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Hydroxylated Metabolites of β - and δ -Hexachlorocyclohexane: Bacterial Formation, Stereochemical Configuration, and Occurrence in Groundwater at a Former Production Site

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ABSTRACT

Although the use of hexachlorocyclohexane (HCH), after the Second World War one of the most popular insecticides, has been discontinued in many countries, problems remain from former production and waste sites. Despite the widespread occurrence of HCH's, the environmental fate of these compounds is not fully understood. In particular, environmental metabolites of the more persistent β -HCH and δ -HCH have not been fully identified. Such knowledge, however, is important to follow degradation and environmental fate of the HCH's. In the present study, several hydroxy metabolites that formed during incubation of β - and δ -HCH with the common soil microorganism *Sphingobium indicum* B90A were isolated, characterized and stereochemically identified by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). The metabolites were identified as isomeric pentachlorocyclohexanols (B1, D1) and tetrachlorocyclohexane-1,4-diols (B2, D2); δ -HCH additionally formed a tetrachloro-2-cyclohexen-1-ol (D3) and a trichloro-2-cyclohexene-1,4-diol (D4), most likely via the hydroxylation of δ -pentachlorocyclohexene (δ -PCCH), initially formed by dehydrochlorination. The dehydrochlorinase LinA was responsible for conversion of δ -HCH into δ -PCCH, and the haloalkane dehalogenase LinB was responsible for the transformation of β -HCH and δ -HCH into B1 and D1, respectively, and subsequently into B2 and D2, respectively. LinB was also responsible for transforming δ -PCCH into D3 and subsequently into D4. These hydroxylations proceeded in accordance to S_N2 type reactions with initial substitution of *equatorial* Cl's and formation of axially hydroxylated stereoisomers. The apparently high reactivity of *equatorial* Cl's in β - and δ -HCH toward initial hydroxylation by LinB of *Sphingobium indicum* B90A is remarkable when considering the otherwise usually higher reactivity of *axial* Cl's. Several of these metabolites were detected in groundwater from a former HCH production site in Switzerland. Their presence indicates that these reactions proceed under natural environmental conditions and that the metabolites are of environmental relevance.

INTRODUCTION

Hexachlorocyclohexane (HCH) was widely used as broad-spectrum insecticide in public health, in agriculture, in forestry, and as a wood preservative. Initially, HCH was applied as a technical mixture consisting of various isomers. Typically, technical HCH consisted of 60-70% α -HCH, 5-12% β -HCH, 10-15% γ -HCH, 6-10% δ -HCH and 3-4% ϵ -HCH, and smaller amounts of other isomers and congeners (1, 2). Among all isomers, only γ -HCH possesses γ -aminobutyric acid (GABA) antagonistic activity and is considered to be the only isomer with insecticidal properties (3). Although the use of technical HCH is now discontinued in most countries, pure γ -HCH (lindane) is still in use in some countries around the world (4).

The former production of technical HCH, and particularly the production of lindane, has lead to significant amounts of waste consisting of the other HCH isomers (α -, β - and δ -HCH) (5).

Although such waste HCH isomers had been sometimes used for production of other chemicals, they were often disposed in landfills, chemical waste, and dump sites (6). Subsequent leaching and run-off from such sites has resulted in heavily contaminated soils and sediments particularly with the chemically and environmentally more stable β - and δ -HCH isomers (7).

The HCH isomers differ from each other not only with respect to the relative orientation (*axial/equatorial*) of the chlorine atoms on the cyclohexane ring but also with respect to physical and chemical properties and persistence (8). In general, degradation rates increase with increasing number of *axial* Cl's in the thermodynamically most stable conformation of an HCH isomer in agreement with a mechanism of anti-periplanar dehydrohalogenation where leaving H and Cl are both *axial* and in antiparallel position ("*trans* HCl elimination") (9, 10).

In spite of their generally perceived persistence, HCH's are biodegradable to some degree and several HCH degrading microorganisms, both anaerobic (11, 12) and aerobic (13-16) have been identified. The aerobic degradation pathway of γ -HCH was studied to some detail in *Sphingobium japonicum* UT26 (17, 18). However, much less is known about the degradation pathways of the other isomers, especially β - and δ -HCH (19). Few aerobic bacteria (16, 20) have been reported to degrade β -HCH. Even though not much information is available about the degradation of β - and δ -HCH, circumstantial evidence indicates that β - and δ -HCH have similar degradation pathways (21, 22). Recently, several research groups have reported the formation of hydroxylated metabolites during biodegradation of β - and δ -HCH in laboratory and field

experiments (22-24). Unfortunately, these metabolites are insufficiently characterized and a thorough description of their stereochemical configuration is missing.

In order to gain more insight into the transformation and the metabolism of β - and δ -HCH, laboratory incubation experiments were carried out with *S. indicum* B90A and with clones expressing *linA1*, *linA2*, and *linB* genes from strain B90A. We report detailed gas chromatographic (GC), mass spectrometric (MS), and nuclear magnetic resonance (NMR) data on the major metabolites and discuss the stereochemistry and mechanisms of their formation. We then confirmed the presence of some of these metabolites in groundwater from a former HCH production site.

EXPERIMENTAL

Materials and Reference Compounds. Pure β - and δ -HCH (98% and 99%, respectively) were obtained from Riedel-de-Haën (Seelze, Germany). Stock solutions of HCH isomers were made in dimethylsulfoxide (DMSO) for bacterial cultures. For analytical purposes solutions were prepared either in hexane or ethyl acetate and diluted according to requirements. δ -PCCH was chemically synthesized by alkaline dehydrochlorination of δ -HCH (9).

Resting Cell Assays of strain B90A and Extraction of HCH Isomers and Metabolites. A preculture of *S. indicum* B90A grown over night in LB medium was transferred (1% v/v) to fresh Luria-Bertani (LB) medium in an Erlenmeyer flask and incubated at 28°C and at 200 rpm on a shaker until optical densities (OD_{600}) reached 1.5-2.0. Cells were harvested by centrifugation at 7000 rpm for 10 min and washed twice with sterile potassium phosphate buffer (10 mM; pH 7). Washed cells were finally resuspended in the same buffer at cell densities of 10^9 cells/mL and divided in 16 batches of 10 mL, spiked with β - or δ -HCH separately and incubated on a shaker at 28°C and at 200 rpm. The final concentrations were 5 and 20 μ g/mL for β -HCH and δ -HCH, respectively, which is slightly below the reported water solubility limits (25). The concentrations were higher than those usually experienced in contaminated waters in order to allow the isolation of sufficient quantities of metabolites for identification. For each time point, a whole flask was extracted with an equal volume of ethyl acetate. The aqueous phase was then adjusted with 1 N HCl to a pH value of 2, and re-extracted again with ethyl acetate. The two extracts were combined and dried by filtration through anhydrous sodium sulphate (Fluka, Buchs,

Switzerland) and evaporated to dryness in a rotary evaporator (Büchi, Flawil, Switzerland) at 40°C. The residue was dissolved in hexane and an appropriate dilution subjected to GC-MS analysis without further cleanup.

Resting Cell Assays with Various Clones. 500 ml LB medium was inoculated with a seed culture (1% v/v) of *E.coli*BL21-pET-3c (containing *linA1*, *linA2* or *linB*) grown overnight. The culture was allowed to grow until OD₆₀₀ reached a value of 0.6 to 0.8 at 37°C and 200rpm. At this point, the culture was induced with isopropyl β-thiogalactopyranoside (IPTG) that was added at a final concentration of 0.5 mM. After 4h, the culture was harvested and washed twice with potassium phosphate buffer (10 mM; pH 7) and finally, the culture pellet (~0.3 mg/ml dry weight) was resuspended in the same amount of buffer as was initially present in the culture flask. These cells were used for incubation experiments to generate metabolites as described above for strain B90A. Resting cells of *E. coli* BL21-pET-3c that do not contain the genes of interest served as controls. Additional controls, in which cells were incubated without HCH, and controls without cells were also analyzed.

GC-MS Analysis. Characterization and identification of HCH metabolites was carried out using a VG Tribrid mass spectrometer (VG Analytical, Manchester, England) operated under electron (EI; 50 eV; ion source, 180°C) or chemical ionization (CI; reagent gas, i-butane) conditions, and a 30 m SE54 capillary column (0.32 mm i.d.; film thickness, 0.25µm). Samples were injected at 50°C and the column was temperature programmed as follows: 50°C, 2 min isothermal, 20°C/min to 120°C, then 5°C/min to 280°C followed by a 10-min isothermal hold at this temperature. Retention time measurements were started at 120°C and reported relative to those of the n-alkanes as retention indices (RI's; e.g. RI = 1800 for n-octadecane), using linear interpolation in the temperature programmed runs.

Acetylation of HCH Metabolites. Sample extracts were carefully brought to dryness in 2 mL glass vials with teflon-lined screw caps. Pyridine (5 µL, Fluka) and acetic anhydride (100 µL, Fluka) were added, the vials closed and heated for 20 min at 80°C. After cooling, 0.5 mL of methylene chloride were added, followed by 0.5 mL of 1M KHCO₃ solution. The samples were mixed with the aid of a pipette and more KHCO₃ solution added until CO₂ evolution ceased, taking care not to loose any of the sample due to foaming. The samples were then extracted 3 times with 1-2 mL of methylene chloride and the combined extracts finally dried over anhydrous sodium sulphate.

Isolation and Purification of Metabolites for NMR Analysis. Overnight grown cultures of wild type *S. indicum* B90A and pLINB-B90A were prepared as mentioned above. The culture pellet (~3 g, wet weight) was washed with potassium phosphate buffer (10 mM; pH 7) and resuspended in 500 mL of the same buffer. β -HCH or δ -HCH were added separately to a concentration of 10 μ g/mL for β -HCH and 20 μ g/mL for δ -HCH and incubated at 30°C for 4 h for formation of the initial metabolites (B1, D1) and between 20-24 h for formation of subsequent metabolites (B2, D2). δ -PCCH was added separately at a concentration of 20 μ g/mL and incubated for 30 s to 1 min for formation of D3 and for 10-12h for formation of D4. After the respective time intervals the whole content of a flask was centrifuged and the supernatant extracted twice with equal volumes of ethyl acetate. Extracts were combined, concentrated to 2ml in a rotary evaporator, dried over a column of anhydrous sodium sulphate, subjected to silica gel chromatography (silica gel 60, 70-230 mesh; Merck, Darmstadt, Germany) and finally concentrated to dryness under a stream of nitrogen. Extracts were dissolved in hexane or ethyl acetate for GC and GC-MS analysis. For NMR analysis, the isolates were dissolved in CDCl_3 (99.9% D). Trace amounts of β - and δ -HCH in the extracts of B1 and D1, respectively, were removed by a second silica gel chromatography using 10% ethyl acetate in hexane followed by 20% ethyl acetate in hexane.

Groundwater Analysis. Groundwater from a former HCH production site near Zürich, Switzerland was sampled on October 17, 2005 using standard equipment. The analytical procedure used was previously described (26). Briefly, 1-L water samples were fortified with $^{13}\text{C}_6$ - γ -HCH (Cambridge Isotope Laboratories, Cambridge, MA) in toluene (spike level, 500 ng/L), and extracted without prior filtration using a macroporous polystyrene adsorbent (Bio-Beads SM-2, 20-50 mesh, Bio-Rad Laboratories, Hercules, CA). The extracts containing HCH and the metabolites were then subjected to a silica gel chromatography (minicolumn) using ethyl acetate as eluent. Finally, the eluates were concentrated carefully to dryness, acetylated and brought to a final volume of 30 μ L; 1- μ L aliquots were then analyzed by GC-MS. A fossil groundwater (zero-contaminant water, (27)) was analyzed for control purposes.

NMR Analysis. Stereochemical information was obtained from ^1H and ^{13}C NMR spectra recorded at 400.13 (100.61) MHz on a Bruker Avance-400 NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland). The ^1H and ^{13}C NMR spectra and the ^1H , ^{13}C 2D correlation

experiments were performed at 297 K using a 5 mm broadband inverse probe with z-gradient (100% gradient strength of 10 G cm^{-1}) and 90° pulse lengths of $6.7 \mu\text{s}$ (^1H) and $14.9 \mu\text{s}$ (^{13}C). All spectra were recorded with the Bruker standard pulse programs and parameter sets and the $^1\text{H}/^{13}\text{C}$ chemical shifts were referenced internally using the resonance signals of CDCl_3 at 7.26/77.0 ppm, benzene at 7.15/128.0 ppm and DMSO at 2.49/39.5 ppm. An overview of the HSQC spectra and the HMBC correlations relevant for the chemical shift assignments is available in the Supplementary Material (Fig. S1 and Tab. S2)

Cloning of *linA1*, *linA2*, *linB* into expression vector pET-3c

To clone *linA1*, *linA2*, and *linB* in an expression vector, PCR primers listed in Table S1 in the Supporting Material were constructed using the already known gene sequence of B90A from the database of the National Center for Biotechnology Information (NCBI, Bethesda, MD). Open reading frames (ORFs) of *linA1*, *linA2* and *linB* genes was amplified by the polymerase chain reaction (PCR) (Techne, Progene, Cambridge, UK) with genomic DNA of B90A (28) as template. The amplification protocol consisted of an initial denaturation step for 3min at 94°C followed by extension of 35 cycles for 30s at 94°C , 30s at 48°C and 1 min at 72°C . The reaction was terminated after a final extension for 4 min at 72°C . *Taq* polymerase (Sigma, Buchs, Switzerland) was used during all PCR amplifications. PCR products were cloned in pGEM-Teasy (Promega, Madison, WI, USA) transformed in *E.coli* DH5 α , sequenced and again cloned in expression vector pET-3c (Novagen, Madison, WI, USA) and finally transformed in *E.coli* BL21(DE3, pLys S) using standard protocols (28). The *E.coli* BL21(DE3, pLys S) - pET-3c plasmids containing *linA1*, *linA2* and *linB* ORFs were named pLINA1-B90A, pLINA2-B90A, pLINB-B90A, respectively.

RESULTS AND DISCUSSION

Formation of polar metabolites from β - and δ -HCH by *Sphingobium indicum* B90A and the clone pLINB-B90A. The laboratory incubation experiments with resting cells of strain B90A indicated rapid degradation of all HCH's. Within minutes, formation of metabolites was observed, even from β - and δ -HCH. Incubation of β -HCH lead to formation of two major metabolites, B1 and B2; several further minor metabolites were observed but not yet investigated. The kinetic data indicated that β -HCH was initially transformed into B1, which subsequently was converted into B2. Incubation of δ -HCH lead to formation of the four major metabolites, D1, D2, D3, and D4. The data indicated initial formation of D1 and D3 which then

were converted into D2 and D4, respectively. The polar nature of these metabolites resulted in significant tailing when analyzed by GC-MS. To improve analysis, derivatives (acetates) were usually prepared which showed much better GC properties. In Figure 1 we show typical EI GC-MS total ion chromatograms from the incubation of β - and δ -HCH, analyzed after acetylation of the metabolites. In Table S3 in the Supporting Material, we list the RI's of these metabolites and their derivatives, and for reference purposes, those of the five most important HCH's. These RI's describe the GC properties best, and are generally helpful in stereochemical considerations.

Incubation experiments carried out with clones expressing *linB* showed that β -HCH was transformed into B1 and B2 and δ -HCH into D1 and D2, respectively; however, formation of D3 and D4 was not observed in these experiments. In the control experiments none of these metabolites were formed.

Identification of the Initial β -HCH and δ -HCH Metabolites B1 and D1 as Pentachloro-cyclohexanols. In Figures 2a, b we present the EI mass spectra of B1 and D1. The spectra are similar but clearly differ in the relative abundances of certain fragment ions (e.g. m/z 156 versus 163). The two compounds possess slightly different RI's (see Table S3), and appear to be stereoisomers.

The EI mass spectra indicate extensive fragmentation with highest mass ions (monoisotopic) at m/z 235 (monoisotopic; Cl_4 -cluster). Under CI conditions, quasi-molecular ($\text{M}^+ + \text{H}$) ions were observed at m/z 271 (monoisotopic, Cl_5). Upon acetylation, both compounds formed acetates (B1-ac; D1-ac), as indicated by 42-Da shifts of the highest mass ions to m/z 277 (EI, Cl_4) (Figures S3a, b), and 313 (CI, Cl_5). Thus both, B1 and D1 must be isomeric pentachlorocyclohexanols.

The EI mass spectra of B1 and D1, and their acetates show even mass radical ions (monoisotopic) at m/z 198, 170, 156 and others. Of particular interest is the ion m/z 156 (Cl_3) with the elemental composition $\text{C}_4\text{H}_3\text{Cl}_3$. It implies the loss of a C_2 -moiety from the cyclohexane ring, most likely via a retro-Diels-Alder (RDA) fragmentation pathway (see Scheme S1 in the Supporting Material, upper part). The m/z 234 (Cl_4) ion likely has the formal structure of a tetrachlorocyclohexenol.

β -HCH and δ -HCH each showed formation of a single major pentachlorocyclohexanol (B1, D1). Whereas this finding is not surprising for β -HCH (all Cl's *equatorial* and equivalent, see Scheme 1), the formation of a single stereoisomer from δ -HCH indicates that the reaction is highly regioselective. The relative configurations of the hetero atoms (*axial* or *equatorial* position of OH and/or Cl) for the isomers B1 and D1 were assigned from NMR spectra based on the magnitude of the ^1H , ^1H coupling constants ($J \approx 10$ Hz corresponds to *axial-axial* and $J \approx 3$ Hz to *axial-equatorial* positions of the involved protons). Although some NMR data on D1 was previously reported (23), a stereochemical assessment was not made. The NMR data of B1 (see Table 1) now indicated the presence of an *axial* OH group at C(1), as depicted in Scheme 1. The NMR data of D1 indicated the presence of an *axial* OH group at C(1) with the *axial* Cl still present at C(4) (Scheme 1). The data indicate that hydroxylation of both HCH's, β - and δ -HCH, proceeded with substitution of *equatorial* Cl's and inversion of configuration such as in a nucleophilic $\text{S}_{\text{N}}2$ type reaction; it is of particular interest that in δ -HCH an equatorial Cl is replaced and not the axial Cl. B1 and D1 assigned in this way are epimers with respect to Cl at C(4). Their structures have a plane of symmetry and both compounds are achiral. The two pentachlorocyclohexanols show a relatively small RI difference (ΔRI , ~ 3), as do the respective epimeric HCH's (δ - and ϵ -HCH, ΔRI , ~ 14 , see Table S3). The data thus indicate that *equatorial* Cl's in β - and in δ -HCH are susceptible toward initial hydroxylation by the enzymes of this soil microorganism.

Identification of the Subsequent β -HCH and δ -HCH Metabolites B2 and D2 as

Tetrachlorocyclohexanediols. In Figure S4c, d we present EI mass spectra of B2 and D2. The spectra are quite similar. Both compounds formed diacetates (B2-ac₂; D2-ac₂), indicated by shifts of the highest mass ions (monoisotopic) from m/z 217 (Cl₃) to m/z 301 (EI, Cl₃) (Figure S3c,d). Diacetate formation is also indicated by a larger RI increase (ΔRI , ~ 170 , as compared to ΔRI , ~ 51 -95 for monoacetates, see Table S3). This identified B2 and D2 as two isomeric tetrachlorocyclohexanediols.

EI fragmentation of the two tetrachlorocyclohexanediols is governed by losses of Cl, HCl, H₂O, and combinations thereof, and that of their diacetates by additional losses of CH₂CO, and CH₃COOH. The highest mass ions correspond to $(\text{M}-\text{Cl})^+$; M^+ ions of the diols (m/z 252) and their diacetates (m/z 336) were not observed. The weak even mass radical ions at m/z 138

(monoisotopic) with the presumed elemental composition $C_4H_4OCl_2$ were likely hydroxy analogs of the RDA type ion m/z 156 observed with B1 and D1 (see above).

The fact that β - and δ -HCH formed single tetrachlorocyclohexanediol stereoisomers indicates that the diol formation is again highly regioselective. NMR data indicated both compounds to be 1,4-diols, with B2 to have the two hydroxyl groups in 1,4-*trans*-*di*axial position ($J_{12} = 0.9$ Hz), and with D2 to have the two hydroxyl groups in 1,4-*cis*-axial/equatorial position (1H , 1H coupling constants of 2.3 and 9.6 Hz, see Table 1; structures, see Scheme 1). This indicates that the introduction of a second hydroxyl group is again of S_N2 type with reaction of the Cl's at C(4) of the pentachlorocyclohexanols, leading to a *trans*-1,4-diol (B2) from B1, but to the *cis*-1,4-diol (D2) from D1. B2 and D2 have an epimeric relationship with respect to OH at C(4); again, for these epimers the difference in RI is again small (ΔRI , ~ 8). The structures of B2 and D2 have a plane of symmetry and both compounds are achiral.

Identification of D3 as a tetrachlorocyclohexenol and D4 as a trichlorocyclohexenediol. In Figure 2a, c we present EI mass spectra of D3 and D4, the two additional major metabolites of δ -HCH. The spectrum of D3 indicated an intense highest mass ion at m/z 199 (Cl_3), shifted to m/z 241 after acetylation (Cl_3 , see Figure 2b). This identified the derivative as a monoacetate (D3-ac; $\Delta RI = 95$) and hence D3 as a tetrachlorocyclohexenol. Again, the highest mass ions under EI conditions correspond to $(M-Cl)^+$. The mass spectrum of D3 revealed an intense even mass radical ion at m/z 138 (Cl_2 ; see below) and the spectrum corresponded well to that for an isomeric tetrachloro-2-cyclohexen-1-ol, identified as a γ -HCH metabolite formed by *Phanerochaete chrysosporium* (29).

The mass spectrum of D4 showed the highest mass ion at m/z 181 (Cl_2), shifted to m/z 265 (Cl_2) after acetylation (Figure 2d). This identified the derivative as a diacetate (D4-ac₂; $\Delta RI = 212$) and hence D4 as a trichlorocyclohexenediol. The spectrum of D4 revealed an intense even mass radical ion at m/z 120 (Cl_1).

The characteristic ions at m/z 138 and 120 in the EI mass spectra of D3 and D4, respectively, were again interpreted as RDA fragment ions from the losses of C_2 -moieties (see Scheme S1, lower part). This suggested D3 and D4 to be a tetrachloro-2-cyclohexen-1-ol and a trichloro-2-cyclohexen-1,4-diol, respectively, with D3 to have one, and D4 to have both allylic positions of

the cyclohexene moiety substituted by OH. The ^1H and ^{13}C chemical shifts of D3 and D4 were unambiguously assigned from the ^1H , ^{13}C correlated spectra. In chloroform, some resonances of both compounds severely overlapped in either the ^1H or the ^{13}C NMR dimension and therefore spectra were recorded in benzene (D3) and DMSO (D4) (see Table 1). The protons H-5 and H-6 of metabolite D3 must have *trans* configuration as in β - and δ -HCH, respectively. From the coupling constants $J_{56} = 9.5$ Hz it is clear, that both H's are in *axial* position. An NOE effect was observed between H-4 and H-6 (and vice versa). Therefore, these two protons must be located on the same side of the ring. Thus, H-4 should be in *pseudo axial* position. This stereochemistry is further supported by the magnitude of the coupling constant ($J_{45} = 6.3$), (30). No NOE effect was observed between H-1 and H-5 in the molecule; this is an indication that they are located at opposite sides of the ring. This relative configuration is further supported by the coupling constants $J_{16} = 3.5$ Hz, indicating an *axial / pseudo-equatorial* configuration of H-1 and H-6 in D3.

The stereochemistry of D4 was established accordingly. From the ^1H , ^{13}C 2D correlated NMR spectra it was confirmed that both OH groups are in allylic position. Again, H-5 and H-6 both must be in *axial* positions (see Scheme 1). The only significant change in the NMR data was observed for J_{45} (decrease from 6.3 Hz for D3 to 3.8 Hz for D4). Thus, the relative configuration of H-4 and H-5 must have changed from *axial* and *pseudo-axial* (D3) to *axial* and *pseudo-equatorial* (D4), whereas the configuration at H-1 was maintained. The structures of D3 and D4 are asymmetric and both compounds are chiral.

Degradation pathways for β - and δ -HCH. Our data clearly show that δ -HCH was initially biotransformed into D1 and then converted into D2; in the same way, β -HCH was initially biotransformed into B1 and subsequently converted into B2. However, the stereochemical assignments also show that the dehydrohalogenated analogs D3 and D4 cannot be formed via D1 or D2, respectively. D1 still has the axial Cl present (see above), and *trans* HCl elimination, the most probable reaction, then would lead to an isomeric 3-cyclohexen-1-ol. The formation of a 2-cyclohexen-1-ol, however, can easily be envisioned by an initial *trans* HCl elimination of δ -HCH to δ -PCCH, followed by hydroxylation of one or both of the quasi-*equatorial* Cl's in allylic position (see Scheme 1). In fact, a close examination of the GC-MS data revealed the formation of δ -PCCH during the initial phase of incubation of δ -HCH with *S. indicum* B90A, suggesting that δ -PCCH is indeed an intermediate for the formation of D3 and D4. Incubations of δ -HCH

with the clones (pLINA1-B90A, pLINA2-B90A) effected formation of δ -PCCH, and incubations of δ -PCCH (chemically synthesized) with pLINB-B90A showed formation of D3 and D4 as metabolites, confirming the above hypothesis. These findings, and the stereochemical considerations discussed above suggest that D1 and D2 were formed by direct hydroxylation of δ -HCH (pathway A), and D3 and D4 by hydroxylation of δ -PCCH (pathway B) as outlined in Scheme 1. Therefore, it seems that biotransformation of the hydroxylated metabolites D1 and D2 by dehydrohalogenation is not possible by *S. indicum* B90A, even though an *axial* Cl is present in D1. The fact that D1 and D2 were formed with similar or higher yields than D3 and D4 indicates that pathways A and B are of comparable importance.

The situation with δ -HCH may be unique in that δ -PCCH is the only PCCH with all Cl's in *equatorial* or *quasi-equatorial* position. Other PCCH's such as β - and γ -PCCH that are formed from α - and γ -HCH, respectively, have *axial* Cl's susceptible to *trans* HCl elimination; this might be the reason why formation of tetrachlorocyclohexadienes, and eventually trichlorobenzene is observed with those HCH's. β -HCH cannot yield a PCCH by *trans* HCl elimination (9), and, consistent with the above mechanisms, cannot yield unsaturated tetra- and trichlorocyclohexenols/diols.

Detection of Hydroxylated Metabolites in Groundwater from a Former Production Site.

To demonstrate the environmental occurrence of these metabolites and document that the same reactions do proceed under natural conditions we analyzed groundwater samples from a former HCH production site near Zurich, Switzerland for the presence of these HCH metabolites. The plant produced technical HCH beginning in the 1950s, and later lindane was produced. Production eventually ceased in the 1960's. Some HCH wastes were initially dumped on or near the production site. Although practically all these wastes have been excavated and incinerated, HCH's are still detectable in some groundwater, particularly β - and δ -HCH with concentrations in 2005 in the range of a few $\mu\text{g L}^{-1}$.

Analysis of groundwater from this site had revealed the presence of α -, β -, γ - and δ -HCH. On a close inspection, GC-MS data of acetylated samples also revealed the presence of the hydroxylated metabolites (B1, D1, B2, D2). In Figure 3a, b we show EI mass chromatograms of this groundwater (upper traces) and, for comparison, those of an uncontaminated fossil groundwater that showed no HCH's and metabolites present (lower traces). In Figure S5 we

further show partial EI mass spectra of the 4 major HCH metabolites detected. A determination of precise metabolite concentrations is difficult because standard reference material is not available. However, an estimation of concentrations taking into consideration the respective ion abundances from the EI mass spectra, indicated metabolite concentrations of similar magnitude as β - and δ -HCH concentrations. When considering metabolite concentrations one should keep in mind that the ratio metabolites/HCH in groundwater may be larger than the ratio initially in soil because the hydroxylated metabolites are more hydrophilic and thus more prone to leaching than the HCH's.

The data clearly indicate that hydroxylated HCH metabolites are present in such groundwater and that the same or equivalent microbial processes (e.g. hydroxylation), as were observed in the laboratory incubation studies, do in fact occur under natural environmental conditions. More detailed analysis will be needed to reveal the importance of these metabolites for risk assessment of HCH-contaminated sites. However, the mere occurrence of these metabolites in concentration ranges similar to those of the δ - and β -HCH in some groundwater shows that the metabolites are of some environmental relevance.

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SUPPORTING INFORMATION

In the accompanying Supporting Information we report further NMR and mass spectral data and other supporting information

LITERATURE CITED

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LEGEND TO FIGURES

FIGURE 1. EI GC-MS total ion chromatograms of acetylated samples from the incubation of β -HCH (a) and δ -HCH (b) with *S. indicum* B90A (incubated for 60 min) showing formation of the respective metabolites B1, B2, D1, D2, D3, and D4. Retention indices of n-alkanes indicated for reference purposes (see text).

FIGURE 2. EI mass spectra of the metabolites D3 (a) and D4 (c) and their respective acetate (b) and diacetate (d). D3 and D4 were identified as a tetrachloro-2-cyclohexene-1-ol and a trichloro-2-cyclohexene-1,4-diol, respectively. Only high mass ($M^+ - Cl$) and RDA fragment ions labelled.

FIGURE 3. EI mass chromatograms (a, b) showing presence of the β - and δ -HCH metabolites B1, B2, D1 and D2 in groundwater from a former HCH production site in Switzerland (upper traces), and absence in a fossil groundwater (lower traces). Samples analyzed after acetylation monitoring the most intense higher mass ions at m/z 241+243 (B1-ac, D1-ac) and m/z 265+267 (B2-ac₂, D2-ac₂), respectively. Note changed absolute retention times due to different column used.

SCHEME 1. General reaction pathways for the formation of hydroxylated metabolites from β - and δ -HCH. (Hydrogen substituents not shown; for abbreviations refer to text, the absolute stereochemistry of D3 and D4 is shown arbitrarily)

Table 1. ^1H and ^{13}C chemical shifts (in CDCl_3 solutions) and ^1H , ^1H coupling constants

Position ^{a)}	$\delta(^1\text{H})$ / ppm and $^3\text{J}(^1\text{H}, ^1\text{H})$ / Hz						$\delta(^{13}\text{C})$ / ppm					
	B1	B2 ^{b)}	D1	D2	D3 ^{b)}	D4 ^{b)}	B1	B2 ^{b)}	D1	D2	D3 ^{b)}	D4 ^{b)}
1	4.36	4.11	4.38	4.33	3.55	4.32	73.8	72.7	73.5	73.8	65.8	65.6
2	3.98	4.34	4.40	3.98	5.44	5.97	62.9	62.3	61.0	62.2	127.9	127.7
3	4.32		4.54	4.21			63.2		58.9	63.2	132.7	133.9
4	3.91		4.72	3.70	4.09	4.22	66.5		67.3	77.7	62.3	70.1
5					4.32	4.58					61.9	61.8
6					3.18	4.45					61.7	61.5
OH-1	^{c)}	6.39	2.84	^{c)}	^{c)}	5.7 (br)						
OH-4					^{c)}	6.2 (br)						
	$J_{12} = 2.6$	$J_{12} = 0.9$	$J_{12} = 2.4$	$J_{12} = 2.3$	$J_{12} = 5.4$	$J_{12} = 5.1$						
	$J_{23} = 10.8$	$J_{1(\text{OH})} = 6.1$	$J_{23} = 10.7$	$J_{23} = 11.1$	$J_{16} = 3.5$	$J_{16} = 3.8$						
	$J_{34} = 10.3$		$J_{34} = 3.0$	$J_{34} = 9.6$	$J_{45} = 6.3$	$J_{45} = 3.8$						
			$J_{1(\text{OH})} = 2.5$		$J_{56} = 9.5$	$J_{56} = 10.7$						

a) For atom numbers, see Scheme 1.

b) B2 and D4 measured in DMSO-d_6 and D3 in benzene-d_6

c) Not observed.

Scheme 1.

Figure 1

Figure 2

Figure 3