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Discovery and widespread occurrence of polyhalogenated 1,1'-dimethyl-2,2'-bipyrroles (PDBPs) in marine biota

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ABSTRACT

Polyhalogenated 1,1′-dimethyl-2,2′-bipyrroles (PDBPs) are halogenated natural products (HNPs) previously shown to bioaccumulate in marine mammals and birds. Since their discovery in 1999, six hexahalogenated and a few lesser halogenated congeners have been identified in diverse marine mammal samples. Here we report the identification of 17 additional hexahalogenated PDBPs in the blubber extract of a humpback dolphin (*Sousa chinensis*) from Queensland, Australia. Thirteen of these new PDBPs were also detected in an Australian sea cucumber (*Holothuria* sp.). Additional samples were also tested positive on several new PDBPs, including an Australian venus tuskfish (*Choerodon venustus*) as well as a white whale (*Delphinapterus leucas*) and a sperm whale (*Physeter macrocephalus*) from the Northern Hemisphere. GC/ECNI-MS-SIM quantification of the molecular ions was carried out with the help of synthesized standards. The sum concentration of PDBPs was 1.1 mg/kg lipid in the humpback dolphin and 0.48 mg/kg lipid in the sea cucumber.

1. Introduction

More than 5000 halogenated natural products (HNPs), mainly produced by sponges, algae and marine bacteria, have been discovered so far (Gribble, 1998, 2010, 2012). Some of these HNPs were shown to share the bioaccumulative properties of anthropogenic pollutants (Tittlemier et al., 1999; Vetter, 2006). Occasionally, these HNPs were detected with higher concentrations in marine biota samples than the man-made pollutants (Melcher et al., 2005). One group of HNPs frequently detected at elevated concentrations in the marine environment are the polyhalogenated 1,1′-dimethyl-2,2′-bipyrroles (PDBPs) (Fig. 1a) (Tittlemier et al., 1999; Vetter, 2012). In addition to the major PDBP representative, i.e. 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (3,3′,4,4′-Br_4-5,5′-Cl_2-DBP, a.k.a. Br_4Cl_2-DBP, BC-10) (Fig. 1d) (Gribble et al., 1999), three further PDBP congeners have been determined in bald eagle liver and marine biota by means of synthesized reference standards (Fig. 1b,c,e) (Gribble et al., 1999; Tittlemier et al., 2001; Haraguchi et al., 2006). In addition, a recently detected PDBP congener with yet unknown structure and a few tetra- to pentahalogenated DBPs were detected in marine mammals (Haraguchi et al., 2006; Hoh et al., 2009, 2012). These are probably metabolites of the hexahalogenated DBPs. Hoh et al. (2012) recently mentioned the presence of a Br_2Cl_4-DBP isomer in an Atlantic dolphin sample. Hitherto, the highest PDBP concentration of 40.7 mg/kg lipids was measured in a bottlenose dolphin from Japan (Haraguchi et al., 2006). Although the natural producer(s) of PDBPs could not be identified up to now, radiocarbon analysis of 3,3′,4,4′-Br_4-5,5′-Cl_2-DBP supported the assignment to biogenic sources (Reddy et al., 2004). In addition, a structurally closely related compound of $C_{10}H_6N_2Br_6$ (Fig. 1b), 3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole, was identified as metabolite of a marine bacterium (Andersen et al., 1974). PDBPs were shown to be persistent and bioaccumulative (Hoh et al., 2009; Tittlemier et al., 2001, 2003a). In addition, PDBPs have been shown to bind to the aryl hydrocarbon receptor (AhR) and induction of cytochrome P4501A (CYP1A) was demonstrated in chick embryo hepatocytes (Tittlemier et al., 2003b).

3,3′,4,4′-Br_4-5,5′-Cl_2-DBP (Fig. 1d) was not only the predominant PDBP congener in samples from the Northern Hemisphere but could also be identified in marine samples from Australia (Vetter et al., 2002). A structurally similar substance class, the polyhalogenated 1′-methyl-1,2′-bipyrroles (PMBPs) and especially the heptachlorinated compound heptachloro-1′-methyl-1,2′-bipyrrole (Q1) were also detected in different marine samples (Vetter et al.,

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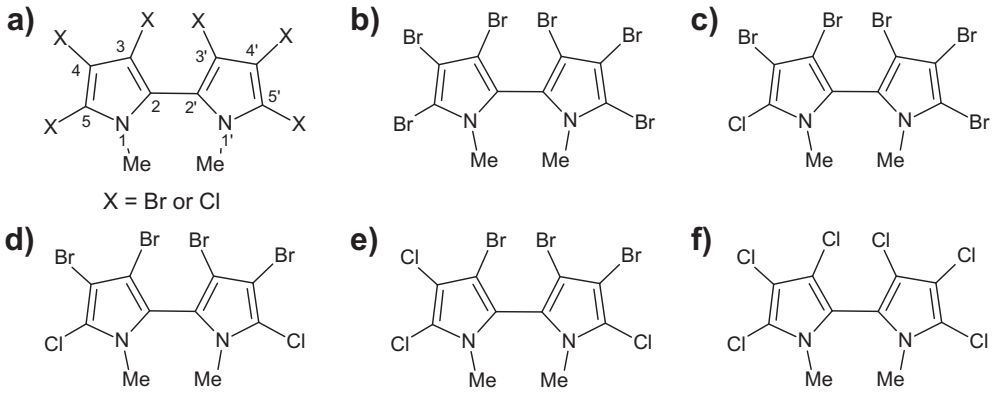

Fig. 1. Chemical structures of PDBPs. (a) General structure (1,1′-dimethyl-3,3′,4,4′,5,5′-hexahalo-2,2′-bipyrrole), (b) 1,1′-dimethyl-3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole, (c) 1,1′-dimethyl-3,3′,4,4′,5-pentabromo-5′-chloro-2,2′-bipyrrole, (d) 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (BC-10), (e) 1,1′-dimethyl-3,3′,4-tribromo-4′,5,5′-trichloro-2,2′-bipyrrole and (f) 1,1′-dimethyl-3,3′,4,4′,5,5′-hexachloro-2,2′-bipyrrole.

2000). Along with Q1, more than 20 PMBPs of the worldwide ~70 PMBPs have been reported in Australian samples (Vetter, 2012). By contrast, the five PDBPs initially detected and the Br_2Cl_4-DBP remained the only hexahalogenated DBPs identified so far (Tittlemier et al., 1999; Gribble et al., 1999; Hoh et al., 2012).

In this study we have searched for more than the few initially detected and structurally known PDBPs in Australian marine mammals and benthic feeding organisms. These samples were selected to cover a broad range with regard to geographic origins and the position in the marine food chain. We used gas chromatography with electron-capture negative ion mass spectrometry (GC/ECNI-MS) in the full scan and selected ion monitoring (SIM) mode for the identification of new PDBPs. Since GC/ECNI-MS responses are known to vary from compound to compound, the response factors of three synthetic standards were examined with different gas chromatographic detectors including electron ionization mass spectrometry (GC/EI-MS), electron-capture detection (GC/ECD), flame ionization detection (GC/FID) and nitrogen-phosphorus detection (GC/NPD).

2. Materials and methods

2.1. Samples

Blubber tissue of a humpback dolphin (*Sousa chinensis*) was obtained from an adult male animal stranded in 2000 at Gladstone near the Great Barrier Reef, in Queensland/Australia. Humpback dolphins are widespread in the West Pacific and Indian Oceans. Their habitat extends from South China and North Australia in the east to South Africa in the west (Jefferson, 2000). The highest population density is found in estuaries. Humpback dolphins were found to feed at least on twenty fish species from thirteen families which often live in estuaries (Jefferson, 2000). Especially around Australia, humpback dolphins occur mostly in waters close to the coast (Corkeron et al., 1997). The sea cucumber (*Holothuria* sp.) was collected from the reef flat off Heron Island (southern Great Barrier Reef, Australia). Sea cucumbers are deposit feeders which incorporate suspended and deposited particles from the sediment (Roberts et al., 2000). The fish liver was from a venus tuskfish (*Choerodon venustus*) which also originated from Heron Island/Australia. The sperm whale sample (*Physeter macrocephalus*) was from an individual stranded specimen at the German North Sea coast in the 1990s and the white whale (*Delphinapterus leucas*) was from Canada (1998). No further details are known for these two samples. Unfortunately, PDBPs could not be quantified in the white whale, the sperm whale, and the venus tuskfish, because these samples had been processed without suitable internal standards. In addition, about 20 samples previously analyzed on halogenated natural products were screened for the novel PDBPs; however, they were detected in none of them.

2.2. Sample clean-up

After sampling, samples were stored frozen until analysis as well as the measuring solutions. The sample clean-up was carried out according to Weichbrodt et al. (2000). Blubber samples and lyophilized tissue of the sea cucumber were extracted with cyclohexane/ethyl acetate (1:1; v/v) by accelerated solvent extraction (ASE, Dionex, Idstein, Germany) with α-PDHCH as internal standard. Lipids were removed from aliquots of the concentrated extracts using gel-permeation chromatography (GPC) on Bio-Beads SX-3 (Bio Rad, USA). The solvent of the collected organohalogen fraction was changed to *iso*-octane and the samples were subjected to further purification by adsorption column chromatography on deactivated silica. Additionally, a group separation on activated silica gel was carried out for the humpback dolphin sample to separate the HNPs from the majority of anthropogenic polyhalogenated compounds. The first fraction was eluted with 48 mL *n*-hexane. For further analysis, only the second fraction eluted with 50 mL *n*-hexane/ethyl acetate (8:2; v/v) was used.

The recovery rate of the entire clean-up method determined with spikes of standards of Cl_6-BDP, Br_4Cl_2-DBP, and Br_6-DBP, into oil was 98%, 88%, and 91%, respectively.

2.3. Chemicals and standards

Cyclohexane (>99%), ethyl acetate (>99%), *n*-hexane (for residue analysis, >95%), *iso*-octane (for residue analysis, >99%), silica gel 60 (pure for column chromatography) and sodium sulfate (anhydrous) were purchased from Fluka/Sigma–Aldrich (Steinheim, Germany). Cyclohexane and ethyl acetate were mixed 1:1 (v/v) and purified by azeotropic distillation before use. The standards Br_6-DBP and Cl_6-DBP were synthesized by Martin et al. (2011). 3,3′,4,4′-Br_4-5,5′-Cl_2-DBP was synthesized according to Gribble et al. (1999). 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (Q1) was synthesized by Wu et al. (2002). Perdeuterated α-1,2,3,4,5,6-hexachlorocyclohexane (α-PDHCH) was synthesized according to Vetter and Luckas (1995).

2.4. Gas chromatography with different detectors

Gas chromatography with electron-capture negative ion mass spectrometry (GC/ECNI-MS) measurements were performed on an Agilent 7890/5975C system equipped with an 7673 GC/SFC automatic injector (Agilent Technologies, Waldbronn, Germany). The ion source and transfer line temperatures were set at 200 °C and 300 °C, respectively. Methane 5.5 (Air Liquide, Bopfingen, Germany) was used as the reagent gas at a flow rate of 40 mL/min. Sample solutions (1 μL) were injected and the sample was transported with a constant He (5.0 quality, Westfalen, Münster, Germany) carrier gas flow of 1.2 mL/min. An HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm d_f; Agilent, Waldbronn, Germany) was installed in the GC oven which was programmed as follows: 60 °C (2 min), at 10 °C/min to 300 °C (24 min). In the GC/ECNI-MS full scan mode, *m/z* 50–800 was recorded after a solvent delay of 8 min. The GC/ECNI-MS-SIM run was divided into four time windows where the monoisotopic peak of $[M]^-$ and three subsequent isotopic peaks ($[M+2]^-$, $[M+4]^-$, $[M+6]^-$) of every possible hexahalogenated PDBP congener eluting in this time range were measured as follows: 8.0–20.6 min: *m/z* 364 (51.9%), 366 (100%), 368 (80.3%) and 370 (34.5%) for Cl_6-DBP, *m/z* 384 (44.6%), 386 (100%), 388 (96.2%) and 390 (51.5%) for Q1; 20.6–22.3 min: *m/z* 408 (38.4%), 410 (99.2%), 412 (100%) and 414 (51.6%) for $BrCl_5$-DBPs, *m/z* 452 (24.4%), 454 (79.2%), 456 (100%) and 458 (63.1%) for Br_2Cl_4-DBPs; 22.3–23.7 min: *m/z* 496 (16.6%), 498 (64.8%), 500 (100%) and 502 (77.5%) for Br_3Cl_3-DBP, *m/z* 540 (11.9%), 542 (54.4%), 544 (100%) and 546 (94.0%) for Br_4Cl_2-DBPs; 23.7–40.0 min: *m/z* 584 (7.99%), 586 (41.7%), 588 (89.3%) and 590 (100%) for Br_5Cl-DBP, *m/z* 628 (5.30%), 630 (31.2%), 632 (76.4%) and 634 (100%) for Br_6-DBP. In addition, *m/z* 79 and *m/z* 81 were measured in all time windows. Concentrations of PDBPs not available as standard compounds were estimated on the basis of the GC/ECNI-MS response factors of available PDBP standards relative to the GC/FID response based on the molar carbon content (Table 1). This measure was necessary because the