b03601](https://doi.org/10.1021/acs.est.6b03601). Additional experimental methods, figures, data tables and mass spectral data (PDF)

Notes

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INTRODUCTION

More than 5000 naturally occurring organohalogen compounds are currently known.^{1,2} These compounds comprise a large number of structure types including halogenated terpenoids, alkaloids, polyketides, peptides, and others. Halogenation is a known biochemical process in some terrestrial organisms, but it is particularly pronounced in marine plants, animals and bacteria, by virtue of their access to the halogens in seawater as building blocks. However, very few, if any of the biosynthetically produced organohalogens are found to accumulate in marine life, presumably due to their naturally evolved biodegradation pathways. Among the molecules that are being accumulated in marine food webs worldwide are a large diversity of conspicuous polyhalogenated *N*-methylbipyrroles, containing randomly mixed bromine and chlorine substituents.^{3–16} These compounds have received focused attention because they have been found in over 100 environmental samples, including air samples,⁴ fish¹¹ and sharks,¹³ dolphin¹² and dugong blubber,⁷ and remarkably, human breast milk⁴ where they are among the most abundant, non-PCB and non-DDT contaminants that bioaccumulate in fatty tissues.^{17,18} Their general absence in freshwater sources, except one report of their occurrence in Lake Baikal seals, has been taken as strong evidence of a marine origin.⁶

Polyhalogenated *N*-methylbipyrroles are of two types, polyhalo-1,1'-dimethyl-2,2'-bipyrroles (DMBPs) and polyhalo-1'-methyl-1,2'-bipyrroles (MBPs), which are related structures in which two pyrrole rings are linked by either a carbon–carbon (C₁–C₁) bond or a carbon–nitrogen (C₁–N) bond (Figure 1). Both classes of polyhalobipyrroles are found in Nature with bromine and chlorine in various ratios and at various substitution sites on the bipyrrole skeletons.

Subsequent to the first observation of these compounds, natural abundance radiocarbon measurements by Reddy and co-workers have provided significant support for a natural rather than anthropogenic origin of the carbon skeletons in the polyhalobipyrroles.¹⁹ Further, the presence of these contaminants has been observed in archived whale oil produced prior to the industrial era.²⁰ Radiocarbon measurements, however, can only identify a biosynthetic origin of carbon, not how and when halogenation occurs. One unique feature of these two

classes of bipyrrroles is their random halogenation patterns ranging from 1 to 7 halogen isomers at various positions on the bipyrrrole rings.^{14,17,18} Another significant feature is that these compounds are always *N*-methylated. To date, no industrial materials even closely related to these bipyrrroles can be identified. The consistent presence of chlorine and bromine, and the completely random regiochemistry of halogen substitution, is *inconsistent* with known biosynthetic halogenation processes which largely target bromination. These processes involve a variety of brominating enzymes that function in a regio- and stereochemically specific manner.^{1,2} Bacteria of the common genus *Pseudoalteromonas*, for example, are known to brominate, but to not chlorinate pyrroles in natural biosynthetic processes.^{21–23} Numerous other halogenation products such as the halomethanes CH₃I, CH₃Br, CH₂I₂, CH₂ICl are known to occur at ppb concentrations in seawater by photochemical halogenation of unknown dissolved organic matter.^{24–29} Other halogenation reactions, involve the production of halomethanes by seaweeds, albeit also at ppm and ppb levels.²⁵ These observations of very low levels of photochemical halogenation reaction products do not explain the worldwide bioaccumulation of the polyhalo-*N*-methylbipyrrroles. That these bipyrrrole contaminants are produced in significant amounts, and bioconcentrated in such a vast diversity of marine life, suggests a prolific and potentially dynamic source in ocean systems.

The exchange of gases across the air-sea interface provides a primary relationship between the chemistry of the ocean and atmosphere. Many gases (CO, CH₄, N₂O, H₂, O₃, methyl halides etc.,) and numerous shorter-lived species (<10⁵s lifetime) are known to exchange across the air–sea interface.^{30–35} Many of these species are transients produced by photochemical and other reactions. Ozone, in particular, is one of the most important shorter-lived species produced under the influence of sunlight on air masses that have become polluted by nitrogen oxides and volatile organic compounds. Further, 10 percent of the global ozone is concentrated in the lower Earth atmosphere (troposphere) where it decomposes and is continually replenished by transport from the stratosphere and by numerous photochemical reactions, which include the photodegradation of NO_x from polluted atmosphere as an indirect product of forest fires, and from lightening strikes on the ocean surface.³⁵ Lightning strikes more than 77 million times per year in the U.S. alone. These and other human activities have doubled the level of low altitude ozone in the last century. The abundance of atmospheric ozone also appears to be increasing as a function of climate change and increasing temperatures.^{33,36–40} Thus, while ozone may play an important oxidative role at the air-sea interface, little is known of its rate of dissolution, its concentration and reactivity in seawater and what the impact is on dissolved and particulate organic matter.

Ozone is a highly reactive gas that has been used, at high concentrations, for wastewater treatment. Under these conditions, ozone is known to oxidize halide ions present in wastewater into hypohalous acids and possibly molecular halogens.^{41–43} These halogen species are known to react in wastewater with dissolved organic matter to generate some known but mostly unknown halogen-containing products. There are some reports that provide evidence, but little structural information, of the formation of halogenated organics by these abiotic processes.^{44–46}

In this paper, we present direct evidence of the rapid abiotic halogenation of 1,1'-dimethyl-2,2'-bipyrrole (DMBP) and 1'-methyl-1,2'-bipyrrole (MBP) in seawater using ozone as the oxidant source. Experiments employing nonhalogenated DMBP and MBP precursors resulted in the conversion of these bipyrroles to a diversity of halogenated congeners, most of which are analogous to those observed to bioaccumulate in marine food webs. By keeping in mind that exchange of ozone takes place across the air-sea interface,³⁰ we performed a series of laboratory seawater-ozone experiments at various temperatures, times, and ozone and precursor concentrations to simulate surface ocean ozone exchange using a closed seawater-air system.

MATERIALS AND METHODS

Precursors 1,1'-dimethyl-2,2'-bipyrrole (DMBP) and 1'-methyl-1,2'-bipyrrole (MBP), were synthesized according to literature procedures (Full details are available in the Supporting Information (SI)).^{9,47} Gaseous O₃ was produced using a commercial ozone generator (model HG-500 Ozone Solutions, Hall, IA). The concentration of ozone being produced was measured by the iodometric titration method and the generator was set to produce ozone at a flow rate of 90 mg O₃/h for all experiments. The outlet of the ozone generator was connected to a 1L round-bottom flask by septum and sealed with parafilm. In a series of experiments, precursor bipyrroles were dissolved in seawater (natural seawater, SIO Pier) by adding the compounds in acetonitrile. Subsequently, ozone was then introduced into the head space of the solution at defined concentrations and the reactions, all at 500 mL seawater volumes, were allowed to stir for intervals of 2 h. When stirring was complete, the seawater solution was extracted with ethyl acetate (2 × 200 mL), the extract was dried using anhydrous sodium sulfate, and the mixtures obtained were analyzed by gas chromatography-mass spectroscopy (GC-MS), electron capture-gas chromatography (EC-GC) and liquid chromatography-mass spectroscopy (LC-MS) methods. These experiments were conducted at 15 and 10 °C to best reflect surface ocean temperatures. Concentrations of bipyrrole precursors were 10, 5, and 1 mg per 500 mL of seawater and ozone concentrations varied from 45 to 4.5 mg per 500 mL seawater, over periods of 6–12 h.

GC-MS analyses of reaction products were performed on an Agilent 7890B gas chromatograph equipped with an automatic injector (Agilent 7683B) and connected to a mass spectrometer (Agilent 5975C) and an electron capture detector (maintained at 300 °C using Argon/methane at a rate of 5 mL/min), a method with high detection capabilities for organohalogenes. A fused silica capillary column, DB-5ht (30 m × 320 μm × 0.1 μm; Agilent J&W), was employed as a nonpolar stationary phase. Operating conditions were as follows: inlet temperature 300 °C; Helium as carrier gas. The column temperature was initially held at 50 °C for 0.5 min and then ramped to 305 °C at a rate of 10 °C/min and held for 5 min. The total analysis time was 31 min. Analysis involved the injection of a 3 μL sample. The mass spectrometric detector (MSD) was operated in electron ionization mode with an ionizing energy of 70 eV, scanning from *m/z* 50 to 1000 with an ion source temperature of 230 °C and a quadrupole temperature of 150 °C. The samples were run with a solvent delay of 7.00 min. The relative abundances of the halogenated isomers were calculated by peak area analysis using the Agilent software Integrator, Chem-Station.

Where appropriate the masses of halogenated reaction products were assigned by interpretation of LC-MS/MS data. Samples were prepared in methanol with concentration of 0.1 mg/mL. Twenty μL of the compound in solution was injected onto a Phenomenex Luna C18 5 μm C18 (4.6 $\times$ 100 mm) analytical HPLC column operating on an Agilent 1260 HPLC coupled to an Agilent 6530 Accurate Mass Q-TOF mass spectrometer. The solvents used were water (A) and acetonitrile (B). The HPLC elution profile was as follows (flow rate 0.7 mL/min): Started with 10% solvent B then linear increase to 100% solvent B across 20 min, 100% solvent B for 3 min, linear decrease to 10% solvent B across 5 min, 10% solvent B for 2 min. Drying nitrogen gas at a flow rate of 11.0 L/min and a temperature of 350 $^{\circ}\text{C}$ was used for mass spectrometry.

NMR spectra, when required for compound identification, were obtained using a Varian Inova 500 MHz spectrometer (500 and 125 MHz for the ^1H and ^{13}C nuclei, respectively) in CDCl_3 (Cambridge Isotope Laboratories, Inc., (99.8% D) containing 0.03% v/v tetramethylsilane (δ_{H} 0.0 and δ_{C} 77.16 as internal standards). Chemical shifts are reported in parts per million (δ values). Splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), broad doublet (bd), double doublet (dd), triplet (t), quartet (q), and multiplet (m).

RESULTS AND DISCUSSION

Synthetic 1,1'-dimethyl-2,2'-bipyrrole (**1**) was subjected to ozone treatment in natural seawater at a variety of temperatures, ozone and precursor concentrations and reaction times (See SI pp S6–S17 for complete details). These experiments included much higher dilutions of precursors and ozone, shorter and longer reaction times and lowered temperatures. In general, highly similar results were observed. We found that halogenation incorporating both bromine and chlorine was a very fast and clean reaction. Little to no starting material remained. The reaction mixtures were analyzed by electron capture GC (EC-GC) and GC-mass spectrometry (GC-EIMS). Since authentic standards did not exist for all halogen congeners, we could not use retention times to assign the halogen regiochemistry of many of the products. Instead, we relied on predicted halogen isotope patterns and parent ion masses to assign the composition of products (See SI pp S32–S42). In some cases, the mass differences and halogen isotopes observed in MS fragment ions enabled further confirmation of these products. The overall product composition is shown in Figure 2 (and SI Table S1). After optimizing the reaction conditions, experiments were performed in duplicate to derive an average composition of the polyhalo-1,1'-dimethyl-2,2'-bipyrroles (polyhalo-DMBPs) reaction mixture. Similar, but not identical results were observed in replicate experiments illustrating a moderate degree of random halogenation. The various halogenated DMBP isomers were clearly resolved and could be definitively differentiated.

Ozone-induced halogenation under these conditions is not halogen regioselective and results in the rapid production of a diversity of polyhalogenated-DMBP products. The fully halogenated compounds DMBP- Br_6 , DMBP- Br_5Cl , and DMBP- Br_4Cl_2 were formed in replicate #1 at 16.60, 9.79, and 4.57% of the overall mixture (by EC-GC peak integration), while partially halogenated DMBPs were produced in the range of 0.20 to 13.71% (Figure 2, SI Table S1). In both replicates, the later eluting DMBP- Br_5 congeners (of the two detected

isomers) were consistently the most abundant product, followed by three other highly brominated DMBPs (DMBP-Br₆, DMPB-Br₅Cl and DMBP-Br₄Cl). The relative abundance of polyhalogenated DMBPs reported here exhibits significant overlap with abundances recently reported for eight dolphin blubber samples.¹⁸ For example, although DMBP-Br₅ was rarely the most abundant polyhalo-DMBP, it was consistently a significant contributor to the polyhalo-DMBP load in dolphins that fed in offshore regions of the Southern California Bight.¹⁸

It is interesting that brominated DMBPs are dominant over chlorinated DMBPs. Logically, this could be because (1) bromide is more easily oxidized than chloride or (2) hypochlorous acid (HOCl) is formed which quickly oxidizes bromide ion to hypobromous acid. Hypobromous acid has been shown to be a more reactive halogenating agent toward aromatic species compared with hypochlorous acid.^{41,42} Another possibility is that chlorine may act preferentially as an oxidant, whereas bromine may act more like an electrophilic agent. However, a mixture of HOCl/HOBr is known in laboratory experiments to lead to the formation of mixed brominated and chlorinated products.⁴¹ In the absence of ozone, the bipyrrole halogenation reactions did not proceed even after prolonged cooling or heating, clearly indicating that ozone is the essential oxidant. Further, we could not detect any halogenated materials by EC-GC analysis of our local seawater.

In order to explore the impact of varying reactant concentrations, temperature and reaction times, the same experiment with precursor **1** was performed under a variety of conditions in six experiments. Even at very low ozone and precursor concentrations (Experiment 6, SI p 17), halogenation proceeded rapidly to generate mixtures of polyhalo-1,1'-dimethyl-2,2'-bipyrroles comparable to the experiment above. Complete details of these seawater halogenations under variable conditions are found in the Supporting Information pp S11–S17.

We next investigated the abiotic halogenation of the other important precursor 1'-methyl-1,2'-bipyrrole (**2**, MBP), also available by synthesis.⁹ Under the same reaction conditions as with **1**, this bipyrrole was also rapidly halogenated to give a complex mixture of brominated and chlorinated products. The reaction mixture was also analyzed using GC-EIMS and EC-GC and the formation of halogenated products confirmed by mass spectrometry and in some cases retention time comparisons with authentic synthetic standards. The relative percentage of halogenated compounds detected by GC-MS is shown in Figure 3 and SI Table S2. The fully halogenated MBP-Br₇, MBP-Br₆Cl, and MBP-Br₅Cl₂ were formed in 3.34%, 8.83%, and 0.23% of the mixture, respectively, as calculated by peak area analysis from EC-GC spectra. The hexahalogenated MBP-Br₆ (two isomers), MBP-Br₅Cl (two isomers), MBP-Br₄Cl₂, and MBP-BrCl₅ (two isomers) were produced in 1.22%, 13.41%, 2.42%, and 1.46%, respectively. In addition, mono-, di-, and penta-halogenated products were formed as 0.08 to 13.08% of the overall mixture. The most abundant products were MBP-Br₄Cl (three isomers) and MBP-Br₂ (four isomers). In Pacific dolphin samples, MBP-Cl₇ and the higher-order chlorinated congeners are the most abundant and ubiquitous,¹⁸ however studies of dolphin blubber from the Atlantic Ocean found that higher order brominated MBPs are most commonly present in this environment.¹⁵

In apex marine biota, the polyhalobipyrroles can be present at similar levels as the brominated flame-retardants, specifically, the polybrominated diphenyl ethers (PBDEs). Abundances of the polyhalopyrroles in cetaceans, for example, can vary widely and reported levels are between 10 ng to 10⁴ ng per gram of extracted lipid. That these occur in human breast milk raises serious questions about their potential health impacts. Tittlemier and co-workers have shown that several polyhaloDMBP congeners bind to the aryl hydrocarbon receptor (AhR), in a fashion similar to the dioxins.⁴⁸ The aryl hydrocarbon receptor (AHR) is known for its essential role in physiologic processes such as cell growth, death, and differentiation. While little is known of the bioactivity of these polyhalobipyrroles, that they are AhR antagonists indicates that they contribute to the overall body burden of toxicants and in high concentrations may produce negative health consequences. The related polyhalo-monopyrroles are well-known to be cytotoxic to mammalian cells and to be genotoxic.^{49,50}

The results described in this work clearly show the nonregiospecific formation of brominated and chlorinated DMBP and MBP products in the presence of ozone in seawater. These reactions, which are rapid and result in high yields of halogenated bipyrroles, indicate that abiotic ozone-induced halogenations of the precursors DMBP and MBP in seawater can be considered as a possible source of these bioaccumulating contaminants. The high levels of halogen variability seen with different samples collected from diverse environments worldwide are not unexpected considering the reactivity of halopyrrole derivatives and the random halogenation observed. Given the significant UV chromophores of the polyhalobipyrroles (UV λ_{max} 240–250 nm), it might also be expected that under select conditions they may be photochemically reactive. We have observed the replacement of bromine by chlorine, presumably via nucleophilic substitution, upon long period natural sunlight illumination of 1,1'-dimethyl-3,3',4,4'-5,5'-hexabromo-2,2'-bipyrrole (DMBP-Br₆) in seawater (SI p S31). Similarly, polyhalo-monopyrroles have been reported to undergo both thermal and photochemical isomerizations resulting in halogen rearrangements.⁵¹ During our GC-based analyses, we also observed that 1,1'-dimethyl-3,3',4,4'-5,5'-hexabromo-2,2'-bipyrrole (DMBP-Br₆) can dehalogenate at high temperatures (SI p S30). Consequently, given the diversity of GC instruments and elevated temperatures involved to analyze these contaminant mixtures, it seems difficult to confidently predict the composition of the natural polyhalobipyrrole mixture. It is also clear that the pathways for the accumulation of polyhalobipyrroles can be complex and that thermal and photochemical degradation, as well as biotic metabolism can impact overall mixtures seen in nature.

Given these results, it seems clear that other dissolved organics, especially those activated to electrophilic substitution, would be expected to undergo ozone-induced halogenations. Under the same experimental conditions described above, we have also observed the rapid halogenation of indole, a major marine bacterial metabolite produced by the metabolism of tryptophan (SI p S21).⁵² The presence in seawater of a high diversity of trace organic compounds, both anthropogenic and natural, further opens the intriguing question whether other organics are halogenated by this abiotic process. Natural phenolics and polyphenolic metabolites from marine algae, for example, and a diversity of metabolites from vertebrates and invertebrates, that are activated for electrophilic substitution, and perhaps concentrated on or near the ocean would be expected to undergo analogous halogenations. To test this

hypothesis, we observed that phenol indeed undergoes bromination and chlorination when exposed to ozone in seawater (SI p S24).

An intriguing question remains as to the biotic origin of the DMBP and MBP carbon skeletons. While there are rare observations of unmethylated bacterial 2,2'-bipyrroles produced by marine bacteria,^{22,23} a ubiquitous source for these *N*-methylated carbon skeletons (DMBP and MBP) is not obvious. A recent paper describing the chlorination of saline wastewater has, however, illustrated that chlorination of chlorophyll yields a diversity of polyhalo-monopyrroles apparently by oxidative degradation.⁵⁰ Thus, it is conceivable that this ubiquitous plant component and other related pigments could be responsible for the generation of these and other small pyrrole derivatives. Demonstrating these processes do occur in surface seawaters, locating their sources, sinks and deciphering the environmental fate of these molecules, on the basis of these preliminary observations, is currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Polyhalo-1,1'-Dimethyl-2,2'-bipyrrole
(Polyhalo DMBP) X = H, Br, Cl

Polyhalo-1'-Methyl-1,2'-bipyrrole
(Polyhalo MBP) X = H, Br, Cl

Figure 1.
Chemical compositions of the major polyhalogenated-*N*-methylbipyrroles observed worldwide that accumulate in marine fatty tissues and human breast milk.

Figure 2. Gas chromatogram (ECD) of the ozone-induced seawater halogenation of 1,1'-dimethyl-2,2'-bipyrrole (**1**), replicate #1 at 15 °C.

Figure 3. Gas chromatogram (ECD) of the ozone-induced seawater halogenation of 1'-methyl-1,2'-bipyrrole (**2**) at 15 °C.