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Identification of a Fungi-Derived Terrestrial Halogenated Natural Product in Wild Boar (*Sus scrofa*)

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ABSTRACT: In this study, we identified and quantitated a tetrachlorinated compound found at high concentrations in some samples of the meat of free-ranging wild boar (*Sus scrofa*) from Southern Germany. Mass spectrometric analysis indicated that the compound was a tetrachloromethoxyphenol isomer, and the subsequently synthesized tetrachloro-*p*-methoxyphenol was identical with the unknown compound in wild boar. Tetrachloro-*p*-methoxyphenol is a known secondary metabolite of basidiomycetous fungi, which in turn are regular feed items of the wild boar. It is extremely likely that the wild boar have accumulated tetrachloro-*p*-methoxyphenol by exploiting basidiomycetes. The highest concentration in the samples ($n = 22$) was ~1 mg/kg lipids tetrachloro-*p*-methoxyphenol. This concentration was higher than that of polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) in any of the samples. Some samples did not contain tetrachloro-*p*-methoxyphenol, which indicates varied preferences in fungi by wild boars. Our data suggest that during their entire evolution, humans have been in contact with the natural product tetrachloro-*p*-methoxyphenol by consuming wild boars.

KEYWORDS: Polyhalogenated compounds, halogenated natural products, tetrachloro-*p*-methoxyphenol, drosophilin A, wild boar

INTRODUCTION

For more than 50 years, environmental residues of anthropogenic polyhalogenated compounds [chloropesticides and industrial chemicals such as polychlorinated biphenyls (PCBs)] are the focus of environmental, food, and analytical chemists, as well as toxicologists. The detection of dichlorodiphenyltrichloroethane (DDT) in biota and remote areas[1,2] and the identification of polchlorinated biphenyls (PCBs) in marine organisms[3] have initiated global research programs on polyhalogenated environmental pollutants. The persistence of high volume-produced DDT and PCBs, paired with the tendency for bioaccumulation in higher organisms, has led to high concentrations in the environment. These adverse properties not only support long-range transportation to remote areas but also pose a health risk to humans and wildlife. Consequently, such compounds were classified persistent organic pollutants (POPs). The presence of these environmental contaminants has to be thoroughly monitored in food and the environment.

During the past decade, the array of polyhalogenated environmental and food contaminants has been extended by diverse brominated flame retardants (e.g., polybrominated diphenyl ethers).[4,5] In addition, several halogenated natural products have been identified in marine mammals and fish whose chemical structures were astoundingly similar to those of anthropogenic POPs.[6,7] While thousands of halogenated natural products have been isolated from marine organisms since the 1970s,[8] only a small share of them has been detected in higher organisms that are not the producers but accumulated them from the environment in an identical or similar way as compared to POPs.[6] Natural source appointment of the residues in marine mammals was brought forth by means of radiocarbon measurements[9] or by the identification of the natural producers.[10] Halogenated natural products have also been detected in human milk, as a consequence of uptake via food items, as well as in Antarctic air.[11] They have been detected in virtually all matrices albeit not with the same frequency or distribution patterns as found for anthropogenic POPs. In all occasions, the abundance of halogenated natural products in higher organisms was associated with marine origins.[6]

In this study, we detected a hitherto unknown environmental contaminant in wild boar (*Sus scrofa*) meat samples collected by routine inspections under the official food control. Wild boars are widespread in forests throughout Germany and Europe, and the meat is officially sold retail. Assessment of the pollution of food including meat with POPs is an important task in food control. On a regular basis, samples are screened for regulated contaminants and pesticides. The abundant compound initiated a detailed study with the goal of structure and source identification of the compound.

MATERIALS AND METHODS

Samples and Standards. Twenty-one meat samples of individual wild boars were collected retail in autumn 2009 and winter 2009/2010 in Bavaria (Southern Germany). An additional sample collected in 2006 was also analyzed. The meat samples were from the food market and thus could not be classified with regard to gender and age. In the last years, about 450 000 wild boars were annually shot during open season for hunting in Germany.[12]

Reference standards of chloropesticides [*p*,*p*′-dichlorodiphenyltrichloroethane (**1**) and *p*,*p*′-dichlorodiphenyldichloroethene (**2**)], hexachlorobenzene (**3**), PCBs [PCB 153 (**4**), PCB 138 (**5**), PCB 180 (**6**)], and tetrachloroguaiacol (**7**) (see Figure 1 for structures) were from Dr. Ehrenstorfer (Augsburg, Germany). High-performance liquid chromatography (HPLC) grade methanol was from Th. Geyer (Renningen,

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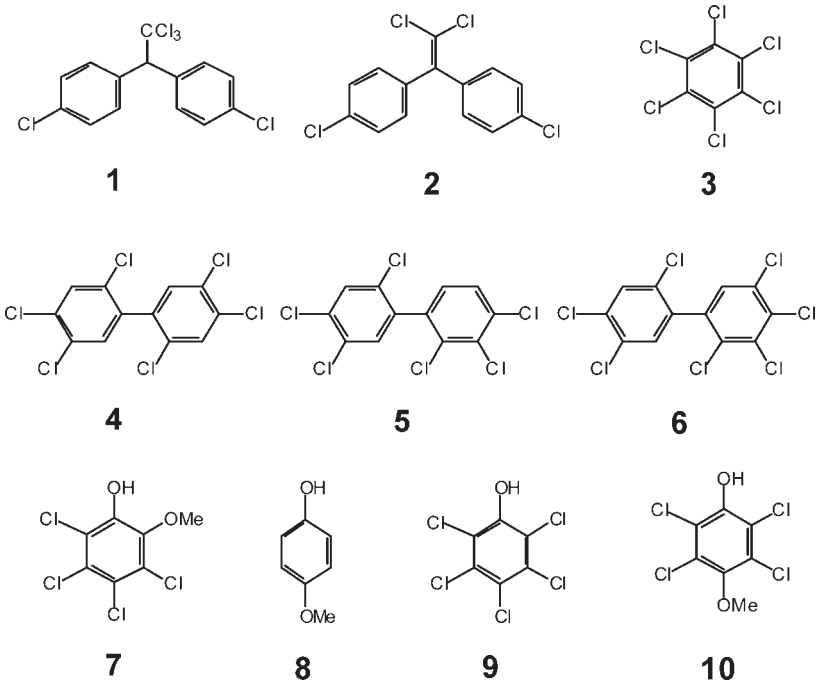

Figure 1. Chemical structures of the compounds and compound classes discussed in this study. The major compound of the chloropesticide DDT, that is, **1** and its major metabolite **2**; **3**; the PCBs 2,2′,4,4′,5,5′-hexachlorobiphenyl (**4**), 2,2′,3,4,4′,5′-hexachlorobiphenyl (**5**), and 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (**6**), tetrachloroguaiacol (**7**), 4-methoxyphenol (**8**), pentachlorophenol (**9**), and tetrachloro-4-methoxyphenol (**10**).

Germany). Solvents used for sample cleanup (*n*-hexane, acetone, cyclohexane, ethyl acetate, isooctane, and toluene) were for residue analysis quality (LGC Promochem, Wesel, Germany). Chemicals for the synthesis were from the following sources: 4-methoxyphenol (**8**), reagent plus, 99% purity, was from Aldrich (Steinheim, Germany). Diethyl ether, for synthesis, >99% purity, was from Roth (Karlsruhe, Germany). Sulfuryl chloride for synthesis was from Fluka (Buchs/Switzerland) and water-free Na_2SO_4, ≥99%. p.a., was from Sigma-Aldrich (Seelze, Germany).

Sample Cleanup. Sample cleanup was performed according to the official German procedure, developed for the determination of chloropesticides and PCBs in animal food as shown previously in detail.[13] In brief, 20 g of meat was ground for column extraction according to Ernst et al.[14] Following this procedure, 0.5 g of the resulting fat and 50 ng of isodrin (internal standard) were dissolved in 5 mL of ethyl acetate/cyclohexane (1:1, v:v). The protocol included gel permeation chromatography with biobeads S-X3 and elution with ethyl acetate/cyclohexane (1:1, v:v). After gel permeation chromatography, the solvent was changed to ethyl acetate and concentrated to 5 mL.

Gas Chromatography/Electron Capture Detection (GC/ECD). Sample extracts were analyzed with a Hewlett-Packard 6890 gas chromatograph equipped with two capillary columns, which both ended in ECDs.[13] Two microliters was injected (splitless time, 1 min) at 285 °C. Helium was used as the carrier gas at a constant pressure of 1.5 bar. The makeup gas argon/methane (90/10) was transported at 40 mL/min. The ECD temperature was set at 300 °C. The GC capillary columns (HP-1 and HP-5, 0.25 μm film thickness, respectively, Hewlett-Packard) were both 30 m long and 0.25 mm internal diameter. The GC oven was programmed as follows. After 2 min at 90 °C, the temperature was raised at 30 °C/min to 150 °C, then at 3 °C/min to 204 °C (hold time, 3 min), and finally at 8 °C/min to 280 °C (hold time, 10 min).

Quantitation of tetrachloro-*p*-methoxyphenol (**10**) was initially carried out with pentachlorophenol (**9**) as an external reference standard, which was analyzed with a blank matrix to exclude matrix effects. After the synthesis of **10**, we compared the responses of **9** and **10** (no correction was made). Additional verification of the reanalyses was obtained from the peak ratio of **10** and **3**, which showed the correct proportions according to initial quantitation. The limit of quantitation was 0.005 mg/kg fat.

Gas Chromatography/Mass Spectrometry (GC/MS). Sample extracts were screened with a 3800/1200 GC/MS system in combination with an 8400 autoinjector (Varian, Darmstadt, Germany). An HP-5 ms column (30 m, 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Agilent) was used in combination with the following GC oven program: after 2 min at 60 °C, the temperature was raised at 10 °C/min to 300 °C (hold time, 8 min). Helium (Sauerstoffwerke, Friedrichshafen, Germany) was used as the carrier gas at a constant flow rate of 1.2 mL/min. The electron energy was set to 70 eV. For electron capture negative ion measurements (GC/ECNI-MS), nitrogen (purity 99.9990%, Sauerstoffwerke, Friedrichshafen, Germany) was used as the reagent gas.[15] In the full scan mode, m/z 50−400 was recorded after a solvent delay of 6 min.

Synthesis of 10 by Chlorination of 8. Compound **8** (0.64 g) and 20 mL of diethyl ether (predried for 24 h over $CaCl_2$) were placed in a reaction flask wrapped in aluminum foil. The system was kept at room temperature by means of a water bath. To the stirred solution, 5 mL of sulfuryl chloride was added dropwise. After 24 h, the flask was placed in an ice bath, and hydrolysis was performed by the dropwise addition of 20 mL of water (demin.). The solution was stirred overnight. The organic phase was separated, washed neutral with water (demin.), and dried overnight over sodium sulfate. The resulting raw product was purified by HPLC (Waters) in combination with three 250 mm × 4.6 mm i.d., 7 μm C_{18} columns coupled in line.[16] Methanol was used as the eluent at a flow rate of 0.3 mL/min. Eight fractions were collected as specified below. The CAS no. of **10** is 484-67-3. The predicted $\log K_{OW}$ of 4.25 ± 0.46 was calculated using Advanced Chemistry Development (ACD/Laboratories) Software V9.04 for Solaris (1994−2009 ACD/Laboratories) via SciFinder.

RESULTS AND DISCUSSION

During the routine analysis of meat from free-ranging, hunted wild boar meat collected on the German food market, we

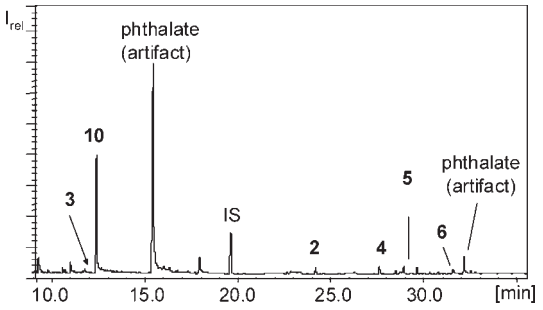

Figure 2. GC/ECD chromatogram of a sample extract of wild boar (*S. scrofa*) from Bavaria (Germany). The phthalate peaks are artifacts of the sample cleanup procedure. The early eluting peak is the halogenated natural product **10** detected and identified in the present study. Further peaks are **3**, **2**, **4**, **5**, and **6**.

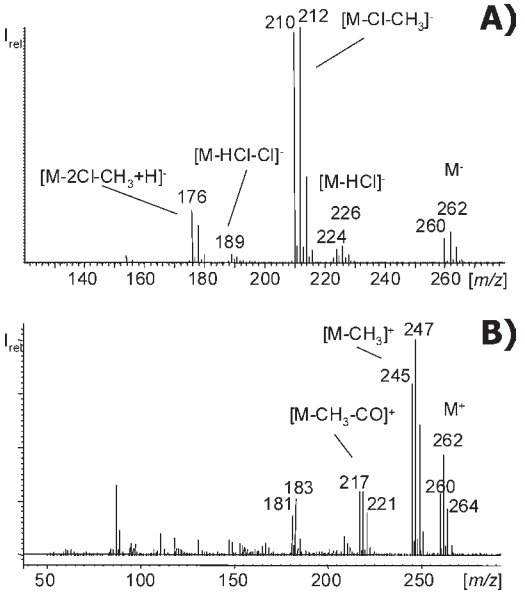

Figure 3. Mass spectra of the wild boar contaminant using (A) electron-capture negative ion (GC/ECNI-MS) and (B) electron ionization (GC/EI-MS) mode.

observed that the dominant peak in several GC/ECD chromatograms could not be traced back to a known pollutant (Figure 2). The compound eluted at comparable short retention time (i.e., shortly after **3**) from the two GC columns used. Notably, known anthropogenic food contaminants such as DDT and PCBs were usually much lower abundant in the lipids gathered from the meat (Figure 2). The abundance of the signal indicated concentrations in the range of residue limits set for polyhalogenated pesticides and contaminants in food. For this reason, we analyzed the sample by GC/MS in the full scan mode. The GC/ECNI-MS (Figure 3A) of the contaminant gave rise to four chlorine substituents. The tailing of the compound on different GC injectors (not shown in this article) indicated the presence of a phenol moiety on the target compound. The missing link between the mass contribution of a tetrachlorinated phenol moiety (140 Da for Cl_4 + 89 Da for C_6HO) and the molecular ion (260 Da) of 31 Da pointed toward an additional methoxy substituent. In accordance with this interpretation, the base ion at m/z 210 ($[M - Cl - CH_3]^-$) included the elimination of a

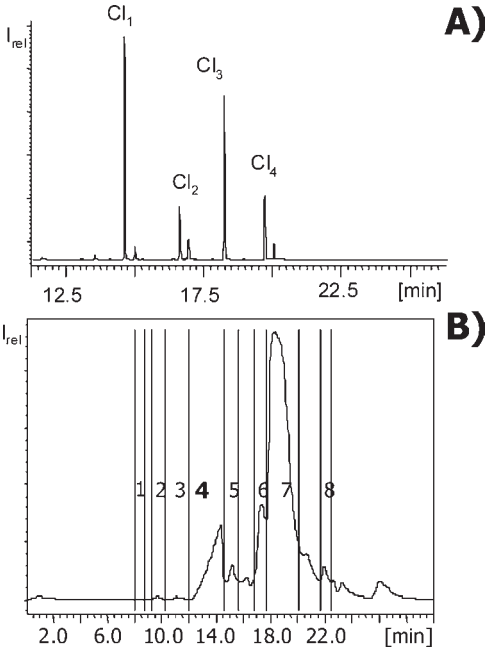

Figure 4. (A) GC/ECNI-MS full scan chromatogram of the synthesis raw product with the structure of the product inserted and (B) HPLC-UV chromatogram with the fractionation labeled. Fraction 4 contained product.

methyl group. Further substantiation of the structural production was derived from the GC/EI-MS, which featured the elimination of a methyl radical from M^+, followed by the elimination of CO (Figure 3B). In GC/EI-MS, the latter fragmentation is characteristic for phenols. The GC/MS measurements indicated that the polychlorinated residue in wild boar was a tetrachloromethoxyphenol. However, the commercial reference standard **7** eluted at higher retention time from different GC columns, so that this compound could not be the contaminant in the wild boar samples. Because the mass spectra were similar, it was assumable that the compound detected in wild boar was isomeric to **7**. A search for the molecular formula $C_7H_4Cl_4O_2$ in SciFinder gave results for **10**. Under the trivial name drosophilin A, **10** had been discovered as a secondary metabolite of basidiomycetous fungi in 1952.[17,18] Although known for ~60 years, a synthetic reference standard of **10** was not commercially available. The given similarity in the mass spectra was motivation to expend effort in the synthesis of the target compound from **8** by exhaustive chlorination. Initial screening of the reaction product by GC/ECD confirmed the presence of the desired compound albeit at low concentration (Figure 4A). Mostly, less chlorinated 4-methoxyphenols were formed in this synthesis, because of the steady deactivation of the molecules with chlorination. We used nonaqueous RP-HPLC for the isolation of the compound because larger amounts could be injected by omitting the use of water in the mobile phase. The loss of selectivity by the higher solvent strength was compensated by using three serially coupled HPLC columns.[16] The fractionation of the crude product was performed as shown in Figure 4B. Subsequent GC analysis verified that fraction #4 contained pure *p*-TCMP. Our synthesis product and the peak detected in wild boar showed identical GC retention times as well as GC/EI and GC/ECNI mass spectra. Thus, the structure of the contaminant in wild boar was verified to be **10** (drosophilin A).

Table 1. Concentrations of 10, 2, PCBs, and 3 in Individual Wild Boar (*S. scrofa*) Meat Samples Collected in 2009/2010 in Northern Bavaria (Germany)[a]

	μg/kg lipids			
no.	**10**[c]	**2**[d]	PCBs[e]	**3**
1	960[1]	53	65[3]	7
2	870[2]	40	53	3
3	92[3]	100	64	10
4	92[3]	29	25	25
5	76	4	9	2
6	76	39	74[2]	44[2]
7	67	36	44	7
8	67	72	38	16
9	30	12	22	4
10	30	11	17	2
11	23	6	13	5
12	23	53	39	6
13	20	6	22	7
14	20	490[1]	25	11
15[b]	19	32	37	2
16	8	11	8	2
17	<5[f]	220[2]	106[1]	5
18	<5	110[3]	47	29[3]
19	<5	9	16	17
20	<5	25	33	110[1]
21	<5	12	14	6
22	<5	3	6	2
mean	120	62	35	15
median	23	31	29	7

[a] Superscript 1−3 numbers denote samples with the highest, second highest, and third highest concentration, respectively, of a given contaminant. [b] Sample from 2006/2007. [c] Listed with a decreasing concentration of **10**. [d] Major residue from dichlorodiphenyltichloroethane (DDT). [e] Sum of **4**−**6**. [f] Detection limit, 5 μg/kg lipids.

Calibrated standards were used to determine the concentrations of **10** in the wild boar samples and to compare them with residues of anthropogenic POPs (Table 1). The range at which **10** was found in the samples spanned over more than 2 orders of magnitude from very high levels of ∼1 mg/kg to nondetectable (Table 1). This feature together with very high maximum concentrations in two wild boar samples effectuated a 5-fold higher mean value than the median (Table 1). These non-Gaussian distributions indicated that **10** was unevenly distributed in the environment. By contrast, the mean and median concentrations of the anthropogenic POPs differed only by the factor of ∼2 or less and thus were in the typical range for POP residues affected by age, gender, and biometric data of individuals in a sample pool. In Table 1, we also labeled the individual samples with the three highest concentrations of **10**, PCBs, DDT, and **3**. Especially, no connection was found between **10** and the anthropogenic POPs. For instance, **10** was below the detection limit in sample #17, which was high in PCB and DDT residues (Table 1). These varied distribution patterns produced clear evidence for different sources of pollution in the habitat of wild boar for **10** and POPs. While anthropogenic POPs are mostly transported to the habitat of the wild boar and thus are rather statistically distributed, halogenated natural products such as **10** are locally produced and available with hot spots in the foraging areal of wild boars. In addition, they reflect direct dietary preferences of individual wild boars. Wild boars are opportunistic omnivores that prefer a wide variety plant (which make up ∼80−90% of the diet) and animal materials.[19] They spend a large amount of time rooting and also commonly exploit fungi. The consumption of fungi has been reported to be local and individually different.[20] Differently constituted habitats (with different density of basidiomycetes) along with individual feed preferences are in conformance with the variety of the **10** residues detected (Table 1). Noteworthy, **10** or drosophilin A shows antibacterial activity, mostly against Gram-positive bacteria.[18] In fact, **10** was the first antibiotic ring-halogenated benzene derivative discovered.[17]

GC/MS screening of the wild boar samples for further chlorinated 4-methoxyphenols or related compounds was not successful. Compound **10** remained the only halogenated natural product identifiable in the samples, although 81 different halogenated natural products have been reported to be produced by 68 genera from 20 different families of basidiomycetes.[21] Basidiomycetous fungi are known to possess a complex palette of relatively nonspecific oxidative enzymes.[21] They serve as the most important source of halogenated compounds in forest litter.[21] Recently, even three natural decachlorinated natural products were identified in basidiomycetes.[22] However, why only **10** and no other halogenated natural product was detected in the wild boar samples remained unclear. The predicted $\log K_{OW}$ of 4.25 ± 0.46 of **10** is at the lower end of values, suggesting bioaccumulation in higher organisms. The $\log K_{OW}$ increases with an increasing number of halogens, so that di- to trichloromethoxyphenols fall below the range required for bioaccumulation. In addition, persistency in higher organisms usually increases with increasing number of halogens. Both features support the exclusive presence of **10** in the samples.

The identification of **10** in wild boars most likely represents the first report of an accumulated terrestrial organohalogen compound in higher organisms. Previously, it was thought that the occurrence of halogenated natural products was linked to marine sources.[6] Actually, the exclusive presence of hexahalogenated 1,1′-dimethyl-2,2′-bipyrroles in top predators of marine food webs as opposed to terrestrial species has even been used to distinguish these marine halogenated natural products from anthopogenic POPs.[23] This finding is also of historic interest. It was long thought that human contact with accumulating polyhalogenated compounds started with the industrial synthesis of the anthropogenic pollutants in the 20th century.[24] While several marine halogenated natural products had been detected in top predators,[6,7] the direct uptake of polyhalogenated contaminants from marine food, air, and seawater by ancient humans was rather irrelevant. Considering that basidiomycetous fungi evolved on earth well over half a billion years ago,[25] it is assumable that the production of halogenated natural products by the terrestrial basidiomycetes commenced soon after their appearance. The appearance of biogenic organohalogen compounds simultaneously necessitated the ability for their degradation in the environment.[26] For instance, **10** can be transformed by *Desulfitobacterium* sp. strain PCE1 into 2,3,5,6-tetrachlorophenol and 2,3,5-trichlorophenol.[27] These recalcitrant chlorophenols are primarily known from anthropogenic syntheses.[27] Hence, the availability of degrading microorganisms may have played an essential role in the (partial) transformation of POPs in soils.[26] However, animals that consume halogenated natural product containing fungi must have had early contact with these

polyhalogenated compounds. It is likely that wild boars bore **10** in their tissues since the very early days of their evolution. Moreover, hunting humans bagged wild boars at all time. By consuming wild boar meat, mankind must have been in contact with **10** throughout the timeline of human evolution (i.e., since 200 000 years ago). In the Mesolithic (~10 000 years ago), wild boar meat covered 40−50% of the human diet.[28] Notably, the second preferred food source of the early modern man, the roe deer, is also a known consumer of fungi with on average proportion of 3.3%.[28] These connections indicate that polyhalogenated compounds were regularly present in the human diet long before the introduction of anthropogenic POPs.[29] According to Vetter and Gribble,[26] the chance for a POP being metabolized by microorganisms increases with its match of the structure of halogenated natural products. For this reason, nonspecific halogenations as used in industrial synthesis should be avoided to minimize the threat caused by polyhalogenated compounds. At least in some positive cases, the degradation of POPs by humans, wildlife, and biotic environments may have its origin in the evolutionary necessity to degrade halogenated natural products.

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