Identification and quantification of the halogenated natural product BC-3

Joachim Melcher[1], Daniel Olbrich[1], Göran Marsh[2], Caroline Gaus[3], Jochen F. Müller[3], Walter Vetter[1]

[1]Institute of Food Chemistry, University of Hohenheim, Germany
[2]Department of Environmental Chemistry, Stockholm University, Sweden
[3]National Research Centre for Environmental Toxicology, Coopers Plains, Australia

Introduction

Halogenated natural products (HNPs) of marine origin are increasingly recognized as critical residues in foodstuff (e. g. fish) and environmental samples (e. g. marine mammals and birds). Some of these HNPs (Q1, MHC-1, BC-2, and HDBPs including BC-10) were detected in diverse fish and marine mammal samples at concentrations sometimes exceeding those of PCBs, DDT, and other anthropogenic pollutants [1,2].

Recent studies with marine mammal samples from Australia led to the detection of six abundant HNPs (Q1, BC-1, BC-2, BC-3, BC-10, and BC-11). In the meantime, Q1 was identified as heptachloro-1´-methyl-1,2´-bipyrrole, BC-2 as 4,6-dibromo-2-(2´,4´-dibromo)phenoxyanisole, BC-10 as 1,1´-dimethyl-3,3´,4,4´-tetrabromo-5,5´-dichloro-2,2´-bipyrrole, and BC-11 as 3,5-dibromo-2-(3´,5´-dibromo,2´-methoxy)phenoxyanisole [1,3,4]. However the identity of BC-1 and BC-3 remained unclear.

The goal of the present study was the identification of BC-3. The tetrabromo compound BC-3 has previously been detected in marine mammals from four continents [5,6]. Furthermore, we attempted establishing quantitative concentrations in diverse marine biota samples.

Materials and Methods

Samples and Chemicals: Australian samples analysed in this study were from Queensland, North-eastern Australia. Commercial shark liver oil from New Zealand was from Lovely Health (Auckland, New Zealand). Additional sample extracts were obtained and purified as described elsewhere [6].

The following solvents were used: cyclohexane (purest; Merck, Germany), ethyl acetate (Acros, Germany), n-hexane (Unisolv®; Merck, Germany), isooctane (ECD tested for pesticide analysis; Acros, Germany). Silica gel (60, purest, for column chromatography) was from Merck, Germany.

Pure 3,5-dibromo-2-(2´,4´-dibromo)phenoxyanisole was recently synthesized by Marsh et al. [7]. 5 mg were accurately weighed into a 100 mL Erlenmeyer flask and stepwise diluted to concentrations ranging from 1 pg/µL to 10 ng/µL (calibration range).

Sample clean-up: Samples except blubber and oils were lyophilized prior to extraction. After spiking with the internal standard perdeuterated α-HCH, samples (0.5 – 30 g) were extracted by accelerated solvent extraction (ASE, Dionex) using the following conditions: temperature 125 °C, pressure 10 MPa, preheat 0 min, heat 6 min, static 10 min, flush 60 %, purge 120 sec, cycles 2. Aliquots were taken for gravimetric determination of the lipid content. Collected extracts were concentrated to 5.0 mL and 4.5 mL were subjected to gel permeation chromatography on Bio-Beads SX-3 (Bio Rad, USA. Cyclohexane/ethyl acetate (1:1, v/v) was used as the mobile phase at a flow of 5 mL/min. The collected organohalogen fraction (20 to 35 min) was concentrated in a rotary evaporator to ~2 mL, and the solvent was changed to isooctane in a gentle flow of nitrogen. The sample in isooctane (~1 mL) was subjected to further purification by adsorption column chromatography on deactivated silica. 3 g of deactivated silica containing 30 % water (w/w) were slurried with hexane into a 1 cm i.d. glass column and covered with dry sodium sulfate. The sample was then rinsed carefully on the column and eluted with 60 mL of hexane. The samples were concentrated to suitable volumes and analyzed by GC/ECD and/or GC/MS.

Gas chromatography with electron capture detection (GC/ECD): GC/ECD analyses were performed with Hewlett-Packard 5890 series II systems equipped with 7673A autosamplers, ECDs, and split-splitless injectors operated in the splitless mode. Helium (purity 5.0) was used as carrier gas and nitrogen (purity 5.0) as make-up gas.
In GC system 1, a CP-Sil 8/10% C18 column (50 m, 0.25 mm i.d., 0.25 µm d_f Chrompack, Middelburg/NL) was used with the following conditions: injector temperature (250 °C), detector temperature (300 °C), carrier gas pressure 25 psi (const.), temperature program: 80 °C (1 min), 40 °C/min to 180 °C (1 min), 2 °C/min to 220 °C (1 min), 4 °C/min to 270 °C (26 min).
In GC system 2, samples were splitted equally after the injector (PAS1, Gerstel) on a CP-Sil 2 column (50 m, 0,25 mm i.d., 0,25 µm d_f) and a CP-Sil 8/20% C18 column (50 m, 0.25 mm i.d., 0.25 µm d_f) both obtained from Chrompack, Middelburg/NL. Thereby these conditions were used: injector temperature (250 °C), detector temperatures (300 °C), coloum head pressure 18 psi (const.), GC oven temperature program: 60 °C (1,5 min), 40 °C/min to 180 °C, 2 °C/min to 230 °C (25 min), 10 °C/min to 270 °C (15 min). The injected volume was always 1 µL of sample.

Gas chromatography-mass spectrometry (GC-MS): GC/ECNI-MS analyses were performed on a Varian 3800 gas chromatograph connected to a Varian 1200 triple quad mass spectrometer. Separations were achieved on a Factor Four® CP-Sil 8ms column from Varian, Darmstadt (30 m, 0.25 mm i.d., 0.25 µm d_f) with a constant flow of 1 mL/min Helium (purity 5.0) and the following temperature program: 70 °C (1.5 min), 30 °C/min to 140 °C, 3 °C/min to 230 °C (25 min), 4 °C/min to 270 °C (36 min), the injector temperature was kept at 250 °C and the transfer line temperature was set at 280 °C. Methane (purity 4.5) was used as reagent gas at a pressure of 8 Torr in the ion source. The injected volume of each sample was 1 µL.
GC/EI-MS analyses were performed on a Varian 3400 gas chromatograph equipped with a HP-DB-5ms column (30 m, 0.32 mm i.d., 0.17 µm d_f) connected to a Varian Saturn 4D iontrap mass spectrometer. The split/splitless injector was operated in splitless mode for 2 min and kept at 250 °C. The carrier gas (Helium, purity 5.0) was used at a constant pressure of 7.5 psi.
The following temperature program was used: 50 °C (1 min), 25 °C/min to 100 °C, 5 °C/min to 300 °C (5 min). The transfer line was heated to 300 °C. The manifold was set to 230 °C, and the

multiplier voltage was 1800 V. At a filament emission current of 60 µA the mass range from 60 to 600 m/z was scanned twice a second.1 µL of sample was injected each run.

Results and Disscussion

Comparison of GC/EI-MS (Figure 1) and GC/ECNI-MS data, as well as identical retention times on four capillary columns of different polarity, prove that BC-3 was identical with 3,5-dibromo-2-(2´,4´-dibromo)phenoxyanisole (Figure 2). BC-3 is an isomer of 4,6-dibromo-2-(2´,4´ dibromo)phenoxyanisole (BC-2) (Figure 3) which was recently found to be produced by sponges from Australia [8]. BC-3 was previously detected in samples from the Baltic Sea [9]. After Q1, BC-2 and BC-11 as well as BC-10, BC-3 was the fifth of the six aforementioned halogenated natural compounds in Australian samples that could be structurally determined. On DB-5 columns, BC-3 eluted slightly prior to BDE 100, which is found in technical mixtures of brominated flame retardants and is a common contaminant in marine biota [10,11].

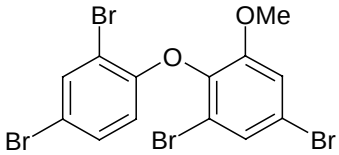

Fig. 2: Molecular formula of 3,5-dibromo-2-(2´,4´-dibromo)phenoxyanisole (BC-3)

Recent semi-quantitative investigations indicated high BC-3 concentrations in biota from Australia [5]. In Australian marine mammal samples, BC-3 was among the dominating halogenated compounds (Figure 4). To verify this hypothesis, a quantitative BC-3 standard was prepared for the analyses of marine samples. An equimolar mixture of BC-2 and BC-3 standard resulted in identical GC/ECD, GC/EI-MS (full scan mode) and GC/ECNI-MS (full scan mode) responses of both analytes. The GC/EI-MS full scan spectrum of BC-3 showed a more intense molecular ion (512 u) than that of BC-2. Therefore, the GC/EI-MS-SIM response based on the most abundant isotopic peak of the molecular ion (m/z 516) of BC-3 was higher by factor ~1.7 (Figure 1a). In GC/ECNI-MS mode, responses of the bromide ions, which may be used for quantification, were equal for BC-2 and BC-3. GC/ENCI-MS of BC-2 showed a weak molecular ion which is in contrast to BC-3, where none was detected. The quantitative solution was used to determine BC-3 concentrations and BC-3/BC-2 ratios in different samples (Table 1).

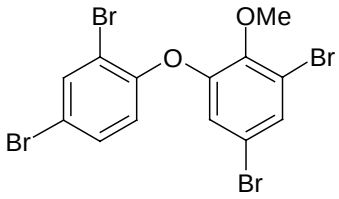

Fig. 3: Molecular formula of 4,6-dibromo-2-(2´,4´-dibromo)phenoxyanisole (BC-2)

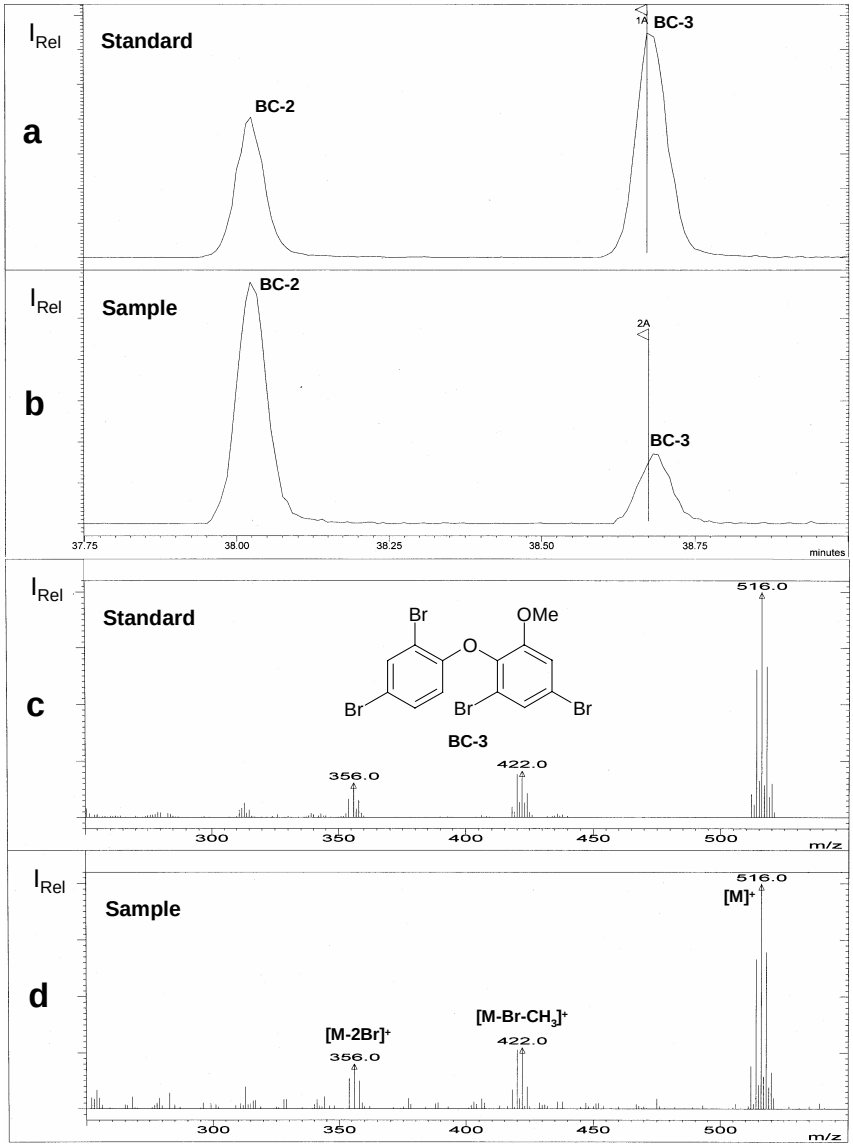

Fig. 1: GC/EI-MS chromatograms (m/z 514+516+518 extracted from the TIC) of a BC-2 and BC-3-standard (a) as well as an extract of the blubber of a bottlenose dolphin *(T. truncatus)* from Australia (b) and the respective EI mass spectra obtained at the retention time of BC-3 (c, d).

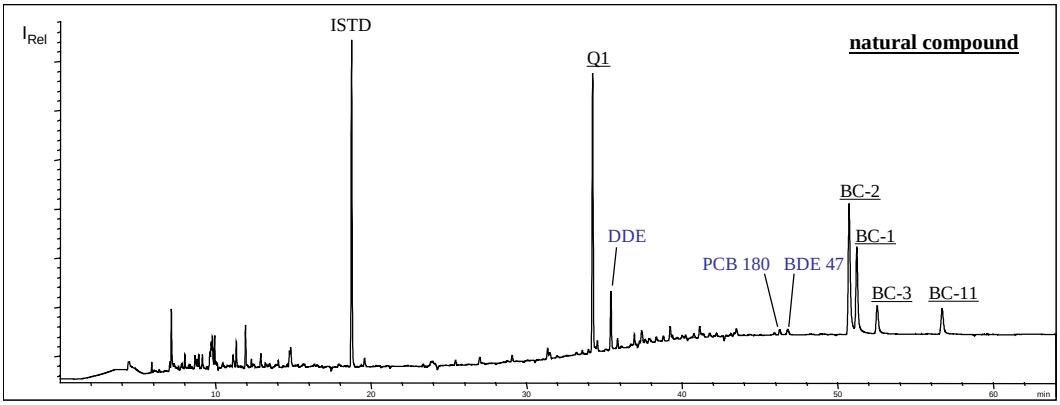

Fig. 4: GC/ECD-chromatogram of the blubber extract of a bottlenose dolphin *(T. truncatus)* from North-eastern Australia. HNPs (Q1, BC-2, BC-1, BC-3, BC-11) were more abundant than anthropogenic compounds (p,p'-DDE, PCB and BDE congeners).

Table 1: Concentrations [µg/kg lipids] of BC-3, p,p´-DDE, and PCB 153 as well as ratios of BC-3 to BC-2 in marine biota

Species	Origin	n	BC-3	BC-3/BC-2	p,p´-DDE	PCB 153
Common dolphin *(D. delphis)*	Australia	1	980	0.22	590	310
Bottlenose Dolphin *(T. truncatus)*	Australia	1	1910	0.17	1980	1060
Melonhead whale *(P. electra)*	Australia	1	790	0.65	610	120
Pygmy sperm whale *(K. breviceps)*	Australia	1	540	0.23	1120	100
Humpback dolphin *(S. chinensis)*	Australia	1	980	0.54	960	1530
Crocodile eggs	Australia	2	200-240	~3.5**	260-1370	n.d.
Shark liver oil (commercial)	New Zealand	1	4	~2.3	174	
Mussel tissue	Mexico	1	*	~0.3	*	*
Pilot whale	Faeore Islands	1	*	~12	*	*
Monk seal *(M. monachus)*	West Africa	5	≤30***	~3.2**	≤130***	≤160***
Weddell seal *(L. weddelli)*	Antarctica	8	≤3***	~4.3**		

* only qualitative determinations were carried out. ** averaged over all samples
*** estimated from earlier data [1]

In accordance with previous estimations, the highest concentrations of BC-3 were found in marine samples from Australia [1,5]. This suggests that a huge population of a natural producer of BC-3 should be located there. As a possible source of BC-3, sponges (*Dysidea sp.*) were described previously in the literature [12]. However, BC-3 was not identified in the Australian sponges described as the producer of BC-2 (see above). The varying concentrations and ratio between BC-3 and BC-2 (Table 1) may be explained due to the different ages, feeding behavior and distribution of the investigated species, but might also point towards different natural producers found in different habitats. In the samples of the Australian marine mammals analysed, the concentrations of BC-3 were equal or higher than the concentrations of the anthropogenic halogenated compounds, i.e. p,p'-DDE (exception: pygmy sperm whale) and PCB 153 (exception: humpback dolphin). The highest concentration of BC-3 determined in this study was ~2 mg/kg fat (Table 1). In samples from other continents BC-3 played only a minor role (Table 1). Unfortunately, no toxicologic data exist for BC-3 and other halogenated natural products. This is deplorable in view of the high concentrations these lipophilic compounds can reach in higher organisms of the marine food web.

References

1 Vetter W., Stoll E., Garson M. J., Fahey S. J., Gaus C., Müller J. F. (2002) Environ. Toxicol. Chem. 21, 2014.

2 Tittlemier S. A., Simon M., Jarman W. M., Elliott J. E., Norstrom R. J. (1999) Environ. Sci. Techn. 33, 26.

3 Wu J., Vetter W., Gribble G. W., Schneekloth J. S., Blank D.H, Görls H. (2002) Angew. Chem. Int. Ed. 41, 1740.

4 Gribble G. W., Blank D. H., Jasinski J. P. (1999) Chem. Commun. 21, 2195.

5 Vetter W., Scholz E., Gaus C., Müller J. F., Haynes D. (2001) Arch. Environ. Contam. Toxicol. 41, 221.

6 Vetter W. (2001) Anal. Chem. 73, 4951.

7 Marsh G., Stenutz R., Bergman Å. (2003) Eur. J. Org. Chem. 2003, 2566.

8 Cameron G. M., Stapleton B. L., Simonsen S. M., Brecknell D. J., Garson M. J. (2000) Tetrahedron 56, 5247.

9 Asplund L., Athanasiadou M., Sjödin A., Bergman Å., Börjeson H. (1999) Ambio 28, 67.

10 Vetter W., Wu J. (2003) Chemosphere 53, 423.

11 Sellström U. (1999) Determination of some polybrominated flame retardants in biota, sediment and sewage sludge (Doctoral Thesis), University of Stockholm Sweden.

12 Anjaneyulu V., Rao K. Nageswara, Radhika P., Muralikrishna M., Connolly J. D. (1996) Indian J. Chem. B 35, 89.