

Biological Activity and Physicochemical Parameters of Marine Halogenated Natural Products 2,3,3',4,4',5,5'-Heptachloro-1'-Methyl-1,2'-Bipyrrole and 2,4,6-Tribromoanisole

W. Vetter,¹ M. E. Hahn,² G. Tomy,³ S. Ruppe,³ S. Vatter,⁴ N. Chahbane,⁵ D. Lenoir,⁵ K.-W. Schramm,⁵ G. Scherer⁶

¹ Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, D-70599 Stuttgart, Germany

² Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

³ Department of Fisheries and Oceans, Winnipeg, Manitoba, R3T 2N6, Canada

⁴ Department of Chemistry, Institute for Food Chemistry and Environmental Toxicology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany

⁵ GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, D-85764 Neuherberg, Germany

⁶ Department of Structure Elucidation NMR, Ciba Specialty Chemicals Inc., CH-4002 Basel, Switzerland

Received: 11 March 2004/Accepted: 28 May 2004

Abstract. Physicochemical parameters (vapor pressure, water solubility, Henry's law constant) and biological activities of two halogenated natural products frequently detected in marine samples and food were determined. Synthetic 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole (Q1) and 2,4,6-tribromoanisole (TBA) were available in pure form. The physicochemical parameters were in the range of anthropogenic chlorinated compounds of concern. The aqueous solubilities at 25°C ($S_{w,25}$) of Q1 and TBA were 4.6 µg/L and 12,200 µg/L, respectively, whereas subcooled liquid vapor pressures were 0.00168 Pa (Q1) and 0.06562 Pa (TBA) as measured by the gas chromatographic–retention time technique. Q1 was negative by established test systems for the determination of ethoxyresorufin-O-deethylase (EROD) induction and by sulforhodamine B assay. EROD induction potency was at least 10^{-7} times lower than that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). At a relatively high concentration (20 µM), Q1 inhibited specific binding of 2 nM [³H]TCDD to the *in vitro*-expressed human aryl hydrocarbon receptor (AHR) by 18%; lower concentrations showed no effect. Molecular modeling showed that Q1 is nonplanar, consistent with its relatively modest affinity as an AHR ligand. When tested for cell-growth inhibitory/cytocidal activity in human tumor cells, Q1 was only marginally, if at all, active with an IC_{50} value >50 µM compared with five to ten times lower IC_{50} values for potent cytotoxins tested in the test system used. Furthermore, standard pesticide tests on insecticidal, herbicidal, and fungicidal activity did not provide any significant activity at highest concentrations. For TBA, the results in all tests were comparable with Q1. The SRB assay was also applied to the halogenated natural product 4,6-dibromo-2-(2',4'-dibromo)phenoxyanisole, but no toxic response was

found. Although it was apparent that Q1 and TBA had been proven to have relatively low biological activity in all tests performed, further research is necessary to clarify whether metabolites of the compounds eventually may possess a risk to humans or other living organisms. Nevertheless, the role of Q1 in nature remains uncertain.

A number of halogenated organic compounds, such as polychloropesticides (dichlorodiphenyltrichloroethane [DDT], chlordane, toxaphene, lindane, and others); polyhalogenated industrial chemicals (polychlorinated biphenyls [PCBs]; brominated diphenyl ethers and others); and polychlorinated dibenzo-*p*-dioxins and dibenzo-*p*-dioxin-furans, are some of the most hazardous environmental contaminants of our times. Several toxic effects to a broad scope of organisms, ranging from insecticidal activity to estrogenic effects in mammals and birds, have been reported. Most of these persistent anthropogenic organohalogen compounds have been banned for this reason. Despite recent efforts with “classic” contaminants (DDT and PCBs among others), new emerging organohalogen compounds of diverse structure are currently under discussion with respect to negative impacts on living organisms (Paasivirta 2000). Most of these halo-organics are persistent and bioaccumulative and might thus be a long-term problem.

It has long been thought that the adverse properties discussed so far are exclusively valid for man-made substances. Thousands of naturally produced organohalogen compounds are known (Faulkner 1980; Gribble 1992, 1999, 2003); these halogenated natural products were thought not to be persistent. Recently, however, halogenated natural products have been detected at high concentrations in marine biota (Asplund *et al.* 1999; Tittlemier *et al.* 1999; Vetter *et al.* 2000, 2001a, 2002), and structural similarities between hazardous anthropogenic pollutants and these natural products have been

demonstrated (Tittlemier *et al.* 1999; Vetter *et al.* 2001a, 2003). One of the marine halogenated natural products of concern, Q1, has been detected at high concentrations (up to 14 ppm [Vetter *et al.* 2003]) in a wide range of biological samples (Vetter *et al.* 2000, 2001b, 2003); this contamination most likely occurred from uptake by way of food. Synthesis and structure elucidation demonstrated that the structure of Q1 is 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole (Wu *et al.* 2002). Recent evaluation clarified that Q1 is identical with a previously unknown compound detected in biological samples for 20 years (Ballschmiter and Zell 1981; Van den Brink 1997; Weber and Goerke 1996). Initial measurements indicated a bioaccumulative behavior of Q1 in some species (Vetter 2000). In addition, the logarithmic octanol-water partition coefficient ($\log K_{ow}$) was estimated from models and experimentally estimated by gas chromatography (GC) to be 5.9 to 6.4 (Hackenberg *et al.* 2003; Vetter 2000). These observations in environmental samples, along with initial determination of properties, pointed toward Q1 being a compound that may be a representative of both the persistent organic pollutants and the persistent bioaccumulative and toxic compounds concepts.

In this article, we present a number of physicochemical, biological, and toxicological properties of synthesized Q1. Some investigations were also accompanied by evaluations of 2,4,6-tribromoanisole (TBA), the residues of which have been ascribed to both natural and anthropogenic sources as well as the halogenated natural product 4,6-dibromo-2-(2',4'-dibromo)phenoxyanisole (BC-2). The structural similarities of the halogenated natural products Q1, TBA, and BC-2 with anthropogenic contaminants such as PCB 149, pentachlorophenol, and BDE 47 are apparent (Fig. 1).

Materials and Methods

Samples

Q1 and BC-2 were synthesized as recently reported (Vetter and Wu 2003; Wu *et al.* 2002), and TBA was ordered from Aldrich (Taufkirchen, Germany). Melting points (mean of reported ranges) of TBA (87°C to 89°C) and Q1 (154°C to 155.5°C) were used as reported in the literature (Aldrich catalogue 2003; Wu *et al.* 2002). Solid or stock solutions were shipped to different institutes for investigation.

Physicochemical Parameters

Aqueous solubility. Aqueous solubilities at 25°C ($S_{w,25}$) of Q1 and TBA were determined with the generator column technique (May *et al.* 1978; Tittlemier *et al.* 2002). Crystals of Q1 (5.1 mg) or TBA (5.6 mg) were dissolved in acetone and sorbed onto glass beads (60 to 80 mesh) that were prepurified by Soxhlet extraction (washed with deionized water, acetone, and 1:1 dichloromethane:hexane [vol/vol]). After careful rotary evaporation followed by blowing down in a gentle flow of nitrogen, the coated glass beads were packed into a stainless steel high-performance liquid chromatography column (250 × 3.9 mm). The column was connected to an HPLC pump (Varian 5000; Varian, Mississauga, Ontario, Canada) and flushed with acetone (5 minutes) and water (flow 5 ml/min). The column was

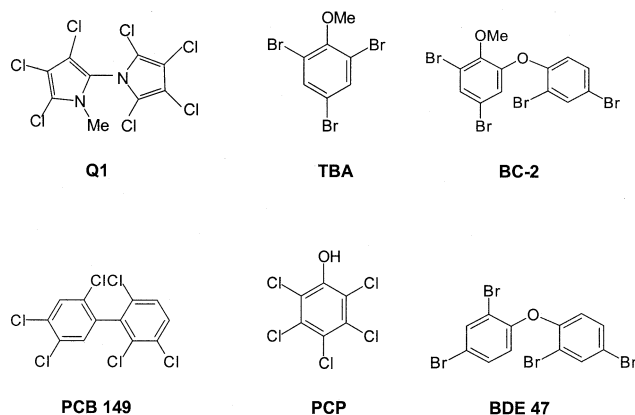


Fig. 1. Structures of the natural halogenated compounds Q1, TBA, and BC-2 (**upper panel**) as well as the structurally related anthropogenic pollutants PCB 149, PCP, and (BDE 47) (**lower panel**). BC-2 = 4,6-dibromo-2-(2',4'-dibromo)phenoxyanisole; BDE 47 = 2,2',4,4'-tetrabromodiphenyl ether; PCB = 2,2',3,4,4',5,6-hexachlorobiphenyl; PCP = pentachlorophenol; Q1 = 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole; TBA = 2,4,6-tribromoanisole

maintained at 25°C ± 1°C. The HPLC column was equilibrated for 30 minutes with water at a flow rate of 1 ml/min to create a saturated solution. Two fractions, 10 ml each, were collected in volumetric flasks. A third sample was collected at 60 minutes to check that the solutions were indeed saturated. Collected water samples were transferred in 250-ml flasks filled with 0.5 g sodium chloride, and the recovery standard PCB 30 was added. The aqueous layer was liquid-liquid-extracted with 1:1 dichloromethane:hexane (three times with 50 ml). Organic fractions were combined and dried with anhydrous Na₂SO₄, and the volume was reduced on a rotary evaporator, transferred into a scaled vial, and blown down with a gentle flow of nitrogen. Before analysis, a volume corrector (aldrin) was added to the samples. Analyses were carried out with a Varian Star 3400 GC/ECD system equipped with a nickel-63 electron-capture detector. Injector and detector temperatures were set at 220°C and 300°C, respectively. Analyses were performed with a 60 m × 0.25-mm inner diameter DB-5 column (film thickness 0.25 μm, J & W Scientific, Folsom, CA) using hydrogen as the mobile phase. The GC oven was programmed in as follows. After isothermal at 100°C, the temperature was increased at 15°C/min to 150°C, followed by a heating rate of 3°C/min to 265°C, which was held for 15 minutes. Samples were introduced with an 8100 autosampler (Varian).

Vapor pressures. Subcooled liquid vapor pressures (P^0_L) were determined using the GC-retention time (GC- t_R) technique described by Hinckley *et al.* (1990). Subcooled liquid vapor pressure, in contrast to vapor pressure, does not include the energy required to overcome attractive forces in the solid state and is the entity measured by the GC- t_R method. The P^0_L values at 25°C ($P^0_{L,25}$) of Q1 and TBA were determined with the GC- t_R method according to Tittlemier *et al.* (2002). The organochlorine pesticides mirex, *trans*-chlordane, and the PCB congeners (PCBs 4, 15, 118, 153, 187, 194, 202, 206, and 209) of known $P^0_{L,25}$ were used as standards (Tittlemier *et al.* 2002). Solutions of all compounds were made in iso-octane in the 50- to 100-pg/μl range and spiked with the reference compounds: *p,p'*-DDT for Q1 and PCB 30 for TBA. Retention times for all compounds were determined with a 10 m × 0.25-mm inner diameter DB-1 column with a film thickness of 0.25 μm (J & W Scientific). The column was installed in the GC system described above (Varian 3400) except for an ECD detector

temperature of 300°C. Single injections of 2 µl were made at 280°C in the split/splitless mode with a Hewlett-Packard 7673A autoinjector. Six isothermal runs were performed for each compound at oven temperatures ranging from 95°C to 165°C at 5°C intervals.

Aryl Hydrocarbon Receptor Binding Assay

2,3,7,8-Tetrachloro[1,6-³H]dibenzo-*p*-dioxin ([³H]TCDD, purity 99%, specific activity 34.7 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Unlabeled 2,3,7,8-tetrachlorodibenzofuran was obtained from Ultra Scientific (Kingston, RI); purity was >98%. Q1 and TBA (see above) were dissolved in dimethylsulfoxide (DMSO).

Human aryl hydrocarbon receptor (AHR) was expressed from cloned complementary DNA (Dolwick *et al.* 1993) by *in vitro* transcription and translation using the TnT Quick-Coupled Reticulocyte Lysate Systems reaction (Promega, Madison, WI) as described previously (Karchner *et al.* 2002). Competitive binding of Q1 and TBA to the human AHR was measured by velocity sedimentation on sucrose gradients in a vertical tube rotor (Tsui and Okey 1981) as modified for TnT-expressed proteins (Karchner *et al.* 1999). The TnT reactions were diluted (eight-fold) in buffer (25 mM MOPS buffer, pH 7.5 at 20°C, with 1 mM dithiothreitol, 1 mM EDTA, 5 mM EGTA, 0.02% Na₃N, 20 mM Na₂MoO₄, and 10% (vol/vol) glycerol); divided into 100-µl aliquots containing one quarter reaction mixture each; and incubated overnight at 4°C with [³H]TCDD (2 nM final concentration) ± Q1, TBA, or DMSO at the concentrations indicated in the figure legends. Q1 and TBA were dissolved in DMSO and added as 1% of the final volume. The nominal concentration of [³H]TCDD for each treatment was verified by sampling each tube for total radioactivity. Subsequently, the remaining portion of each incubation mixture (95 µl) was layered on gradients of 10% to 30% sucrose, spun for 140 min at 60,000 rpm at 4°C in a VTi 65.2 rotor, and fractionated (150 µl/fraction) before measurement of radioactivity in each fraction by liquid scintillation counting using a Beckman LS5000TD scintillation counter. Specific binding in the absence of competitor was calculated as the difference between total binding ([³H]TCDD bound to human AHR in the absence of a competitor) and nonspecific binding ([³H]TCDD in the same fractions when the TnT reaction contained an empty vector rather than the human AHR cDNA expression vector). Only the radioactivity in the 9–10-S peak (typically fractions approximately 10 to 20) was considered. [¹⁴C]catalase (11.3 S) and [¹⁴C] ovalbumin (3.6 S) were used as sedimentation markers and typically eluted in fractions 3 to 4 and 15 to 17, respectively.

In vitro EROD Induction Bioassay

The H4-IIIE rat hepatoma cells were cultured in a Dulbecco's MEM medium containing 1.0 g/L α-D-glucose, 3.7 g/L NaHCO₃, and 1.03 g/L N-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany) supplemented with fetal bovine serum, penicillin, and streptomycin. The cell culture and EROD assay were based on Donato's method (Donato *et al.* 1992). Cells were seeded into individual wells of a 96-well microtiter tissue culture plate. Cells were grown for 24 hours to approximately 60% to 70% confluency and then exposed with Q1 and TBA (10 mg dissolved in 500 µl DMSO/isopropanol, 4:1 vol/vol; stock concentration in medium 258.1 µM Q1; and 290.0 µM TBA, respectively), for 24 or 72 hours. The plate was incubated (Kenreo, Hanau, Germany) at 37°C and 7.5% carbon dioxide. For comparisons, cultures were treated with various concentrations (0.4 to 12.4 pM in media) of TCDD.

Ethoxyresorufin-*o*-Dealkylase Assay

EROD enzyme activity was determined directly in intact rat hepatoma cells cultured on 96-well plates according to Donato *et al.* (1992). The medium was removed, and the assay was started by the addition of 100 µl/well fresh culture medium containing 8 µM 7-ethoxyresorufin (Merck, Darmstadt, Germany) as substrate and 10 µM dicumarol (Boehringer, Ingelheim, Germany). After 30 minutes of incubation at 37°C, a 100-µl aliquot of cell medium was withdrawn from each well and transferred to another 96-well plate containing 200 µl ethanol/well. Fluorescence of the product resorufin was recorded directly on a fluorescence microplate reader (Fluorspektra; Kendro, Langensfeld, Germany) at 550 nm excitation and 585 nm emission wavelengths, which could be regarded as relative EROD activity.

Sulforhodamine B Assay

The human large-cell lung carcinoma xenograft tumor cell line LXFL529L (Fiebig *et al.* 1992) was selected because of its high sensitivity against standard cytotoxic agents. It was cultivated in RPMI 1640 (supplemented with 10% fetal calf serum and 1% penicillin/streptomycin) at 37°C and 5% carbon dioxide. Cells were routinely tested for absence of mycoplasma contamination. LXFL529L cells were seeded in 24-well tissue-culture plates at a density of 4000 cells/well. After 24 hours, the medium was removed and the cells were incubated with test compounds (Q1, TBA, or BC-2) for 72 hours. The assay was performed according to Skehan *et al.* (1990) with slight modifications. Briefly, incubation was stopped by addition of 100 µl trichloroacetic acid (50% solution, weight:vol). After incubation at 4°C for 1 hour, the plates were rinsed four times with water. Thereafter, the dried plates were stained with sulforhodamine B (SRB, 0.4% solution). After 30 minutes, the plates were washed with water and acetic acid (1% solution, vol/vol). The dye was then eluted from the dried plates with Tris-Base and quantified photometrically at λ = 570 nm. Cytotoxicity was calculated as percent survival as determined by the number of treated over control cells × 100 (% T/C).

Pesticide Activity

Pesticide tests were performed using standard methods according to international agreements (K. Naumann and H Schubert, Bayer AG and Bayer Cropscience AG, personal communication to W. Vetter, 2003).

Molecular Modeling

Semiempirical calculations were performed using the PM3 method (Dewar *et al.* 1985) as implemented in the MOPAC package version 6.0 (Stewart 1985). Initially, the MOPAC data set for the 1,2'-bipyrrole backbone and the respective substituents were set up. To get the starting point, a minimization of the structure was performed using the PM3 option in MOPAC. To obtain structures with minimum ΔH^o's, energy profiles were recorded by rotation of the two pyrrole rings in steps of 2.5° to 10° about the central N-pyrrole-C-pyrrole single bond. Further details on our calculation method are presented elsewhere (Vetter and Scherer 1998, 1999).

Results and Discussion

Physicochemical Parameters

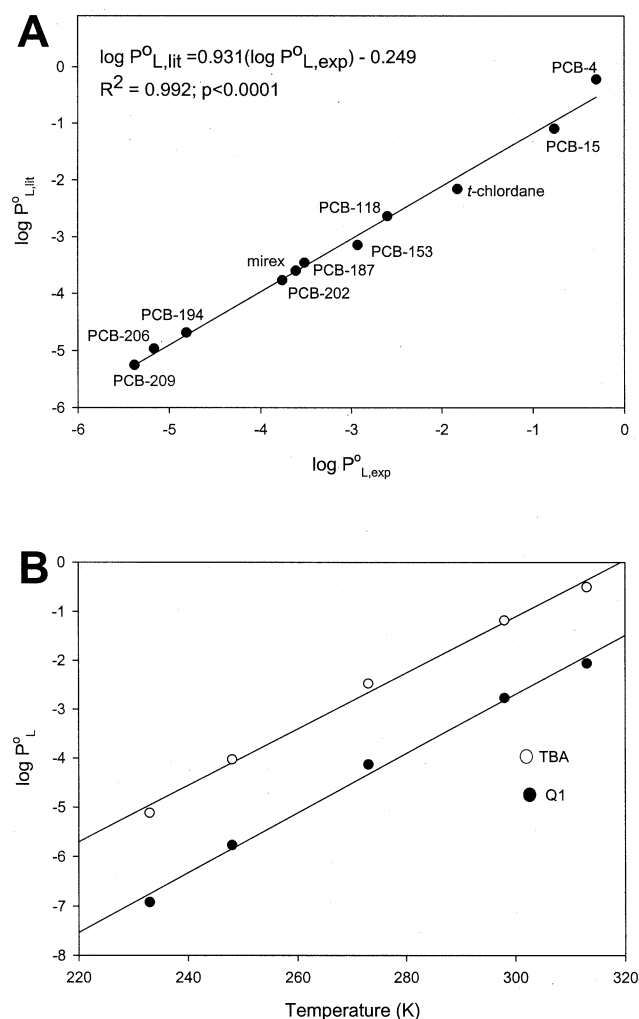
Aqueous solubility (S_{w,25}) of Q1 was three orders of magnitude lower than that of TBA (Table 1). The subcooled liquid

Table 1. Physicochemical parameters of Q1 and TBA

Compound	Aqueous solubilities at 25°C, $S_{w,25^\circ\text{C}}$ (mol m^{-3}) ^a	Subcooled liquid vapor pressure (P_{L}^0 [Pa]) ^{a,b}	Henry's law constant (H [$\text{Pa m}^3 \text{mol}^{-1}$]) ^b	$\log K_{\text{ow}}$
Q1	2.38×10^{-4}	0.00168 (0.00154–0.019) ^c	7.06	6.3 ^c
TBA	0.152 (0.02) ^d	0.06562 (0.8128) ^d	0.432 (74.7) ^d	4.4

^a Measured in this study.^b Values are at 25°C.^c Taken from Hackenberg *et al.* 2003; Vetter 2000.^d Values in parentheses are from Pfeifer *et al.* (2001).

Q1 = 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrrole; TBA = 2,4,6-tribromoanisole.

**Fig. 2.** (A) Correlation between experimentally determined and literature liquid subcooled vapor pressures at 25°C for standard compounds. (B) Variation of liquid subcooled vapor pressure with temperature for Q1 and TBA. TBA = 2,4,6-tribromoanisole; Q1 = 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrrole

vapor pressures (P_{L}^0) of Q1 and TBA were found to be 0.00168 and 0.06562 Pa, respectively. Figure 2A shows a good 1:1 correlation between the P_{L}^0 of the reference compounds

determined in this study and the reported literature values ($r^2 = 0.992$). Figure 2B illustrates the temperature variation of P_{L}^0 for both Q1 and TBA. Henry's law constants (H) at 25°C of TBA and Q1 were estimated from the parameters above according to the following equation:

$$H = P_{\text{L},25}^0 S_{\text{L},25}^{-1}$$

$P_{\text{L},25}^0$ is experimentally derived directly from GC measurements, but $S_{\text{L},25}$ had to be calculated from $S_{\text{w},25}$ because the generator-column technique measures the solid rather than the subcooled liquid. This was carried out according to Kan and Tomson (1996) by using means of melting points (T_{m} , °C) to correct for the energy of fusion required to move from the solid to the subcooled liquid phase (Tittlemier *et al.* 2002):

$$S_{\text{L},25} = S_{\text{w},25} (e^{-0.023(T_{\text{m}}-25)})^{-1}$$

The T_{m} values were obtained with a Reichert hot-stage melting-point apparatus. The T_{m} values used to calculate $S_{\text{L},25}$ were taken as the midpoint of the melting range. The H for Q1 and TBA were 7.06 and 0.432 $\text{Pa m}^3 \text{mol}^{-1}$, respectively.

Fugacity Modeling. The distribution of Q1 at equilibrium in a simple four-compartment model was estimated by following an approach taken by Mackay *et al.* (1992) with the vapor pressure and water solubility from this work. The $\log K_{\text{ow}}$ for Q1 was taken and estimated to be 6.0 in accordance with literature data (Hackenberg *et al.* 2003; Vetter 2000). The organic carbon partition coefficient (K_{oc}) was estimated by using the relation $K_{\text{oc}} = 0.411 \times K_{\text{ow}}$ developed by Karickhoff (1981). The model environment consisted of air ($1000 \times 1000 \times 6000$ m), water ($1000 \times 700 \times 10$ m), bottom sediment ($1000 \times 700 \times 0.03$ m, 5% organic carbon content by volume), and fish (2×10^5 m, 1000 kg/m^3 , 0.05 g/g lipid by weight). Soil was purposely omitted in the model environment because Q1 is a marine natural product and is unlikely to interact with this compartment. The results of the model suggest that >91% of Q1 was distributed in the sediment compartment, 3.3% in air, 2.8 % in suspended sediment, and approximately 0.2% in fish.

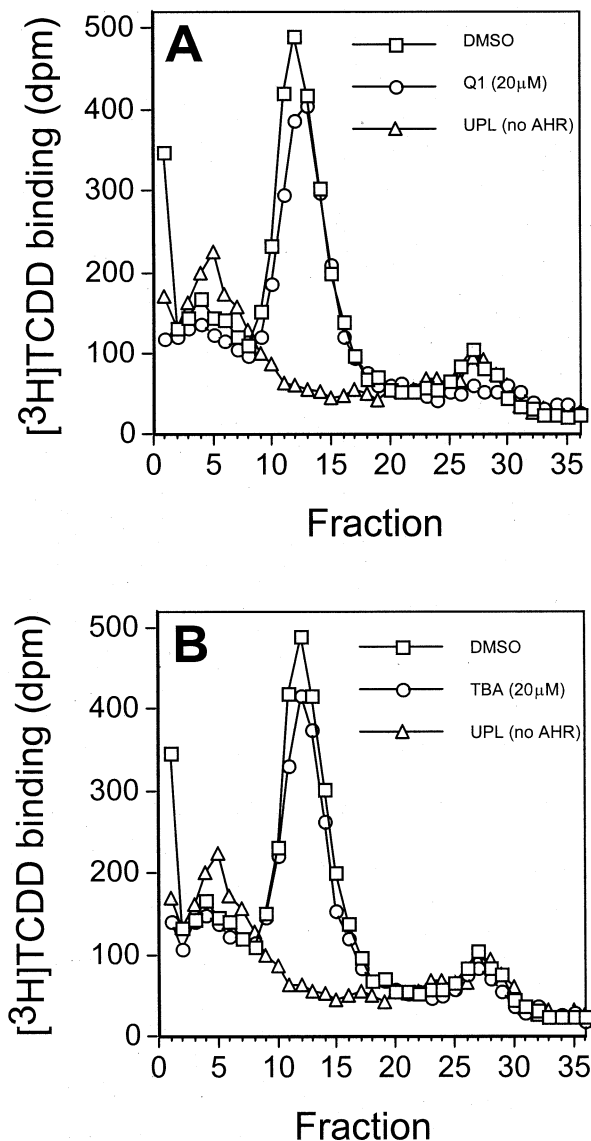


Fig. 3. Inhibition of [³H]TCDD-specific binding to AHR by Q1 (A) and TBA (B). *In vitro*-synthesized human AHR was incubated with [³H]TCDD (2 nM) plus competitor (20 μM) overnight at 4°C and followed by analysis of specific binding by velocity sedimentation on sucrose gradients as described in Materials and Methods. Nonspecific binding was measured using unprogrammed lysate (*in vitro* transcription and translation in the absence of AHR expression plasmid). AHR = aryl hydrocarbon receptor

Pesticide Activity. In addition to the lack of a known natural producer, the ecologic function of Q1 in nature is also mysterious. One potential option would be that the producing organism uses Q1 as a defense chemical to deter or kill predators. Because a series of polyhalogenated compounds constitutes strong pesticides (anthropogenic chloropesticides but also natural products such as telfairine and procememe) (Gribble 1998), Q1 was tested for insecticidal, herbicidal, and fungicidal activity using established screening methods for new potentially active compounds.

Nematicidal, acaricidal, and insecticidal activity on sucking and biting insects was tested, but no activity was found with

Q1 concentrations up to 1000 ppm. Herbicidal activity was tested on different grasses and broadleaf weeds, but none of the tested organisms yielded any response. Furthermore, fungicidal testing on powdery mildew, downy mildew, and rust at 500/1000 ppm gave no hint of any activity on these plant pathogens. In summary, even in the high tested dose ranges, Q1 exhibited no pesticidal activity at all.

Competitive Binding of Q1 and TBA to AHRs

AHR was initially discovered by virtue of its ability to bind planar aromatic molecules such as halogenated dioxins, halogenated biphenyls, and polynuclear aromatic hydrocarbons, leading to the induction of cytochrome P4501A1 (CYP1A1) (Poland *et al.* 1976). The set of compounds capable of binding to AHR is now known to be large and structurally diverse (Denison and Nagy 2003). A few of these, including non-halogenated chemicals such as brevetoxin (Washburn *et al.* 1997) and retene (Billiard *et al.* 2002), are natural products found in marine or freshwater environments. Previously, the only natural organohalogen shown to bind to AHR were the halogenated dimethyl bipyroles (Tittlemier *et al.* 2003) and several brominated indoles (Hahn *et al.*, manuscript in preparation). Given the enormous variety and structural diversity of halogenated marine natural products (Gribble 1992), it was of interest to determine if the prominent marine organohalogen Q1 and TBA are able to interact with the AHR.

Q1 at 20 μM (the highest concentration that could be tested) caused an 18% inhibition of the specific binding of [³H]TCDD (2 nM) to the *in vitro*-expressed human AHR (Fig. 3A). At 2 μM and 0.2 μM, Q1 had a minimal effect on [³H]TCDD specific binding (data not shown). TBA produced a concentration-dependent inhibition of [³H]TCDD specific binding: 4%, 14%, and 18% inhibition at 0.2, 2, and 20 μM TBA (Fig. 3B), respectively.

The results of the competitive AHR binding assay suggest that Q1 and TBA are weak ligands for the human AHR. In this regard, these compounds appear similar to the halogenated dimethyl bipyroles, which at concentrations of 10 to 20 μM inhibited the specific binding of [³H]TCDD to chicken AHR by 3% to 26% (Tittlemier *et al.* 2003). Whether Q1 and TBA act as full agonists, partial agonists, or antagonists cannot be determined from this assay.

In vitro EROD Induction Bioassay

The *in vitro* EROD assay indicates the ability of compounds to act as agonists of the AHR. This bioassay uses cultured H-4-II-E rat hepatoma cells to assess the cytochrome P450 1A inducing potencies of planar aromatic hydrocarbons and/or contaminated environmental samples. The response of the cells to pure test chemicals or extracts of mixtures is compared with their response to the standard TCDD (Schwirzer *et al.* 1998; Schramm *et al.* 1999).

In this study, the fluorescence value was used directly to express the relative EROD induction level and compared it with those of the standard curve of 2,3,7,8-TCDD (see experimental). The results showed that both Q1 (Fig. 4) and

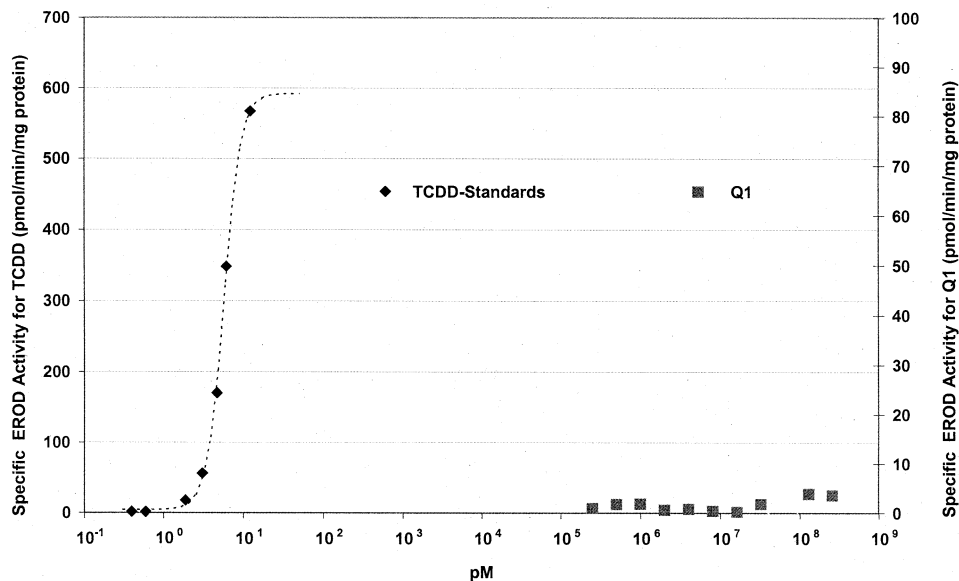


Fig. 4. Specific EROD activity for Q1 and TCDD

TBA at a used concentration of 15 $\mu\text{g}/\text{well}$ both have a very low response, e.g., the fluorescence signal for TBA was 703 ± 13 (blank 689 ± 13) and for Q1 was 480 ± 13 (blank 443 ± 13). Related to the EC_{50} value of 2,3,7,8-TCDD (0.2 pg or 6.2 pM/well), both substances were, in this test, at least around a factor 7.5×10^7 less active.

SRB Assay

The SRB assay is a standard test system used in the National Cancer Institute 60 Cell Line Panel System to measure cytotoxicity and growth inhibition of tumor cells (Skehan *et al.* 1990). The activity pattern in the 60-cell line panel, when compared with that of standard agents with known mechanisms of action, can provide hints as to whether the action mechanism of a new agent might be dissimilar to known ones and thus new. When used in a single-cell system, it provides only information on growth inhibition and cytotoxicity.

The compounds of interest were diluted in DMSO (five concentrations) to ascertain a final DMSO concentration of 0.5% in the assay. A corresponding solvent control was carried along with each plate. Compounds Q1, BC-2, and TBA were tested in duplicate in concentrations of 1, 10, 50, 100, and 200 μM . IC_{50} values were $>50 \mu\text{M}$ (Q1, BC-2) or $>100 \mu\text{M}$ (TBA). Consequently, none of the substances showed substantial cell-growth inhibition in LXFL529L cells, and further SRB assays were not considered of value. For a substance to be classified as active in the test system, IC_{50} values should be in the range of approximately $\leq 20 \mu\text{M}$. For reference, certain highly active derivatives of natural compounds based on the pteridine or bisindol ground structure achieve IC_{50} values of $<5 \mu\text{M}$ in LXFL529L cells (Marko *et al.* 2001; Merz *et al.* 1998).

Evaluation

It is difficult to compare the physicochemical parameters of Q1 with published values for structurally similar compounds

because that data do not exist. However, comparisons can be made with other polyhalogenated biaryl systems such as PCBs. For example, the $S_{w,25}$ of Q1 ($2.38 \times 10^{-4} \text{ mol}/\text{m}^3$) lies within the range of the S_w (20°C to 25°C) for the monochlorinated to dichlorinated PCBs, whereas the P^0_L (0.00168 Pa) falls within the range of the monochlorinated to tetrachlorinated PCBs (Mackay *et al.* 1992). Pfeifer *et al.* (2001) have reported on the physicochemical properties of TBA. The P^0_L value for TBA as measured by the GC-correlation technique (0.8128 Pa) in that study was approximately 10 times higher than that determined in our study (0.06562 Pa). Differences of this magnitude are often observed in the P^0_L literature values of PCBs. Conversely, the $S_{w,25}$ for TBA in our study was approximately eight times higher than that measured by Pfeifer *et al.* It should be noted that S_w in the Pfeifer *et al.* study was correlated to retention time off a reverse-phase column (Table 1). Not surprisingly, the H determined for TBA in this study ($0.432 \text{ Pa m}^3 \text{ mol}^{-1}$) was much lower than that determined by Pfeifer *et al.* ($74.7 \text{ Pa m}^3 \text{ mol}^{-1}$) and is likely related to the large discrepancy in $S_{w,25}$ between the two studies. The H of Q1 ($7.06 \text{ Pa m}^3 \text{ mol}^{-1}$) puts it in the range of some tetrachlorinated PCB congeners (Mackay *et al.* 1992).

The low water solubility of Q1 was surprising in view of the free electron pair on the two nitrogens. Obviously, they are fully drawn into the ring system to build the aromatic character of the compound. It is noteworthy that the heptachloro compound Q1 ($M = 384$) eluted in the typical range of pentachlorobiphenyls ($M = 324$), whereas heptachlorobiphenyls ($M = 392$) eluted significantly later. One key parameter that strongly influences the GC retention time on DB-5-like columns (but on others as well) is the number of *ortho*-substituents. For instance, tetra-*ortho* substituted PCBs elute much earlier than PCBs with fewer *ortho*-substituents. The non-planarity of the two pyrrole units in Q1 has been found in the solid state where both rings formed an angle of approximately 70° (Wu *et al.* 2002). All observations are in agreement with the conclusion that Q1 is not a planar molecule in all phases. This was also confirmed by molecular modeling with the PM3 method. This technique is based on behavior in the gas phase

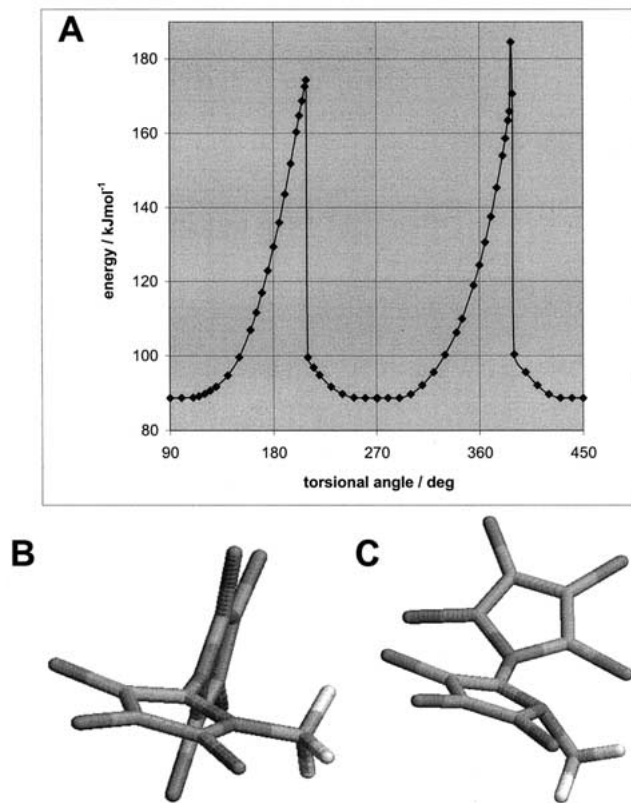


Fig. 5. (A) Modeled structure and rotational energy profile about the pyrrole–pyrrole single bond of Q1. (B) Rotational profile (torsion angle between $N_1-C_2'-N_1-C_2$) for the central C–N single bond. Due to the swift of the nitrogens from planarity, the maximum of the angle between the atoms $N_1-C_2'-N_1-C_2$ is not found at an angle of 180° . Note also that the rotational profile is valid for a passage from left to right. Passage from right to left would be accompanied with a mirroring of the profile at a plane orthogonal to 180° or 360° line (not shown). Furthermore, the two maxima of the rotation (170 and 184 kJ/mol, respectively) are slightly different. This arises from the orientation of the N-methyl group, which may be oriented upward as shown in (B) or downward. (C) Orientation of Q1 at lowest energy (angle between the two rings is 90°). Orientation of Q1 at highest energy with tetrahedral conformation of the nitrogens; see explanation for (A)

and has previously used by some of us to determine rotational barriers of chlorinated bornanes (Vetter and Scherer 1998). The rotational profile of Q1 is shown in Figure 5A. Lowest energy was found when both rings were orthogonal to each other (torsion angle 90° and 270° , respectively) as was expected (Fig. 5B). As the angle between the rings becomes smaller, the energy increases to a maximum because of steric hindrance. However, when the strain in the molecule was surmounted, the molecule quickly relaxed. This fast relaxation pointed toward a change in conformation during the building up of energy. In fact, the PM3 modeling provided that both nitrogens are changing their conformation from planar to pyramidal during the passage of the barrier (and then they immediately switch back into the planar unstressed system). This change of conformation makes the methyl group move outside of the plane of the ring (Fig. 5C). In addition, the second five-membered ring moves out of the plane of the first

ring. Both effects support passage of the rings in the presence of bulky substituents adjacent to the N-pyrrole–C-pyrrole bond, which is not possible in the unstressed system. Our semiempirical calculations clarified that the rotational barrier is high (Fig. 5A) and in the range of that of tri-*ortho*-PCBs (Andersson *et al.* 1996) and thus cannot be surmounted under typical environmental conditions (temperature $<100^\circ\text{C}$). This is supported by the GC enantiomer separation of a chiral hexachloro congener of (the nonchiral) Q1 (Vetter and Wu 2002). The hexachloro congener of Q1 possesses only one additional hydrogen atom in β -position to the N-pyrrole–C-pyrrole single bond. Because of the long distance between the central N-pyrrole–C-pyrrole-bond and the β -position, substitution of the latter has no steric influence on the rotational profile. Thus, it is clear that the pyrrole units of Q1 cannot occupy planar conformation. Consistent with that, EROD induction potency and human Ah receptor binding, which typically (though not always) are a function of planarity, were low.

The results of the tests previously described may be interpreted to suggest that Q1 is not acutely toxic compared with known compounds that show these effects. We also demonstrated that the effects of Q1 on typical pesticide test organisms (fungi, some plant bacteria, arthropods, and herbs) were negligible. This natural compound exhibited little biological activity in the assays employed. Thus, the role of Q1 in nature is still mysterious. Pharmaceutical and other bioactivities need to be investigated, and the biotransformation of Q1 along with the toxic evaluation of potential metabolites should be explored as well. It cannot be excluded that initially formed metabolites may have effects on the systems applied (AHR binding, EROD, and SRB assays and pesticides tests). In light of the novel 1,2'-bipyrrole backbone, a prediction of the ecotoxicological fate of the environment is difficult. Similar work needs also to be carried out with TBA and other halogenated natural products.

Acknowledgments. We are grateful to Klaus Naumann and Hans Schubert (Bayer AG and Bayer Cropscience AG, Germany) for the organization and performance of the pesticide tests. Gerhard Eisenbrand (University of Kaiserslautern, Germany) is acknowledged for supporting the work on SRB assay. This part of the work was supported by NOAA National Sea Grant College Program Office, Department of Commerce, under Grant No. NA86RG0075, and Woods Hole Oceanographic Institution (WHOI) Sea Grant Project No. R/P-64. We thank Diana Franks (Woods Hole Oceanographic Institution) for assistance with the AHR binding assays. This is contribution no. 11169 from the Woods Hole Oceanographic Institution. We are grateful to two anonymous reviewers for their valuable comments on an earlier draft of this manuscript.

References

- Andersson P, Haglund P, Rappe C, Tysklind M (1996) Ultraviolet absorption characteristics and calculated semi-empirical parameters as chemical descriptors in multivariate modelling of polychlorinated biphenyls. *J Chemometr* 10:171–185
- Asplund L, Athanasiadou M, Sjödin A, Bergman Å, Börjeson H (1999) Organohalogen substance in muscle, egg and blood from healthy Baltic salmon (*Salmo salar*) and Baltic salmon that produced offspring with the M74 syndrome. *Ambio* 28:67–76

- Ballschmiter K, Zell M (1981) Baseline studies of the global pollution. *Intern J Environ Anal Chem* 8:15–35
- Billiard SM, Hahn ME, Franks DG, Peterson RE, Bols NC, Hodson PV (2002) Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs). *Comp Biochem Physiol* 133:55–68
- Denison MS, Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* 43:309–334
- Dewar MJS, Zöbisch EG, Healy EF, Stewart JJP (1985) Development and use of quantum mechanical molecular models. 76. AM1: A new general purpose quantum mechanical molecular model. *J Am Chem Soc* 107:3902–3909
- Dolwick KM, Schmidt JV, Carver LA, Swanson HI, Bradfield CA (1993) Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol* 44:911–917
- Donato MT, Castell JV, Gómez-Lechón J (1992) A rapid and sensitive method for measuring monooxygenase activities in hepatocytes cultured in 96-well plates. *J Tissue Cult Methods* 14:153–158
- Faulkner DJ (1980) Natural organohalogen compounds. In: Hutzinger O (ed) *The handbook of environmental chemistry*. Volume 1, Part A. Springer Verlag, Berlin, Germany, pp 229–254
- Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR (1992) Combined *in vitro/in vivo* test procedure with human tumour xenografts for new drug development. In: Fiebig HH, Berger DP (eds) *Contributions to oncology*. Volume 42: Immunodeficient mice in oncology. Karger, Basel, Switzerland, pp 321–351
- Gribble GW (1992) Naturally occurring organohalogen compounds—a survey. *J Nat Prod* 55:1353–1395
- Gribble GW (1998) Naturally occurring organohalogen compounds. *Acc Chem Res* 31:141–152
- Gribble GW (1999) The diversity of naturally occurring organobromine compounds. *Chem Soc Rev* 28:335–346
- Gribble GW (2003) The diversity of naturally produced organohalogenes. *Chemosphere* 52:289–297
- Hackenberg R, Schütz A, Ballschmiter K (2003) High-resolution gas chromatography retention data as basis for the estimation of K_{ow} values using PCB congeners as secondary standards. *Environ Sci Technol* 37:2274–2279
- Hinckley DA, Bidleman TF, Foreman WT, Tuschall JR (1990) Determination of vapor pressures for nonpolar and semipolar organic compounds from gas chromatographic retention data. *J Chem Eng Data* 35:232–237
- Kan AT, Tomson MB (1996) UNIFAC prediction of aqueous and nonaqueous solubilities of chemicals with environmental interest. *Environ Sci Technol* 30:1369–1376
- Karchner SI, Powell WH, Hahn ME (1999) Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitus*. Evidence for a novel subfamily of ligand-binding basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) factors. *J Biol Chem* 274:33814–33824
- Karchner SI, Franks DG, Powell WH, Hahn ME (2002) Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2. *J Biol Chem* 277:6949–6959
- Karickhoff SW (1981) Semi-empirical estimation of sorption of hydrophobic pollutants on natural sediments and soils. *Chemosphere* 10:833–846
- Mackay D, Shui WY, Ma KC (1992) *Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals*. Volume I. Lewis, Boca Raton, FL
- Marko D, Schätzle S, Friedel A, Genzlinger A, Zankl H, Eisenbrand G (2001) Inhibition of cyclin-dependent kinase 1 (CDK1) by indirubin derivatives in human tumour cells. *Br J Cancer* 84:283–289
- May WE, Wasik SP, Freeman DH (1978) Determination of the aqueous solubility of polynuclear aromatic hydrocarbons by a coupled column liquid chromatographic technique. *Anal Chem* 50:175–179
- Merz KH, Marko D, Regiert T, Reiss G, Walter F, Eisenbrand G (1998) Synthesis of 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine and novel derivatives free of positional isomers. Potent inhibitors of cAMP-specific phosphodiesterase and of malignant tumor cell growth. *J Med Chem* 41:4733–4743
- Paasivirta J (ed) (2000) *The handbook of environmental chemistry: New types of persistent halogenated compounds*. Volume 3, Part K. Springer Verlag, Berlin, Germany
- Pfeifer O, Lohmann U, Ballschmiter K (2001) Halogenated methylphenyl ethers (anisoles) in the environment: Determination of vapor pressures, aqueous solubilities, Henry's law constants, and gas/water- (K_{gw}), n-octanol/water- (K_{ow}) and gas/n-octanol (K_{go}) partition coefficients. *Fresenius J Anal Chem* 371:598–606
- Poland A, Glover E, Kende AS (1976) Stereospecific, high-affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. *J Biol Chem* 251:4936–4946
- Schramm K-W, Hofmaier A, Klobasa O, Kaune A, Kettrup A (1999) Biological *in vitro* emission control. *J Anal Appl Pyrol* 49:199–210
- Schwirzer SMG, Hofmaier AM, Kettrup A, Nerdinger PE, Schramm K-W, Thoma H (1998) Establishment of a simple cleanup procedure and bioassay for determining 2,3,7,8-tetrachloro-p-dioxin toxicity equivalents of environmental samples. *Ecotoxicol Environ Safety* 41:77–82
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 82:1107–1112
- Stewart JJP (1993) MOPAC 6.0: A semi-empirical molecular orbital program 10. *QCPE* 455
- Tittlemier SA, Simon M, Jarman WM, Elliott JE, Norstrom RJ (1999) Identification of a novel $C_{10}H_6N_2Br_4Cl_2$ heterocyclic compound in seabird eggs. A bioaccumulating marine natural product? *Environ Sci Technol* 33:26–33
- Tittlemier SA, Hallderson T, Stern GA, Tomy GT (2002) Vapor pressures, aqueous solubilities, and Henry's law constants of some brominated flame retardants. *Environ Toxicol Chem* 21:1804–1810
- Tittlemier SA, Kennedy SW, Hahn ME, Reddy CM, Norstrom RJ (2003) Naturally-produced halogenated dimethyl bipyroles bind to the Ah receptor and induce cytochrome P4501A and porphyrin accumulation in chicken embryo hepatocytes. *Environ Toxicol Chem* 22:1497–1506
- Tsui HW, Okey AB (1981) Rapid vertical tube rotor gradient assay for binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin to the Ah receptor. *Can J Physiol Pharmacol* 59:927–931
- van den Brink N (1997) Early warning for organochlorines entering the (sub-) Antarctic ecosystem using seabirds. In: *Probing for the invisible*. DLO Institute for Forestry and Nature Research (IBN-DLO), Wageningen, The Netherlands, pp. 67–77
- Vetter W (2000) Characterization of Q1, an unknown major organochlorine contaminant in the blubber of marine mammals from Africa, the Antarctic, and other regions. *ACS Symp Ser* 773:243–259
- Vetter W, Scherer G (1998) Variety, structures, GC properties, and persistence of compounds of technical toxaphene (CTTs). *Chemosphere* 37:2525–2543
- Vetter W, Scherer G (1999) Persistency of toxaphene components in mammals that can be explained by molecular modeling. *Environ Sci Technol* 33:3458–3461
- Vetter W, Wu J (2002) Elucidation of a polychlorinated bipyrole structure using enantioselective GC. *Anal Chem* 74:4287–4289

- Vetter W, Wu J (2003) Nonpolar halogenated natural products bioaccumulated in marine samples. II. Brominated and mixed halogenated compounds. *Chemosphere* 52:423–431
- Vetter W, Alder L, Kallenborn R, Schlabach M (2000) Determination of Q1, an unknown organochlorine contaminant, in human milk, Antarctic air and further environmental samples. *Environ Poll* 110:401–409
- Vetter W, Hiebl J, Oldham NJ (2001a) Determination and mass spectrometric investigation of a new mixed halogenated persistent component (MHC-1) in fish and seal. *Environ Sci Technol* 35:4157–4162
- Vetter W, Scholz E, Gaus C, Müller JF, Haynes D (2001b) Anthropogenic and natural organohalogen compounds in blubber of dolphins and dugongs (*Dugong dugon*) from North-eastern Australia. *Arch Environ Contam Toxicol* 41:221–231
- Vetter W, Stoll E, Garson MJ, Fahey SJ, Gaus C, Müller JF (2002) Sponge halogenated natural products found at parts-per-million levels in marine mammals. *Environ Toxicol Chem* 21:2014–2019
- Vetter W, Wu J, Althoff G (2003) Nonpolar halogenated natural products bioaccumulated in marine samples. I. Chlorinated compounds (Q1). *Chemosphere* 52:415–422
- Weber K, Goerke H (1996) Organochlorine compounds in fish off the Antarctic Peninsula. *Chemosphere* 33:377–392
- Washburn BS, Rein KS, Baden DG, Walsh PJ, Hinton DE, Tullis K (1997) Brevetoxin-6 (PbTx-6), a nonaromatic marine neurotoxin, is a ligand of the aryl hydrocarbon receptor. *Arch Biochem Biophys* 343:149–156
- Wu J, Vetter W, Gribble GW, Schneekloth JS Jr, Blank DH, Görls H (2002) Structure and synthesis of the new natural heptachloro-1'-methyl-1,2'-bipyrrole Q1. *Angew Chem Int Ed Engl* 41:1740–1743