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Ichthyotoxic brominated diphenyl ethers from a mixed assemblage of a red alga and cyanobacterium: Structure clarification and biological properties

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ABSTRACT

Primary fractions from the extract of a tropical red alga mixed with filamentous cyanobacteria, collected from Papua New Guinea, were active in a neurotoxicity assay. Bioassay-guided isolation led to two natural products (**1**,**2**) with relatively potent calcium ion influx properties. The more prevalent of the neurotoxic compounds (**1**) was characterized by extensive NMR, mass spectrometry, and X-ray crystallography, and shown to be identical to a polybrominated diphenyl ether metabolite present in the literature, but reported with different NMR properties. To clarify this anomalous result, we synthesized a candidate isomeric polybrominated diphenyl ether (**3**), but this clearly had different NMR shifts than the reported compound. We conclude that the original isolate of 3,4,5-tribromo-2-(2,4-dibromophenoxy)phenol was contaminated with a minor compound, giving rise to the observed anomalous NMR shifts. The second and less abundant natural product (**2**) isolated in this study was a more highly brominated species. All three compounds showed a low micromolar ability to increase intracellular calcium ion concentrations in mouse neocortical neurons as well as toxicity to zebrafish. Because polybrominated diphenyl ethers have both natural as well as anthropomorphic origins, and accumulate in marine organisms at higher trophic level (mammals, fish, birds), these neurotoxic properties are of environmental significance and concern.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have recently attracted considerable attention due to an increasing awareness that these compounds accumulate in higher trophic level animals, including sperm whales (Marsh et al., 2005; Vos et al., 2003), sea gulls (Verreault et al., 2007), seals (Vos et al., 2003), polar bears (Kelly et al., 2008), as well as in humans (Betts, 2002; Athanasiadou et al., 2008). Some PBDEs are industrially produced in large quantities for use as flame retardants, and therefore their presence in animals has generally been assumed to be of anthropogenic origin (Hale et al., 2003). Hydroxylated congeners of PBDEs (OH-PBDEs) have also been found in many of these same higher animals (Verreault et al., 2007; Kelly et al., 2008; McKinney et al., 2006a), and recent studies have shown that the OH-PBDEs found in large marine-associated animals may be of mixed origins with some deriving from natural sources and the others being derivatives of anthropogenic PBDEs (McKinney et al., 2006b; Kelly et al., 2008). Moreover, a variety of anthropogenic PBDEs have been shown to undergo hydroxylation in rats via oxidative metabolism, thereby producing OH-PBDE congeners (Malmberg et al., 2005; Marsh et al., 2006). Nevertheless, a number of OH-PBDEs are known to be natural products of various marine organisms, such as sponges, tunicates, and cyanobacteria (Carte and Faulkner, 1981; Carte et al., 1986;

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Unson et al., 1994). Environmental concerns have been raised over the occurrence of PBDEs in higher animals because PBDEs have been linked to endocrine disruption (Vos et al., 2003; Mariussen and Fonnum, 2006). Activities similar to those of chlorinated aromatic compounds have been observed with PBDEs, such as aryl hydrocarbon-receptor agonist and antagonist activities, thyroid toxicity, and effects on the immune system (Hwang et al., 2008). Neonatal exposure to PBDEs has been shown to cause neurotoxicity in adult animals (Mariussen and Fonnum, 2006). OH-PBDEs have also been shown to disrupt thyroid hormone homeostasis presumably due to their structural similarity to the thyroxin-type endogenous thyroid hormones (Kelly et al., 2008). Recently, BDE-47 and 6-OH-BDE-47 have been shown to trigger an increase in cytosolic Ca^{2+} concentration as well as exocytosis of catecholamines in neuronal cells within a few minutes (Dingemans et al., 2008, 2007). These findings indicate that PBDEs and OH-PBDEs have the potential to acutely disrupt normal neuronal communication in animals.

Our program has been systematically screening marine algae and cyanobacteria for secondary metabolites with neuropharmacological properties, partially because of the environmental impacts these substances may impose, and partially to detect agents with potentially useful biomedical properties (Berman and Murray, 2000; Li et al., 2001; Rogers et al., 2002; Dravid and Murray, 2003). Because a variety of cellular events can trigger an increase in cytosolic Ca^{2+} levels in neuronal cells, we have employed a FLIPR-based fluorescence assay which detects cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse neocortical neurons. Herein, we describe the assay-guided isolation, structural determination and neurotoxicological evaluation of two OH-PBDE congeners (**1** and **2**, Fig. 1) from a mixed assemblage of marine cyanobacteria and a red alga. In the course of this work, we have also corrected the assignment of a ^{13}C NMR signal for **1** in the literature which could be a source of confusion in future efforts with OH-PBDE congeners.

2. Materials and methods

2.1. Reagents and algal material

All solvents used were of HPLC grade from EMD and were used without purification. TLC grade silica gel from Sigma–Aldrich was used for vacuum liquid chromatography (VLC). Flash chromatography was performed using EMD silica gel (230–400 mesh). Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals. Minimum essential medium, deoxyribonuclease (DNase), poly-L-lysine, cytosine arabinoside, veratridine, aconitine, and deltamethrin were from Sigma–Aldrich. The fluorescent dye Fluo-3, and pluronic acid F-127 were obtained from Invitrogen Corporation.

Algal material was collected at Grabo Reef in Papua New Guinea (5° 28.664 S by 150° 12.524 E) at 12–18 m depth by SCUBA on August 24, 2000 (voucher specimen available as PNG-GR 8/24/00-3). The algae was identified in the field as *Vidalia* sp., but subsequent microscopic examination of the voucher sample identified it to likely be *Leptofaucia* sp. Additionally, a small abundance of cyanobacterial filaments similar to *Oscillatoria* was observed in the sample by light microscopy.

2.2. Bioassay-guided isolation of OH-PBDEs

A total of 2 L of the red algal and cyanobacterial assemblage (dry weight 128 g) were extracted with CH_2Cl_2/MeOH (2:1). Upon removal of the solvents *in vacuo*, a portion (4.0 g) of the crude extract (4.6 g) was fractionated into nine fractions using silica gel vacuum liquid chromatography (hexanes to EtOAc to MeOH), one of which (308 mg, eluted with 2:3 EtOAc/hexanes) possessed Ca^{2+} modulating activity in mouse neocortical neurons. A portion (306 mg) of the active fraction was further fractionated by flash column chromatography (Still et al., 1978) on silica gel (hexanes/Et_2O 1:9 to 1:8) to obtain seven sub-fractions, of which two (2 mg and 77 mg) were associated with Ca^{2+} modulation activity. The larger sub-fraction was further purified by HPLC using a Jupiter 10 μ C18 300A column (250 × 10 mm) with a gradient solvent system (2.5 mL/min, 3:2 MeCN/H_2O to 4:1 over 30 min, then to MeCN over 10 min) to obtain 15 mg (0.38% of extract) of compound **1** (R_t = 52 min) and ~0.5 mg (0.01% of extract) of compound **2** (R_t = 55 min), both of which were confirmed to elevate neuronal $[Ca^{2+}]_i$.

2.3. Neocortical neuron cultures

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice. Briefly, pregnant mice were euthanized by CO_2 asphyxiation and their neocortices were collected. Isolated neocortices were then removed of their meninges, minced by trituration using a Pasteur pipette, and treated with trypsin for 25 min at 37 °C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and resuspended in Eagle's minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 10% fetal

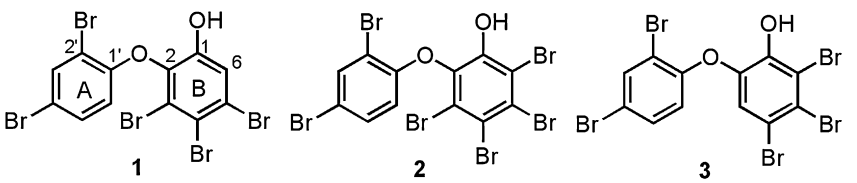

Fig. 1. OH-PBDE congeners isolated from the red alga/cyanobacteria assembly (**1** and **2**) and an alternative regioisomer of **1** (**3**).