STRUCTURE AND ORIGIN AND CONCENTRATIONS OF THE NATURAL HALOGENATED MONOTERPENE MHC-1 IN MARINE MAMMALS AND FISH

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Introduction

Some of the >4500[1] halogenated natural products (HNPs) behave in a manner similar to the anthropogenic POPs[2,3]. The bioaccumulating HNPs include two tetrabromophenoxyanisoles[4], two polyhalogenated hexahydroxanthene derivatives[5] and polyhalogenated 1,2´- and 2,2´-bipyrrole derivatives[6,7] and a dibromotrichloro-monoterpene initially named mixed-halogenated compound 1 (MHC-1)[8]. Of all key-HNPs reported with high concentration in marine biota, MHC-1 remained the only one whose exact structure remained unknown. The primary goal of this study was to elucidate the structure of this compound which has been detected in samples from several continents. GC/EI-HRMS analysis of MHC-1 had led to the elemental composition $C_{10}H_{13}Br_2Cl_3$ [8].

A search of the literature for structurally known isomers only pointed to four natural products isolated since the 1970s, whereas no synthetic lab-made compound with this elemental composition has been reported (Vetter et al. 2001). The most potential source is *Plocamium cartilagineum* because occurrence of the algae and detection of MHC-1 in marine biota correlated (**Figure 1**). Over 20 polyhalogenated monoterpenes (PHMTs) have been described in this seaweed but only one, MHC-1, was regularly detected at elevated concentrations in food and environmental samples[8]. We therefore attempted to isolate MHC-1 from *P. cartilagineum* from a sample from Helgoland (Germany).

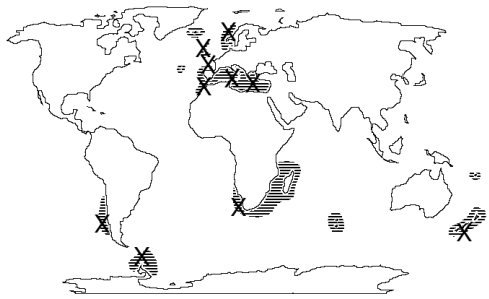

Figure 1: Global map with distribution of *P. cartilagineum* (shaded areas) and sites (marked with X) where samples were positively tested for MHC-1

Material and methods

Gas chromatography in combination with mass spectrometry (GC/MS) parameters

GC/MS analyses were performed with a Varian CP-3800/1200 system (Darmstadt, Germany) in combination with a 30 m x 0.25 mm i.d. x 0.25 µm CP-Sil 8MS column (Varian). He was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The GC oven temperature program started at 50 °C (2 min), ramped at 10 °C min^{-1} to 300 °C (18 min). Injections were performed in splitless mode (split opened after 2 min). In the electron capture negative ion (ECNI) mode, CH_4 (purity 99.995%, Air Liquide) was used as reagent gas at 8.1 Torr. The ion source temperature was set at 150 °C. A scan time of 0.5 s/cycle was used, and the SIM peak width was 0.5 u. For GC/ECNI-MS analysis in full scan mode, the detector voltage was set at 1200 V. In the SIM-mode we monitored *m/z* 35 and 37($[Cl]^-$), *m/z* 79 and 81 ($[Br]^-$), *m/z* 158, 160 ($[Br_2]^-$), $[HBr_2]^-$ (*m/z* 159, 161), $[BrCl]^-$ (*m/z* 114, 116) (Vetter, 2001). GC/EI-MS full scan measurements (*m/z* 50-500) were performed with the same instrument under the same conditions.

GC in combination with electron capture detection (GC/ECD) and flame ionization detection (GC/FID)

GC/ECD analyses were performed with systems recently described in detail[8,9]. GC/FID analyses were performed with a HP 5890 series II system equipped with a HP 7673 autosampler. The injector (splitless) and detector temperatures (FID) were 250 °C, respectively. A HP-5MS column (30 m x 0.25 mm i.d., 0.25 µm d_f; Agilent) was used with the following GC oven program: 100 °C (2 min), at 10 °C min^{-1} to 270 °C (15 min). He 5.0 was the carrier gas (head pressure 1.25 bar), whereas H_2 (5.0) and synthetic air were used as detector gasses.

RP-HPLC with diode array detector (DAD) parameters

A Waters (Eschborn, Germany) system consisted of a 717plus auto sampler, a 616 pump, a 600 S controller, and a 969 DAD (operated at 210 nm). The system was operated in the reversed phase mode (RP-HPLC) using a

C_{30} column (250 mm length, 4.6 mm i.d., 5 µm particle size; Trentec Analysentechnik, Gerlingen, Germany) and acetonitrile (HPLC gradient grade, Roth, Karlsruhe, Germany) as solvent at a flow rate of 0.5 mL min^{-1}.

Nuclear magnetic resonance spectroscopy (NMR) parameters

^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectra were recorded with a Varian Inova 300 MHz instrument. A 5 mm ATB probe was used. Chemical shifts were referenced to residual undeuterated $CDCl_3$ used as solvent.

^{13}C NMR (75 MHz, CHCl3, ppm): 135.7, 119.4, 71.2, 64.9, 51.5, 48.9, 42.4, 40.7, 39.4 and 27.7.

^{1}H NMR (300 MHz, CHCl3, ppm): 6.57 (d), 6.08 (d), 4.49 (dd), 3.96 (d), 3.70 (dd), 3.58 (d), 2.79 (ddd), 2.59 (ddd), 2.41 (d), 2.28 (d), and 1.29 (s).

Isolation of MHC-1 from *P. cartilagineum* from Helgoland/Germany

1.06 g air-dried material was collected by scuba diving in May 2007. The sample was cold-extracted with 2x 60 mL *n*-hexane. The combined extracts were condensed in a rotary evaporator to 2 mL. The condensed cold-extract was transferred to a 4 mL-vial and 2 mL H_2SO_4 was added. The vial was shaken and kept for 24 h at room temperature. The organic phase was separated, washed with distilled water, and condensed (re-extracts of the sulphuric acid phases with *n*-hexane were free of MHC-1). The concentrated organic phase containing MHC-1 was subjected to adsorption chromatography (3 g silica deactivated with 30% water, w/w, eluted with 60 mL *n*-hexane (Weichbrodt et al., 2000). The extract mainly contained MHC-1 and squalene as major compounds, according to GC/MS. The latter was separated twice by adsorption chromatography on activated silica (8 g silica, fractions of 48 mL, 50 mL *n*-hexane/ethyl acetate 9:1, v/v, and 50 mL *n*-hexane; second: after 198 mL *n*-hexane (five fractions), and twice 50 mL of *n*-hexane/ethyl acetate 9:1, v/v, fractions 6 and 7). Fraction 6 which contained MHC-1 was concentrated and carefully transferred into a solution of 4 mL acetonitrile. MHC-1 was isolated by RP-HPLC. A total of 82 subsequent injections of 50 µL in acetonitrile were distributed into seven fractions based on manual fractioning according to the UV detector response. The fractionation was started after ~5.8 min at which the baseline increased and 98% of MHC-1 eluted between 1.00 and 1.41 minutes after this starting point. This fraction was virtually free of impurities and was analyzed by NMR.

Calibration solution

The NMR-solution was transferred to a vial, evaporated to dryness and brought to 2 mL. Aliquots were subsequently diluted until the peak area matched a solution of 50 ng/µL of *trans*-chlordane as determined by GC/MS in the full scan mode and GC/FID. The mix and dilutions were repeatedly analyzed by GC/FID, GC/EI-MS, and GC/ECD in order to establish the response of MHC-1.

Sample clean-up for biological samples

The sample clean-up procedures for marine mammals[9] and fish[8] were reported elsewhere.

Results and discussion

Sample preparation and isolation of MHC-1

Diluted samples of the cold extract were directly analyzed by GC/ECD. These extracts showed a peak at the retention time of MHC-1. Subsequent GC/ECNI-MS-SIM analysis verified the presence of all low mass fragment ions (Br^-, $BrCl^-$, and Br_2^-) suitable for the identification of MHC-1 in environmental samples. Thus, it was concluded that MHC-1 was present in the seaweed samples. Experience with MHC-1 in food samples showed that the extracts could be treated with concentrated sulphuric acid without loss of the compound while non-halogenated compounds were decomposed. Therefore this technique was also applied to the seaweed sample (see Materials and methods). Aliquots were subjected to adsorption chromatography with activated silica and MHC-1 eluted in the more polar second fraction. The GC/ECNI-MS full scan spectrum of MHC-1 (**Figure 2a**) was virtually identically with the one previously obtained from fish samples[8].

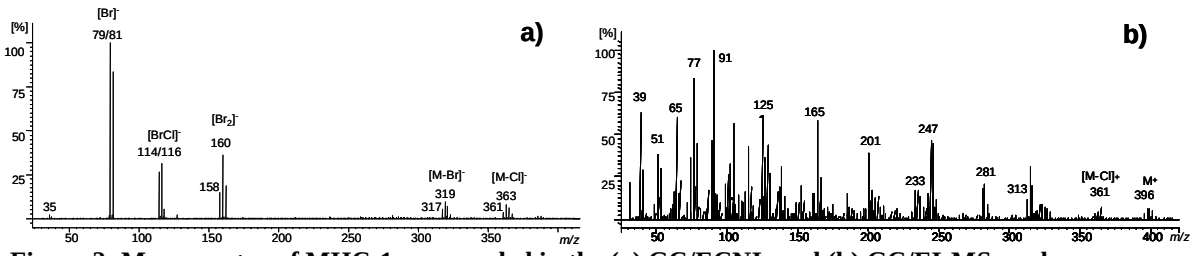

Figure 2: Mass spectra of MHC-1 as recorded in the (a) GC/ECNI- and (b) GC/EI-MS modes

In GC/EI-MS, the molecular ion was detected albeit at low relative abundance (**Figure 2b**). The most abundant fragment ions were again found in the low mass range, where the tropylium cation (*m/z* 91) and the chlorotropylium cation (*m/z* 125), phenyl (*m/z* 77) and pentadienyl (*m/z* 65) fragment ions as well as the corresponding products formed by the elimination of (neutral) acetylene (*m/z* 51 and *m/z* 39, respectively) dominated. These and all other fragment ions at higher mass were identical with those determined in GC/EI-MS of MHC-1[8]. These GC/MS measurements confirmed that the compound detected in the seaweed samples was identical with MHC-1 previously described in fish. However, the GC/EI-MS spectrum was not easy to interpret. For instance, *m/z* 313 was suspected to arise from elimination of a $CHCl_2$ radical[8]. However, this could not be verified by GC/ECNI-MS since the $[M-83]^-$ fragment ion and the corresponding fragment ion at *m/z* 83 (charge remaining on the proposed $CHCl_2$ group) were not found. These fragment ions are readily detected in the mass spectra of chlorobornanes bearing a $CHCl_2$ moiety[10].

Isolation and structure elucidation of MHC-1

The final purification steps included repeated adsorption chromatography on silica and RP-HPLC (see Materials and methods). The combined RP-HPLC fractions contained 1.9 mg of MHC-1 which was virtually free of impurities according to NMR. Both ^1H and ^{13}C NMR data (see experimental) were verified by two dimensional experiments (HSQC) analysis and fully matched the initial structural proof of the natural products chemists for ((*1R,2S,4R,5R,1´E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2´-chloroethenyl)-5-methylcyclohexane) (**Figure 3)**. The stereochemistry was determined by x-ray analysis[11]. Noteworthy, this compound does not contain a $CHCl_2$ moiety as indicated by the fragment ion at *m/z* 313 in the GC/EI-MS (see **Figure 2b**). It is evident that structural proof of halogenated monoterpenes can only be obtained by NMR spectroscopy.

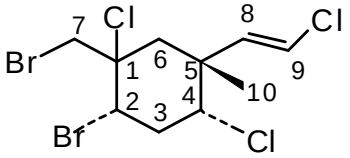

Figure 3: Structure of MHC-1

Calibration of MHC-1 and quantification of MHC-1 in environmental and food samples

The isolate from *P. cartilagineum* was used for preparation of a solution suitable for quantitative determination of MHC-1 in food and wildlife. Previous reports on MHC-1 were based on the GC/ECD response of the known C_{10}-cyclodiene chloropesticide *trans*-chlordane[8]. In this study, we recalibrated MHC-1 and *trans*-chlordane by GC/FID. GC/FID responses of comparable compounds are almost exclusively dependent on the carbon content. Both, the molecular weight (m.w.) and carbon content of MHC-1 (m.w. 399.38, carbon content 30.1%) and *trans*-chlordane (m.w. 409.78, carbon content 29.3%) agree very well. We then prepared a mixture of both compounds which resulted in the same peak area for both compounds. This was also found for GC/EI-MS in the full scan mode. This mix was diluted to different concentrations and analyzed by GC/ECD. The signal of MHC-1 was 2.4-fold lower than of *trans*-chlordane. Thus, the concentrations of MHC-1 reported previously on the basis of *trans*-chlordane underrated the relevance of MHC-1 by this factor. Corrected concentrations for samples positively tested on MHC-1 and new samples are summarized in **Table 1**. The MHC-1 concentrations in marine mammals also reached up to 0.14 mg/kg blubber (wet weight) in samples from the habitat of the seaweed. contained 1-2 ng/g whereas MHC-1 was not detected in blubber of dolphins from Australia (data not shown, detection limit, ~0.25 ng/g). It appears that MHC-1 is more relevant in the Northern than in the Southern hemisphere.

The highest concentrations were determined in salmon and pollack from aquaculture. Three individual fish had concentrations exceeding 1 ppm two of which contained more than 2 mg MHC-1 per kg lipids (**Table 1**). While it is plausible that farmed fish will contain higher concentrations of MHC-1 and other HNPs because the fish farms are located in direct proximity to the natural habitat of *P. cartilagineum* and other natural producers, the detection in cultivated freshwater fish – albeit on a low level – confirms that MHC-1 may also be present in

commercial fish feed[12]. The previous qualitative detection of MHC-1 in human milk[13] and air samples from Norway [14] indicate that the environmental behaviour of MHC-1 is similar to anthropogenic POPs.

Table 1: Concentration of MHC-1 (ng/g) in various environmental and food samples

Species	Origin (year)	(n)	MHC-1 [ng/g] [a,b]
Free living marine biota			
seal blubber (1991)	Greenland Sea, European Arctic	4	82 - 140
monk seal (*Monachus monachus*)	Mauretania, NW Africa (1997)	14	14 – 79
Weddell seal (*Leptonychotes weddelli*)	Weddel Sea Antarctica (1990)	1	2.4
saithe (*Pollachius virens*), free living	Norway (2003)	2	2.9 – 13 w.w.
Farmed fish			
pollack (*Pollachus pollachus*)	Denmark (2001)	1	2,260
salmon (*Salmon salary*)	Europe (2001, 2004, 2007)	34	2.4 – 2,250
sea bass (*L. lupus*) and gilt head bream (*S. aurata*)	Mediterranean Sea (2006-2007)	14	<2 – 125
mackerel (*Scomber scombrus*)	Unknown	16	10 – 65
other fish	Europe or unknown (1999-2006)	22	2.4 – 41

[a] lipid weight except where noted; w.w. = wet weight
[b] previous estimates of MHC-1 were corrected with the response factor determined in this study

Conclusions

In this study, we provided the structure of the halogenated monoterpene MHC-1 previously determined as a potential organic pollutant in marine mammals and fish. While other species are also producing MHC-1, the most potential source is *Plocamium cartilagineum* species, since this red seaweed is present at many European local shores and intertidal areas (**Figure 1**). The high concentrations determined in marine mammals and fish along with the biological activity of MHC-1 verify the conclusion that the ecological and potential ecotoxicity of MHC-1 but also of other HNPs needs to be studied in more detail.

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