Journal of Chromatography A, 1217 (2010) 2050–2055

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Enantioseparation and absolute configuration of the atropisomers of a naturally produced hexahalogenated 1,1′-dimethyl-2,2′-bipyrrole

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ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 21 January 2010
Accepted 28 January 2010
Available online 4 February 2010

Keywords:
Halogenated dimethyl bipyrrole
Halogenated natural products
Enantiomer separation
Enantiomer fractions
Marine mammals

ABSTRACT

Hexahalogenated 1,1′-dimethyl-2,2′-bipyrroles (HDBPs) are a group of marine halogenated natural products (HNPs) that have been detected in environmental samples from all over the world. The most frequently described congener is the 5,5′-dichloro-1,1′-dimethyl-3,3′,4,4′-tetrabromo-2,2′-bipyrrole (DBP-Br_4Cl_2, BC-10). This compound is axially chiral, by virtue of hindered rotation about the interannular pyrrole–pyrrole bond forming stable atropisomers. This effect was proven by the separation of synthesized racemic DBP-Br_4Cl_2 by enantioselective high performance liquid chromatography (HPLC). Pure enantiomers were isolated using enantioselective HPLC. Crystallization led to white crystals studied by X-ray analyses to determine the absolute configuration. Subsequent polarimetric measurements verified the first eluting enantiomer on HPLC as R_a-(+)-DBP-Br_4Cl_2 and the second as S_a-(−)-DBP-Br_4Cl_2. We also investigated the gas chromatography (GC) enantioseparation of DBP-Br_4Cl_2. However, too high temperatures in the injector port led to partial racemization at temperatures >150 °C. GC coupled to mass spectrometry was used to study DBP-Br_4Cl_2 in marine mammal samples. All samples contained both atropisomers of the natural product DBP-Br_4Cl_2 with enrichment of the levo (−) enantiomer. This led to the assumption that both enantiomers of DBP-Br_4Cl_2 were already produced in the environment.
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1. Introduction

To date, more than 4500 halogenated natural products (HNPs) are known to be produced by marine sponges, algae and bacteria [1]. During the last decade, a few of these HNPs were also detected in marine biota from different sites throughout the world. Their presence in top predators of the food web that are not the natural producers shows that they are persistent and bioaccumulative. In some studies, the concentrations of these HNPs were comparable with those of anthropogenic persistent organic pollutants (POPs) [2,3]. Many HNPs are either exclusively brominated or chlorinated, but several mixed halogenated compounds have also been identified [4]. One group of such mixed halogenated naturally produced compounds are the hexahalogenated 1,1′-dimethyl-2,2′-bipyrroles (HDBPs) [5–7]. The most relevant HDBP congener is the 5,5′-dichloro-1,1′-dimethyl-3,3′,4,4′-tetrabromo-2,2′-bipyrrole (DBP-Br_4Cl_2, BC-10) (Fig. 1a). Although natural producers of HDBPs have not been identified, their assignment to natural sources is widely accepted owing to the following facts: (i) HDBPs were detected and widely distributed in non-industrial areas, which does not match the behavior of anthropogenic POPs with similar physical–chemical properties; (ii) the mixed halogenation pattern is not typical for anthropogenic compounds but is frequently found for marine natural products [4,8]; (iii) radiocarbon measurements allowed for the identification of ^{14}C in DBP-Br_4Cl_2 isolated from the lipids of marine mammals [9]; and (iv) the structurally related hexabromo-2,2′-bipyrrole (Fig. 1b) is known to be produced by the marine bacterium *Chromobacterium sp.* [10]. HDBPs were detected in various environmental samples including eggs of seabirds from Canadian Pacific and Atlantic coastal areas [5–7]. The concentrations in samples from the northern Pacific Ocean were higher compared to those from the Atlantic Ocean. Although the highest amounts were described in the Northern Hemisphere, HDBPs were also detected in samples from Japan and Australia [11,12].

Interestingly, DBP-Br_4Cl_2 is axially chiral due to its non-symmetric substituent pattern on both pyrrole units (Fig. 1a). When this feature is linked with voluminous substituents in *ortho*-position, the rotation about the interannular C–C bond is hindered. This leads to the formation of stable atropisomers (stereoisomers

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doi:10.1016/j.chroma.2010.01.083

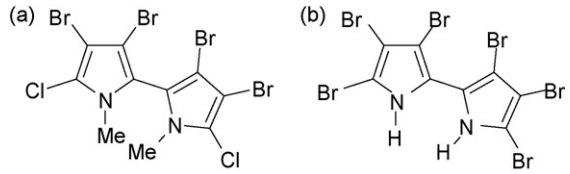

Fig. 1. Structure of (a) 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (DBP-Br_4Cl_2, BC-10) and (b) 3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole.

of axially chiral compounds). The enantioseparation of chiral compounds does not only allow distinguishing the pure enantiomers from the racemate but it also provides insights into their environmental fate. The enantiomer of a chiral molecule may interact differently with other chiral molecules and thus express different bioactivity.

Thus, enantioselective GC analysis involving chiral stationary phases (CSPs) is an interesting tool increasingly applied in environmental sciences. It allows researchers to determine the enantiomeric distribution of chiral compounds and helps to visualize changes in the enantiomeric composition as a consequence of metabolism [13,14]. However, the enantioseparation of polybrominated compounds is difficult due to their high molecular weights. It requires comparable high GC elution temperatures that are detrimental to the enantiomeric resolution. Nevertheless, the enantioselective analysis of the anthropogenic flame retardants polybrominated biphenyls (PBBs) and 2,3-dibromopropyl-2,4,6-tribromophenyl ether (DPTE) in environmental samples was recently described [15,16]. Both, PBBs and DPTE were applied as racemates but their composition was altered in biological samples due to enantioselective metabolism [15,16].

In case of the natural product DBP-Br_4Cl_2 it was not clear if it does form stable atropisomers. There is also the associated question of whether or not the compound is formed in nature as pure enantiomers or as a mixture of both enantiomers. In this study we searched for answers to these questions by attempting to separate the enantiomers from a synthesized racemic standard by enantioselective high performance liquid chromatography (HPLC) and enantioselective gas chromatography (GC).

2. Materials and methods

2.1. Chemicals and samples

Isooctane (SupraSolv, for gas chromatography) was from Merck (Darmstadt, Germany). *n*-Hexane and 2-propanol (both HPLC grade) were from Fisher Scientific (Leicestershire, UK). Racemic DBP-Br_4Cl_2 was synthesized as described by Gribble et al. [17]. DBP-Br_4Cl_2 standard solutions, 10 ng μL^{-1}, 1 ng μL^{-1}, 0.5 ng μL^{-1} were prepared from isooctane stock solutions (5.3 mg 50 mL^{-1}) by dilutions with an appropriate volume of isooctane. Solutions for the polarimetric determinations were prepared separately (Section 2.3).

Processed blubber sample extracts of a pygmy sperm whale (*Kogia breviceps*, newborn, female) and a melon-headed whale (*Peponocephala electra*, adult, female), both from Queensland, Australia and a pygmy sperm whale (*Kogia breviceps,* adult, male) from Moreton Bay, Brisbane, Australia were available from previous studies [12]. Blubber of a sperm whale (*Physeter macrocephalus*) was from an individual stranded in the 1990s at the North Sea coast. Further information was not available.

2.2. Enantioselective high performance liquid chromatography (HPLC)

HPLC analyses were performed with a Waters HPLC system consisting of a 616 pump, a 600 s controller, a 717 plus auto sampler and a 969 photodiode array detector (Eschborn, Germany). Atropisomers of DBP-Br_4Cl_2 were separated on a NUCLEOCEL DELTA S column (250 mm length × 4.6 mm internal diameter, particle size 5 μm) from Macherey-Nagel (Düren, Germany). The stationary phase of this column is composed of a cellulose-modified (cellulose-*tris*(3,5-dimethylphenyl)-carbamate) silica phase according to Okamoto et al. [18]. Enantioseparations were carried out in the normal phase mode (NP-HPLC) with a mixture of *n*-hexane and 2-propanol of 95:5 (v/v) as the mobile phase. The mobile phase was transported with an isocratic flow rate of 0.3 mL min^{-1}, held at ambient temperature. The detection wavelengths were set at 211 nm and 254 nm. Approximately 200 μg (in 50–200 μL) of racemic DBP-Br_4Cl_2 were repeatedly injected without overloading the column. In total ∼5 mg (5 mL with a concentration of 1.03 μg μL^{-1}) of DBP-Br_4Cl_2 could be fractionated. Pressure and temperature varied in the laboratory so that preparation of neat enantiomers was better achieved by manual fractioning according to the UV-detector response. Three fractions were collected manually. More details are shown in Section 3.1. The corresponding fractions with the separated enantiomers were combined and stored in the fridge for crystallization and subsequent analysis.

2.3. Polarimetric determination of the DBP-Br_4Cl_2 enantiomers

Analyses were carried out with a Perkin Elmer polarimeter, Model 341 (Rodgau, Germany). Polarimetric determination was performed with 250 μL cells (cell length 0.1 dm) at 589 nm and 23 °C. Crystals of Enantiomer 1 (∼300 μg mL^{-1}) and Enantiomer 2 (∼1.4 mg mL^{-1}) were dissolved in isooctane.

2.4. X-ray analysis

A colourless crystal prism (0.7 mm × 0.2 mm × 0.15 mm) of (−)-DBP-Br_4Cl_2 was measured with MoK_α = 0.71073 Å wavelength on a Nicolet P3 diffractometer in a Wyckoff-scan modus to a maximum scattering angle of 2Theta = 56°. 3650 unique reflections were collected of which 2941 were observed with respect to the criteria $I > 2\text{sigma}(I)$. The compound crystallizes in the monoclinic acentric space group P 2_1 with one independent molecule in the asymmetric unit. The unit cell dimensions are: $a = 8.9716(12)$ Å, $b = 8.4828(9)$ Å, $c = 9.9938(10)$ Å, $\beta = 93.597(9)°$ and $V = 759.07(15)$ Å^3.The structure was refined by the full-matrix least-squares method against F^2. The refinement was converged with following *R*-values: $R(F) = 0.0466$ and $R(F^2) = 0.0900$ for data with $I > 2\text{sigma}(I)$ and $R(F) = 0.0673$ and $R(F^2) = 0.0966$ for all data. H atoms were located on difference Fourier map, but refined with fixed individual displacement parameters, using a riding model with a d(C–H) distance of 0.96 Å. The methyl groups were allowed to rotate but not to tip. The X-ray structure of the title compound is showing an axial chirality characterized by atropisomerism. The absolute configuration is S_a or M indicated by the absolute structure determination by X-ray data characterized by the Flack parameter of $x = 0.035(17)$. The steric hindrance of the pyrrole substituents forced the pyrrole ring systems out of their co-planarity characterized by the torsion angle N1-C4-C5-N2 of 112.7(8)°. The dihedral angle between the pyrrole planes is 66.1(3)°. A weak intermolecular hydrogen bond contact is evident between the methyl group C9 as donor and Br4 as acceptor. The H9C. . .Br4 distance is 2.96 Å and the angle C9-H9C. . .Br4 is 127°. We observe also an intermolecular Br. . .Br contact between Br2 and Br3 with 3.58(1) Å which is remarkably short.

2.5. Enantioselective gas chromatography in combination with electron capture detection (GC/ECD)

Both racemic DBP-Br_4Cl_2 as well as separated atropisomers of DBP-Br_4Cl_2 were analyzed on a Hewlett-Packard 5890 Series

II plus GC/ECD. One microliter of sample solution was injected splitless with a GC PAL autosampler (CTC Analytics, Zwingen, Switzerland) onto a CP-Chirasil-DEX CB (β-PMCD) column (23.5 m, 15 m and 8.5 m, respectively, 0.25 mm i.d.) from Varian Chrompack (Middelburg, The Netherlands). The stationary phase is composed of *heptakis*(2,3,6-tri-*O*-methyl)-*β*-cyclodextrin chemically bonded to the polysiloxane backbone according to Schurig et al. [19]. Helium and nitrogen (both, 99.9990% purity, Sauerstoffwerke, Friedrichshafen, Germany) were used as the carrier gas (constant column head pressure of 1.25 bar) and as the make up gas, respectively. The ECD temperature was set to 270 °C. The GC oven program for the analysis of racemic DBP-Br_4Cl_2 standard started at 80 °C (1 min) and was then increased at 5 °C/min to 120 °C (640 min) and finally with 5 °C/min to 200 °C (10 min). The total run time was 675 min. Injector temperature was set to 250 °C. During analysis of single enantiomers the GC oven was programmed as follows: 80 °C (hold time 1 min), then at 5 °C/min to 120 °C (hold time 150 min) and finally at 5 °C/min to 200 °C (hold time 10 min) with a total run time of 185 min. Injector temperatures were set to 140 °C to 220 °C with steps of 10 °C.

The chiral resolution of the peaks was calculated as follows according to the International Union of Pure and Applied Chemistry (IUPAC): $R_s = 2\,\Delta t/\sum w_{1/2}$, Δt = retention time difference, $\sum w_{1/2}$ = sum of the peak widths at the baseline.

2.6. Enantioselective gas chromatography in combination with mass spectrometry (GC/EI-MS)

Sample analyses were carried out with an HP GCD Plus System (Hewlett-Packard, Waldbronn, Germany). Helium (99.9990% purity, Sauerstoffwerke, Friedrichshafen, Germany) was used as the carrier gas with a constant column head pressure of 0.02 bar. The electron energy was set to 70 eV. The injector temperature was set to 170 °C, in order to accept only lowest possible contamination of the injector port. Two microliters were injected in splitless mode by means of an HP 6890 autosampler (Hewlett-Packard). The GC oven temperature program started for 1 min at 60 °C, then was increased with 5 °C/min to 120 °C (hold time 300 min) and finally at 5 °C/min to 200 °C (hold time 11 min). Additionally, fast oven programs and blanks between the runs were used to avoid interference from memory peaks. Measurements were carried out using GC/EI-MS in the SIM mode using specific ion traces of the molecular ion ($[M]^+$) of DBP-Br_4Cl_2 throughout the run: *m/z* 538.7 (blank), *m/z* 539.7 (monoisotopic peak, $^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_4{}^{35}Cl_2$), *m/z* 541.7 ($^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_3{}^{81}Br^{35}Cl_2$, $^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_4{}^{35}Cl^{37}Cl$) and *m/z* 543.7 (most abundant isotopic peak, $^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_2{}^{81}Br_2{}^{35}Cl_2$, $^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_4{}^{37}Cl_2$, $^{12}C_{10}{}^{1}H_6{}^{14}N_2{}^{79}Br_3{}^{81}Br^{35}Cl^{37}Cl$).

Chromatograms were smoothed using a Savitzky-Golay calculation [20]. Enantiomer fractions were calculated from raw data as follows: $EF_{(\pm)}$ $(\pm 0.02) = A_+/A_+ + A_-$ with A being the area and the indices (+,−) are referring to the dextro (+) and levo (−) enantiomers, respectively.

3. Results and discussion

3.1. Preparation of stable atropisomers with enantioselective HPLC

In order to determine whether DBP-Br_4Cl_2 exists as stable atropisomers at ambient or even elevated temperatures, we initially chose to use enantioselective HPLC. A NUCLEOCEL DELTA S column was selected because it recently enabled the enantioseparation of DPTE [16]. This column also provided a full separation of the atropisomers of DBP-Br_4Cl_2 in combination with a slightly more polar eluent of *n*-hexane and 2-propanol than used for DPTE (Fig. 2).

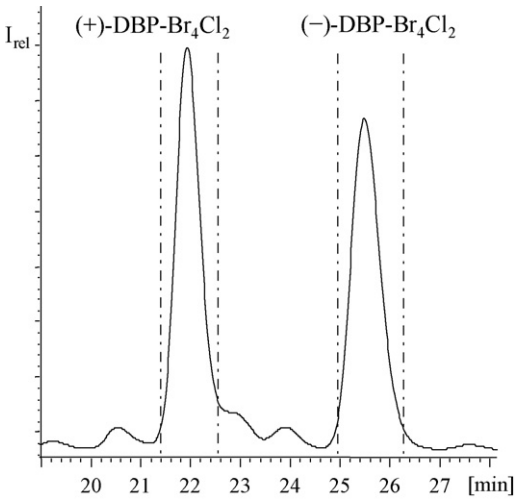

Fig. 2. HPLC/UV chromatogram (NUCLEOCEL DELTA S) of racemic DBP-Br_4Cl_2 and intersections of the manual peak collection. The fraction between Peak 1 and Peak 2 contained low amounts of both enantiomers.

Injections of up to 200 μg of racemic DBP-Br_4Cl_2 could be handled without overloading the column. Peaks were collected separately in two vials and the subsequent analysis of aliquots of the isolated enantiomers proved the compounds to be enantiopure. The solutions of the enantiopure DBP-Br_4Cl_2 enantiomers were stored in the fridge until white (monoclinic) crystals precipitated on the vial walls after several days.

X-ray studies of single crystals of the pure enantiomers showed the absolute configuration of the atropisomer eluted second from the chiral HPLC column to be S_a-DBP-Br_4Cl_2 (Fig. 3), with S denoting the sinister conformation and the subscript (a) referring to axial chirality. Complementary to this, the first eluted enantiomer from the HPLC was R_a-DBP-Br_4Cl_2. The dihedral angle between the pyrrole planes in the solid state is 66.1(3)° (Fig. 3). Polarimetric analysis of solutions of S_a-DBP-Br_4Cl_2 in isooctane showed levorotation (−). Likewise, the other enantiomer was found to be R_a-(+)-DBP-Br_4Cl_2. However, enantiomer concentrations in both solutions were too low for an exact determination of the $[\alpha]_D$-value which was in the range $\sim\pm 55 \pm 20$. The racemate of DBP-Br_4Cl_2 was in-between, as expected.

3.2. Enantioselective separation of DBP-Br_4Cl_2 atropisomers by GC/ECD

Different CSPs were tested but the best gas chromatographic resolution of racemic DBP-Br_4Cl_2 was obtained on *β*-PMCD. Initial separations on *β*-PMCD started isothermally at 150 °C and provided a noticeable separation of the DBP-Br_4Cl_2 enantiomers. This separation confirmed that the energy barrier is high enough to prevent full rotation about the pyrrole–pyrrole bond under typical GC conditions as it was also described for PCB 132 [21]. Due to incomplete resolution, we lowered the isothermal temperature gradually by 10 °C to 120 °C. The best resolution of DBP-Br_4Cl_2 atropisomers was obtained at this temperature. However, The GC run time was very high (~675 min) and the peaks were broad. Since these conditions could not be used in the analyses of environmental samples we split the column (originally 23.5 m) into pieces of 8.5 m and 15 m length, respectively. This strategy has been described before in the literature [22,23]. Both columns provided a good enantioseparation. Baseline enantioseparation on the shorter column (R_s = 0.98) was achieved within approximately two hours and the peaks were comparably sharp.

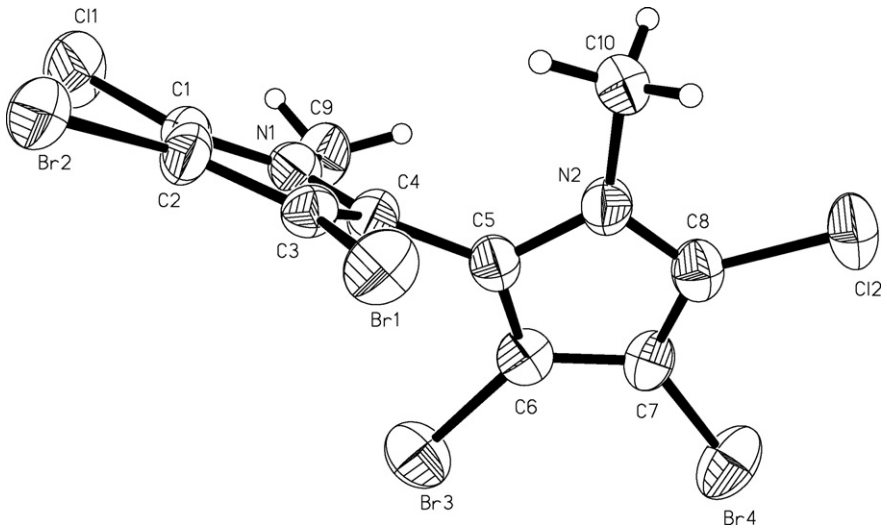

Fig. 3. ORTEP-view of the X-Ray structure of S_a-(−)-DBP-Br_4Cl_2. The dihedral angle between the pyrrole ring planes is 66.1(3)°.

Injection of pure enantiomers resulted in reversed elution order compared to the chiral HPLC column with (−)-DBP-Br_4Cl_2 eluting first (Fig. 4a). Peak reversals by switching from enantioselective HPLC to GC were described earlier for α-HCH and some other organochlorine compounds [13]. The GC/ECD chromatogram of pure (+)-DBP-Br_4Cl_2 obtained by HPLC (Section 3.1) showed traces of its enantiomer and the same was found for (−)-DBP-Br_4Cl_2 (Fig. 4b). This was in contrast to our HPLC and X-ray measurements and therefore we assume that the relatively high injector temperature initiated a partial racemization of the pure enantiomers due to the temperature-dependent rotation about the interannular C–C bond [24]. With the goal of analyzing environmental samples in mind, we were interested in using the highest possible injector temperature in order to exclude discrimination of high-boiling analytes in the injector port. Thus, the GC injector temperature was lowered stepwise from 220 °C to 140 °C (Table 1). No racemization was observed at 150 °C or lower. Between 160 °C and 220 °C the percentage of the formed enantiomer increased from 1% (EF = 0.99) to 9% (EF = 0.92) (Table 1).

3.3. Enantioselective analysis of DBP-Br_4Cl_2 in the blubber of marine mammals

When the β-PMCD column (8.5 m) was installed in the GC/MS system, the racemic DBP-Br_4Cl_2 standard was better resolved than in the GC/ECD system (Fig. 5a). At an injector temperature of 170 °C, both (+)-DBP-Br_4Cl_2 and (−)-DBP-Br_4Cl_2 could be analyzed without significant racemization. For instance, racemization of pure (−)-DBP-Br_4Cl_2 was <2% and this injector temperature was chosen for injections of samples containing DBP-Br_4Cl_2. The samples were shown to contain both enantiomers (Fig. 5). The correct peak assignment on the short GC column was verified as follows: spiking of racemic DBP-Br_4Cl_2 standard into the sample produced non-split peaks at the expected retention times and the ratio of m/z 543.7 (quantification ion) to m/z 541.7 (verification ion) was correct for both peaks. Further, we recorded the retention times for other brominated compounds known to be present in the sample extracts on the β-PMCD column but none of them co-eluted with the DBP-Br_4Cl_2 enantiomers.

Table 1
Investigations on the partial racemization of DBP-Br_4Cl_2 by injection of the pure (+)-enantiomer at different temperatures into the hot gas chromatography injector.

Injector temperature [°C]	Amount of (−)-DBP-Br_4Cl_2 [%]	Enantiomer fraction ($EF_{(\pm)}$)
140	0	1.00
150	0.1	1.00
160	1.0	0.99
170	2.0	0.98
180	2.4	0.98
190	5.7	0.95
200	6.5	0.94
210	7.2	0.93
220	8.9	0.92

Both samples shown in Fig. 5 had an $EF_{(\pm)}$ = 0.33, and thus a significant enrichment of (−)-DBP-Br_4Cl_2 (Fig. 5b, c). Spiking of sample 2 with the racemate (see above) resulted in an $EF_{(\pm)}$ = 0.27 after subtraction of the added amounts, respectively. Two further samples were analyzed as well but the intensities of the targeted ions were too low for an exact determination of the EF. However, the pygmy sperm whale sample from Australia (newborn, female) and a sperm whale sample of an individual found stranded at the North Sea coast also contained both atropisomers with a clear dominance of (−)-DBP-Br_4Cl_2. Thus, all blubber samples analyzed were enriched in (−)-DBP-Br_4Cl_2 but the (+)-DBP-Br_4Cl_2 was present in all cases.

The detection of both DBP-Br_4Cl_2 atropisomers at temperatures >100 °C is clear support for the formation of stable atropisomers of DBP-Br_4Cl_2 in the environment. Rotational profiles of DBP-Br_4Cl_2 have not been established, but the heptachloro-1′-methyl-1,2′-bipyrrole Q1 was previously investigated by PM3 modelling [25]. It was found that surmounting the rotational barrier of Q1 required that the conformation of the nitrogens changed from planar to pyramidal (and back) [25]. This process demands a high input of energy that is unlikely to be available under environmental conditions. This fact indirectly supports the stability of the atropisomers of the structurally related DBP-Br_4Cl_2. Thus, partial racemization during accumulation in the food chain is very unrealistic. Hence, the presence of both enantiomers of DBP-Br_4Cl_2 in environmental samples indicates formation of both atropisomers in nature (either a non-racemic mixture or a racemic mixture followed by stereoselective transformation into a non-racemic mixture) as opposed to stereospecific synthesis of one enantiomer followed by transformation into its enantiomer.

Noteworthy, the formation of stable atropisomers requires bulky substituents in *ortho*-positions of the pyrrole–pyrrole bond. On DBP-Br_4Cl_2, these positions are substituted with bromine (C-3) and methyl (N-1), respectively. Substitution of the methyl groups with smaller hydrogens on both rings will significantly decrease the

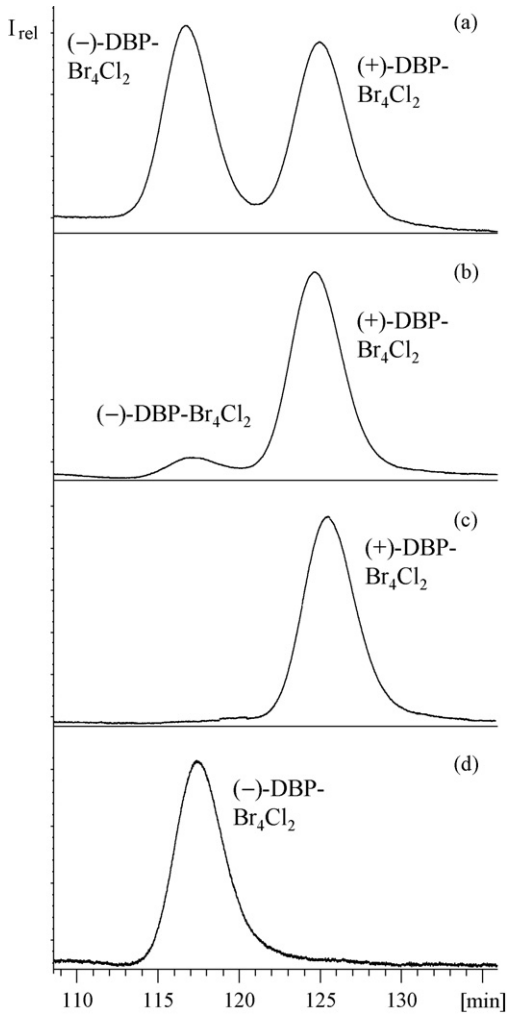

Fig. 4. GC/ECD chromatograms (β-PMCD, 8.5 m, 0.25 mm i.d.) of (a) the enantioseparation of racemic DBP-Br_4Cl_2 (oven temperature, 80 °C (1 min) to 120 °C (640 min) at 5 °C/min and finally to 200 °C (10 min) at 5 °C/min; carrier gas helium, constant column head pressure 1.25 bar; injector temperature 250 °C), (b) partial racemization of (+)-DBP-Br_4Cl_2 during injection at an injector port temperature of 220 °C (oven temperature, 80 °C (1 min) to 120 °C (150 min) at 5 °C/min and finally to 200 °C (10 min) at 5 °C/min; carrier gas helium, constant column head pressure 1.25 bar), and the single enantiomers (c) (+)-DBP-Br_4Cl_2 and (d) (−)-DBP-Br_4Cl_2 (oven temperature, 80 °C (1 min) to 120 °C (150 min) at 5 °C/min and finally to 200 °C (10 min) at 5 °C/min; carrier gas helium, constant column head pressure 1.25 bar; injector temperature 140 °C).

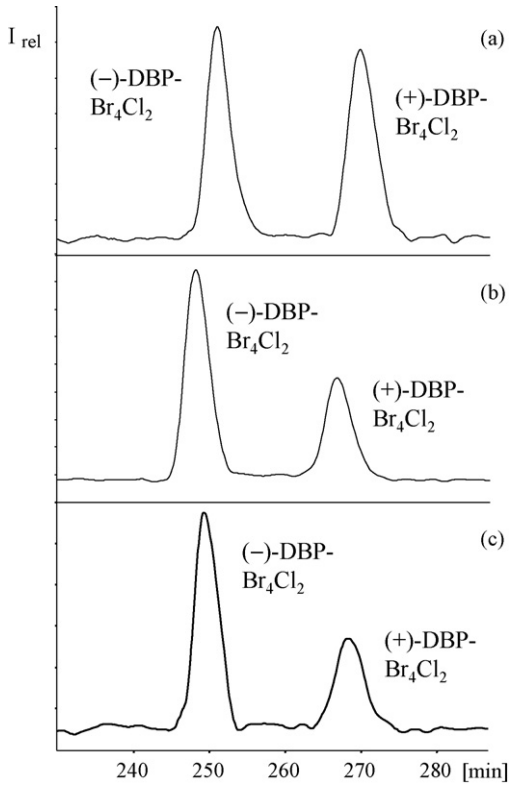

Fig. 5. GC/EI-MS chromatograms (β-PMCD, 8.5 m, 0.25 mm i.d.; m/z 543.7) of the enantioseparation of DBP-Br_4Cl_2 atropisomers, oven temperature, 60 °C (1 min) to 120 °C (300 min) at 5 °C/min and finally to 200 °C (11 min) at 5 °C/min; carrier gas helium, constant column head pressure 0.02 bar; injector temperature 170 °C. (a) Synthesized racemic DBP-Br_4Cl_2 standard solution, (b) melon-headed whale extract and (c) pygmy sperm whale extract.

stability of the atropisomers and it is likely that rotation about the interannular pyrrole–pyrrole bond will not be hindered under this condition [26]. For instance, tri-*ortho* substituted PBBs are rotationally hindered at elevated temperature, whereas the atropisomers of the di-*ortho* substituted PBBs could only be partly separated at 5–25 °C [26].

The natural producers of DBP-Br_4Cl_2 and other HDBPs are still unknown (see above), but the structurally related hexabromo-2,2′-bipyrrole (Fig. 1b) is known to be naturally produced by the marine bacterium *Chromobacterium sp.* [10]. Methylation reactions are common in nature, and it was suggested that DBP-Br_4Cl_2 may be the conversion product of the didesmethylated analogue [2]. In this case, the methyl group is added to the molecule after the formation of the hexahalogenated 2,2′-bipyrrole backbone. The introduction of the methyl group could be the decisive step towards the formation of stable atropisomers. If DBP-Br_4Cl_2 was formed in this manner, the presence of both atropisomers in environmental samples would suggest that this methylation step is not highly enantioselective. It could even be possible that the double methylation – if occurring on a low trophic level – leads to racemic DBP-Br_4Cl_2 and that the $EF_{(\pm)}$ observed in the top predators would then be the result of the gradually faster transformation of (+)-DBP-Br_4Cl_2. Such enantioselective transformation occurring within a food web has been documented for a wide range of chiral polyhalogenated analytes [13,14]. The scenario of methylation producing a racemic or non-racemic mixture of both enantiomers followed by enantioselective transformation in the environment is more realistic than formation of a single atropisomer followed by partial racemization due to the high energy barrier of interconversion. In light of this situation, it would not be required that the methylation reaction – i.e. the last step in the DBP-Br_4Cl_2 synthesis – is carried out by the same organism which produces the hexahalogenated 2,2′-bipyrrole backbone.

Acknowledgements

We are grateful to Prof. Dr. Uwe Beifuss and Mihaela-Anca Constantin (University of Hohenheim, Institute of Chemistry, Department of Bioorganic Chemistry) for the opportunity to perform polarimetric measurements.

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