This document is the accepted manuscript version of the following article:
V. Raina, D. Rentsch, T. Geiger, P. Sharma, H.R. Buser, C. Holliger, R. Lal, H.-P.E. Kohler; New metabolites in the degradation of alpha- and gamma-hexachlorocyclohexane (HCH), Pentachlorocyclohexenes are hydroxylated to cyclohexenols and cyclohexenediols by the haloalkane dehalogenase LinB from *Sphingobium indicum* B90A; J. Agr. Food Chem; 2008, 56, 6594-6603.
<http://doi.org/10.1021/jf800465q>

New metabolites in the degradation of α - and γ -hexachlorocyclohexane (HCH): Pentachlorocyclohexenes are hydroxylated to cyclohexenols and cyclohexenediols by the haloalkane dehalogenase LinB from *Sphingobium indicum* B90A

Journal:	<i>Journal of Agricultural and Food Chemistry</i>
Manuscript ID:	jf-2008-00465q.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Raina, Vishakha; Eawag, Environmental Microbiology Rentsch, Daniel; Empa, Functional Polymers Geiger, Thomas; Empa Sharma, Poonam; University of Delhi, Department of Zoology Buser, Hans-Rudolf; Swiss Federal Research Station Wädenswil Holliger, Christof; EPFL, ENAC-ISTE; CHB Ecublens, EPFL-Genie Biologique Lal, Rup; University of Delhi, Department of Zoology Kohler, Hans-Peter; Eawag

1 New metabolites in the degradation of α - and γ -hexachlorocyclohexane (HCH):
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5 Vishakha Raina^{1†}, Daniel Rentsch², Thomas Geiger², Poonam Sharma³, Hans Rudolf Buser⁴,
6 Christof Holliger⁵, Rup Lal³, and Hans-Peter E. Kohler*¹

7
8 ¹Swiss Federal Institute of Aquatic Science and Technology, Eawag, CH-8600 Dübendorf,
9 Switzerland.

10 ²Laboratory for Functional Polymers, Empa, Swiss Federal Laboratories for Materials Testing
11 and Research, CH-8600 Dübendorf, Switzerland.

12 ³Department of Zoology, University of Delhi, Delhi-110007, India.

13 ⁴Swiss Federal Research Station, Agroscope Changins-Wädenswil, CH-8820 Wädenswil,
14 Switzerland.

15 ⁵EPFL, ENAC-ISTE, Laboratory of Environmental Biotechnology, CH-1015, Lausanne,
16 Switzerland

17 †Present address: KIIT School of Biotechnology, Campus 11, Bhubaneswar-751024, Orissa,
18 India

19 *Corresponding author, Telephone: +41-44-823-55-21. FAX: +41-44-823-55-47. E-mail:
20 kohler@eawag.ch

21
22 **Keywords:** HCH, LinA, LinB, dehydrochlorinase, halidohydrolase *Sphingobium indicum* B90A,
23 haloalkane dehalogenase, dehydrohalogenation, hydroxylation, pentachlorocyclohexene, PCCH,
24 tetrachlorocyclohexenol, trichlorocyclohexenediol

25 **Running title:** New metabolites in the degradation of α - and γ -hexachlorocyclohexane (HCH)

1 ABSTRACT

2 Technical hexachlorocyclohexane (HCH) and lindane are obsolete pesticides whose
3 former production and use led to wide spread contaminations posing serious and lasting health
4 and environmental risks. Out of nine possible stereoisomers, α -, β -, γ -, and δ -HCH are usually
5 present at contaminated sites and research for a better understanding of their biodegradation has
6 become essential for the development of appropriate remediation technologies. Because
7 haloalkane dehalogenase LinB was recently found responsible for the hydroxylation of β -HCH,
8 δ -HCH and δ -pentachlorocyclohexene (δ -PCCH), we decided to examine whether β - and γ -
9 PCCH, which can be formed by LinA from α - and γ -HCH, respectively, were also converted by
10 LinB. Incubation of such substrates with *Escherichia coli* BL21 expressing functional LinB
11 originating from *Shingobium indicum* B90A showed that both β -PCCH and γ -PCCH were
12 direct substrates of LinB. Furthermore, we identified the main metabolites as 3,4,5,6-tetrachloro-
13 2-cyclohexene-1-ols and 2,5,6-trichloro-2-cyclohexene-1,4-diols by nuclear magnetic resonance
14 spectroscopy and gas chromatography-mass spectrometry. In contrast to α -HCH, γ -HCH was not
15 a substrate for LinB. Based on our data, we propose a modified γ -HCH degradation pathway in
16 which γ -PCCH is converted to 2,5-cyclohexadiene-1,4-diol via 3,4,5,6-tetrachloro-2-
17 cyclohexene-1-ol and 2,5,6-trichloro-2-cyclohexene-1,4-diol.

1 INTRODUCTION

Hexachlorocyclohexane (HCH) was one of the most popular organochlorine pesticides. Its production by chlorination of benzene under suitable conditions leads to a mixture of isomers and congeners. Theoretically, there are nine HCH stereoisomers including one pair of enantiomers (α -HCH), but only one isomer, γ -HCH, possesses insecticidal properties. The technical mixture, consisting mainly of α -, β -, γ -, δ - and ϵ -HCH (1), was introduced as a pesticide in the 1940's and then widely used in agriculture and for malaria control (2). Later, it was partially replaced by pure γ -HCH (lindane) and eventually was banned in most countries due to the environmental persistence of some of the isomers. However, lindane is enriched from technical mixtures by fractional crystallization resulting in large amounts of isomeric waste, some of which has been dumped over the past 50 years (3). In fact, γ -HCH is currently under review for addition to the Stockholm Convention on persistent organic pollutants (POPs) (<http://epa.gov/oppt/ar/20052006/managing/stockholm.htm>). Nevertheless, lindane continues to be produced and still has restrictive use in some developing countries.

Several strains of the genus *Sphingobium* (previously *Sphingomonas*) with the ability to degrade HCH under aerobic conditions were isolated and identified. These strains originated from different geographical locations such as Japan (*S. japonicum* UT26), India (*S. indicum* B90A), and France (*S. francense* Sp+) (4) and slight variations in the degradation of various HCH isomers were observed. Among these strains, *S. japonicum* UT26 is studied best in terms of the metabolic pathway of γ -HCH and the putative *lin* genes involved (5). It is widely accepted that the three enzymes LinA, LinB, and LinC catalyze the reactions of the upper pathway and that LinD, LinE, and LinF catalyze those of the lower one. It is suggested that LinA converts γ -

HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) in two dehydrochlorination steps with γ -pentachlorocyclohexene (γ -PCCH) as an intermediate and that LinB acts on 1,4-TCDN to form 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL) and subsequently 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) (5). However, it should be emphasized that up to date neither 1,4-TCDN nor 2,4,5-DNOL were actually isolated and rigorously identified, due to their alleged inherent instability.

Recently, different LinB enzymes were found to be responsible for the conversion of β - and δ -HCH into pentachlorocyclohexanols and tetrachlorocyclohexanediols (6-9). Furthermore, we and others were able to show that δ -HCH was metabolized to 3,4,5,6-tetrachloro-2-cyclohexene-1-ol (D3) and 3,5,6-trichloro-2-cyclohexene-1,4-diol (D4) via δ -PCCH in the presence of LinA and LinB from strains B90A and BHC-A and that δ -PCCH was a good substrate for LinB (Scheme 1) (10, 11).

LinB is an enzyme with a broad substrate range. However, metabolites formed during the degradation of HCH isomers other than δ -PCCH have not yet been tested as substrates for LinB (5, 12, 13). Since δ -PCCH was a good substrate for LinB, we decided to examine whether β - and γ -PCCH, which can be formed by LinA from α - and γ -HCH, respectively, were also converted by LinB. Here, we document that, both β - and γ -PCCH, are direct substrates of LinB from strain B90A, we identify the reaction products, and we propose a modification of the established γ -HCH degradation pathway.

1 MATERIALS AND METHODS

2 **Chemicals and Reference Compounds.** Pure α -, γ - and δ -HCH (98%-99%,
3 respectively) were obtained from Riedel-de-Haën (Seelze, Germany). γ - and δ -PCCH were
4 chemically synthesized from γ - and δ -HCH by alkaline dehydrochlorination according to
5 published procedures (14), β -PCCH was synthesized from α -HCH according to (1). Typically,
6 reaction products were percolated through silica gel (silica gel 60, 230-400 mesh; Merck,
7 Darmstadt, Germany; glass column; 25 cm x 2 cm i.d.) with gradient elution of hexane/diethyl
8 ether mixtures 100:0 (100 mL), 40:1 (100 mL) and 20:1 (100 mL). The combined extracts were
9 washed with equal volumes of dilute HCl, 5% NaHCO₃ solution and water, and finally dried
10 over a column of anhydrous Na₂SO₄. The purities of the PCCHs were established by GC-MS and
11 the relative stereochemistries by 1D and 2D NMR spectrometry (see below). Whereas both, γ -
12 and δ -PCCH were pure (>99%), the synthesized β -PCCH contained additional PCCHs and
13 remaining starting material (β -PCCH mixture 1; structures and typical sample composition, see
14 Results section). For some resting cell assays (see below) this crude reaction product was further
15 purified by flash chromatography (see Supporting Information) resulting in a mixture of β -PCCH
16 and θ -PCCH (~80:20) (β -PCCH mixture 2). PCCHs are chiral and all the chemically
17 synthesized material was used as racemate.

18
19 **Cloning of *linB* into Expression Vector pET-3c.** To clone *linB* in an expression vector,
20 we constructed PCR primers using already known gene sequences of B90A from the database of
21 the National Center for Biotechnology Information (NCBI, Bethesda, MD) as described in (10).

Resting Cell Assay with Various Clones and Extraction of Metabolites. 500 mL LB

medium was inoculated with an overnight seed culture (1% v/v) of *E. coli* BL21-pET-3c (containing *linB*) to OD₆₀₀ ~0.6 - 0.8 at 37°C and 200 rpm. At this point, the culture was induced with isopropyl β-thiogalactopyranosid (IPTG), at final concentration of 0.5 mM at 37°C and 200 rpm. After 4 h, the culture was harvested and washed twice with potassium phosphate buffer (10 mM; pH 7) and the culture pellet (~0.3 mg/mL dry weight) was re-suspended in the same amount (500 mL) of buffer. To this suspension β-, γ- or δ-PCCH were added separately and incubated at 30° at 200 rpm (appropriate incubation times were chosen between 1 and 20 h) Subsequently, the resting cells were extracted twice with equal volumes of ethyl acetate. The combined extracts were dried over Na₂SO₄ (Fluka, Buchs, Switzerland), percolated through silica gel (230-400 mesh) and the solvent evaporated at 40°C. The dry extracts were subjected to NMR analysis and small sample aliquots were dissolved in hexane or ethyl acetate for GC-MS. Control experiments were performed as described in (10).

GC-MS Analysis. The samples were analysed on a VG Tribrid double focusing magnetic sector hybrid mass spectrometer (VG Analytical, Manchester, England) operated under electron ionization conditions (EI; 50 eV; ion source, 180°C), using a 30 m BGB-5 capillary column (0.32 mm i.d.; film thickness, 0.25µm; BGB Analytik, Adliswil, Switzerland). This column showed very similar column characteristics as the earlier used SE54 or DB-5 MS columns (10). It allowed the samples to be analyzed without prior derivatization (acetylation), resulting in peaks with acceptable tailing of the polar metabolites. Samples were injected in ethyl acetate at 70°C and the column was temperature controlled as follows: 70°C, 2 min isothermal, 20°C/min to 120°C, then 5°C/min to 280°C followed by an 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. Some samples were reanalysed after acetylation, which was carried out as reported earlier (10).

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 298 K using a 5 mm broadband inverse probe with z-gradient (100% gradient strength of 53.5 G cm^{-1}) and 90° pulse lengths of $6.8\text{ }\mu\text{s}$ (^1H) and $14.9\text{ }\mu\text{s}$ (^{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 acetone- d_6 at 2.05/29.8 ppm, CDCl_3 at 7.26/77.0 ppm, benzene- d_6 at 7.15/128.0 ppm and DMSO- d_6 at 2.49/39.5 ppm.

RESULTS

Stereochemical and Conformational Analysis of Pentachlorocyclohexenes (PCCHs) Produced from Different Hexachlorocyclohexane (HCH) Isomers by Chemical

Dehydrogenation. Because only limited NMR spectroscopic data exist for PCCHs (14, 15), we undertook a systematic study of the various PCCHs in order to determine the exact stereochemistry of the compounds. In Table 1 the NMR data (chemical shifts and coupling constants) of the five PCCHs used as substrates for degradation experiments with LinB are summarized and their structures are shown in Scheme 2. Note that the reactions were carried out

with racemic α -HCH, but to simplify matters only reactions with (+)- α -HCH are shown in the schemes. The numbering of carbon atoms used for the NMR signal assignments of PCCHs and metabolites are depicted in Scheme 1. Besides reporting NMR-data for β -, γ -, and δ -PCCH, we also report data for two additional PCCHs that we observed as minor products when α -HCH was chemically dehydrohalogenated. The two PCCHs were named θ - and η -PCCH because they can be envisioned to form by *trans-diaxial* dehydrohalogenation of θ - and η -HCH, respectively. They had been observed earlier but were not further characterized (1, 16).

In the following, we abbreviate the metabolites from β - and γ -PCCH as A- (from α -HCH, the precursor of β -PCCH) and G-compounds, respectively, and those from θ - and η -PCCH as T- and I-compounds, respectively. We already have introduced the B- and D-compounds as metabolites of β - and δ -HCH, respectively (10).

Depending on the solvent, overlap of resonances was observed (^1H and/or ^{13}C NMR spectra), inhibiting appropriate signal assignment and/or exact determination of ^1H , ^1H coupling constants. Thus, the determination of the relative conformation of the hydrogen atoms in the molecules often failed and e.g. for η -PCCH the coupling constant J_{56} was determined indirectly from a 1D TOCSY experiment. Owing to coalescence effects, in the case of γ -PCCH some ^1H NMR signals were significantly broadened and incomplete resolution of the coupling pattern in the ^1H NMR spectra was observed. The *trans diaxial* configurations of H-4 and H-5 of β -, δ -, η - and θ -PCCH were established with $J_{45} \approx 11$ Hz (Table 1), whereas for the γ -isomer the dihedral angle between these protons must be almost 90° ($J_{45} = 2.6$ Hz). From the magnitudes of J_{34} and J_{56} the relative conformations of H-3 and H-6 with respect to H-4 and H-5 were derived.

Supplementary NMR experiments supported the assignment of the relative stereochemistry. While for β -PCCH an NOE enhancement between H-4 and H-6 indicated that these protons must be located at the same side of the molecular plane, the absence of a NOE effect between H-6 and H-3 pointed to a *pseudo equatorial* configuration of H-3 (this was further supported by $J_{34} = 3.7$ Hz). Also for δ -PCCH a NOE enhancement was found between H-4 and H-6 and from the magnitudes of the coupling constants ($J_{34} = 8.4$ Hz; $J_{45} = 11.1$ Hz; $J_{56} = 8.0$ Hz) we concluded that the neighboring protons in the saturated part of the ring must be located *trans* to each other (Schemes 1 and 2). The significant deviation in the magnitudes of J_{56} between δ -PCCH (8.0 Hz) and θ -PCCH (3.6 Hz) resulted from the opposite configuration of the chlorine atom at C-6 (Table 1, Scheme 2). Similarly, β -PCCH and its epimer η -PCCH differed in the magnitude of J_{56} (7.4 and 3.9 Hz). It must be noted that chemically synthesized batches of β -PCCH always contained some β -PCCH, δ -PCCH, θ -PCCH, η -PCCH (typical composition 78:17:3:2), and traces of γ -PCCH besides remaining α -HCH (ca. 40%) (the relative amounts corresponded to those determined earlier by GC-MS (1)). For γ -PCCH, a J_{34} value of 7.6 Hz showed that the pair of protons H-3 and H-4 had the relative configuration *pseudo axial* – *pseudo axial*. Therefore, H-5 must be located in *pseudo equatorial* position ($J_{45} = 2.6$ Hz).

β -PCCH was the major dehydrochlorination product of α -HCH. The formation of additional PCCHs requires some consideration. In Scheme 2 we show the structures of the five PCCH that can theoretically be formed by an 1,2-elimination of HCl from α -HCH. Owing to the C2 symmetry axis through the carbon bonds C-1/C-2 and C-4/C-5, the positions 1 and 2, 3 and 6, and 4 and 5 are equivalent. From vicinal pairs of H and Cl atoms, a total of 6 HCl eliminations can be envisioned. The major product β -PCCH confirms that a *trans diaxial* HCl elimination

from the more stable conformer I (two axial Cl atoms) is favored. With a *cis* H(eq)/Cl(ax) elimination, the formation of δ -PCCH could be explained, and a *trans* H(eq)/Cl(eq) elimination, or more likely, an alternate *trans diaxial* HCl elimination of the α -HCH conformer II (four Cl atoms in *axial* position) would be a plausible explanation for the formation of θ -PCCH. Formation of γ -PCCH and η -PCCH possibly result from a *cis* H(ax)/Cl(eq) elimination. However, γ -PCCH was only observed in very small amounts (< 0.3%) and could also be formed from conformer II by a *cis* H(eq)/Cl(ax) elimination.

Degradation of β -PCCH by Clones of *E. coli* Expressing LinB from *S. indicum*

B90A. Resting cell incubations with *E. coli* expressing LinB were carried out with β -PCCH mixtures 1 and 2. The major product observed by GC-MS was a trichlorocyclohexenediol A4 (Scheme 3), apparently a stereoisomer of the earlier identified δ -PCCH-metabolite D4 (10). Although low in concentration, a small peak of the tetrachlorocyclohexenol A3 (an analog of the δ -PCCH-metabolite D3) was also detected. In Figure 1, we present EI mass spectra of A3 and A4 (also included are spectra of the related metabolites A1 and A2, see Discussion). As expected, the mass spectra of A3 and A4 are similar to those of D3 and D4 (10), respectively. In Table 2a we report the RI's for the A metabolites. When samples were acetylated and reanalyzed, the corresponding mono- and diacetates were observed with appropriate shifts in retention times and masses (data not shown). Further, traces of a dichlorophenol ($M^+ = 162$, Cl₂) and a trichlorobenzene ($M^+ = 180$ Cl₃) were also detected.

The relative stereochemistry of metabolite A3 was established from the magnitudes of the relevant ¹H, ¹H coupling constants (8.2, 11.5 and 8.0 Hz, Table 3). Thus, each of the protons H-1,

6, 5, and 4 must be located in *pseudo axial* position with H-4 and H-6 at one and H-1 and H-5 at the opposite side of the ring plane. Taking into account the stereochemistry of the substrate β -PCCH, we conclude that the OH group at C-1 of A3 was introduced in a S_N2 type of reaction, as observed earlier for the corresponding metabolites from δ -PCCH.

The relative stereochemistry of the main component A4 was established from the scalar coupling constants observed in NMR experiments (Table 3). The main deviation between A3 and A4 (note the different numbering of the carbon atoms in Scheme 1) concerned $J_{45} = 8.0$ Hz (A3) and $J_{16} = 3.4$ Hz (A4), whereas all other coupling constants were of the same magnitude. This again indicates that the OH group at C-1 of A4 was introduced in a S_N2 type of reaction (Scheme 3). The relative configuration of an additional trichlorocyclohexenediol (T4) was established by NMR experiments (Table 3 and Scheme 3) with material extracted from incubations with β -PCCH mixture 2.

E. coli expressing LinB was able to transform all five PCCHs present in β -PCCH-mixture 1. From GC-MS data, we conclude that several bis-hydroxylated metabolites were present. As expected, one additional metabolite was identified as the already known compound D4 (identical ^1H and ^{13}C chemical shifts and scalar coupling constants, (10)). In the ^1H NMR spectra, the signals of at least one additional compound could be detected. Although the correlations observed in the ^1H , ^{13}C 2D NMR experiments were quite weak, the chemical shifts and the stereochemistry of I4 could be deduced. The relevant coupling constants found for I4 and δ -PCCH (Tables 1 and 3) indicate that the two compounds have the same relative stereochemistry. Typically, an overnight incubation of β -PCCH mixture 1 resulted in complete elimination of all

1 PCCHs. Besides remaining α -HCH, these reaction mixtures contained A4, D4, T4, and I4
2 (typical composition 58:10:3:2). Furthermore, detailed analysis of the aromatic region of the ^1H
3 NMR spectra revealed the presence of small amounts of 1,2,4-trichlorobenzene and 2,5-
4 dichlorophenol. These data also clearly show that PCCHs were more rapidly converted than α -
5 HCH. Incubation of pure α -HCH with *E. coli* expressing LinB mainly led to the formation of
6 A4 and T4 (see Discussion).

8 **Degradation of γ -PCCH by Clones of *E. coli* Expressing LinB from *S. indicum***

9 **B90A.** Incubation of γ -PCCH with *E. coli* expressing LinB lead to the formation of several
10 metabolites; the EI mass spectra of the most prominent ones are presented in Figure 2a-d. The
11 major metabolite observed by GC-MS was the tetrachlorocyclohexenol G3, which is a
12 stereoisomer of D3 and A3 but eluting somewhat earlier (See Table 2b for RI values). Smaller
13 amounts of further tetrachlorocyclohexenols and a trichlorocyclohexenediol (G4) were also
14 detected. The prominent peak of a dichlorophenol ($M^+ = 162$, Cl_2) and small peaks of
15 trichlorophenols ($M^+ = 196$, Cl_3), chlorophenol ($M^+ = 128$, Cl), 2,5-dichlorohydroquinone ($M^+ =$
16 178 , Cl_2) and trichlorobenzenes ($M^+ = 180$, Cl_3) were also observed. Upon acetylation of
17 appropriate samples, the corresponding mono- and diacetates were observed with the appropriate
18 shifts in retention time and masses (data not shown).

19
20 The identification of 2,5- and 2,6-dichloro-2,5- cyclohexadiene-1,4-diol (2,5- and 2,6-
21 DDOL) by GC-MS was not straightforward (see spectrum for 2,5-DDOL in Figure 2d) because
22 molecular ions (M^+) at m/z 180 are not easily recognized. However, the fragment ions
23 (monoisotopic) at m/z 178, 162, and 145, interpreted as $M^+ - 2\text{H}$, $M^+ - \text{H}_2\text{O}$ and $M^+ - \text{Cl}$,

1 respectively, and the formation of a diacetate eventually confirmed this identification (data not
2 reported). In the ^1H NMR spectra of samples from γ -PCCH incubation experiments with *E. coli*
3 expressing LinB the resonances of five unsaturated metabolites were observed. These
4 metabolites were identified as G3, G3b, G4, 2,5-DDOL, and 2,6-DDOL (Scheme 4).

5
6 In the case of G3, it is evident from the ^1H , ^1H coupling constants that H-4 and H-5 are in
7 *axial* and *pseudo axial* positions ($J_{45} = 6.5$ Hz, Table 3), whereas from $J_{56} = 2.3$ Hz and $J_{16} = 3.7$
8 Hz, we conclude that the relative stereochemistry indicated in Scheme 4 must be correct (for
9 atom numbering, see Scheme 1). In benzene solution NOE enhancements were observed from H-
10 $5 \rightarrow \text{H-1}$ and from $\text{H-1} \rightarrow \text{H-2}$, 5, 6. This proved that H-1 and H-5 are on the same side of the
11 cyclohexane ring plane and H-4 is on the opposite side. Again, the data indicate that the Cl/OH
12 substitution reaction from γ -PCCH to G3 proceeded in a $\text{S}_{\text{N}}2$ type reaction under inversion of the
13 configuration at C-3.

14
15 When the metabolite mixture was fractionated by flash chromatography, we
16 characterized an additional metabolite (G3b) in the fraction containing mainly G3 ($\text{G3/G3b} \approx$
17 $97/3$). The relative stereochemistry at carbons 1, 4, 5, and 6 in G3b was readily established from
18 the coupling constants observed, and the DQF-COSY and ^1H , ^{13}C correlation experiments
19 revealed that the carbon bearing the OH group must be next to the “quaternary” (non-
20 hydrogenated) carbon at the double bond. Therefore, the minor metabolite G3b was most likely
21 formed by hydroxylation of γ -PCCH at C-6. This is in contrast to the general route observed for
22 the other cyclohexenols, which always started with hydroxylation at C-3.

Two further products showing ^1H -signals at 5.63 and 5.67 ppm revealed 3 and 4 carbon resonances only in the ^1H , ^{13}C correlated spectra (Table 4). Therefore, these signals must have originated from symmetric molecules (assuming six-membered carbon rings). The ^1H NMR data of one of the products, 2,5-DDOL, correspond to earlier findings (17).

Only traces of the intermediate product G4 were detected by NMR spectroscopy. Nevertheless, evidence for the relative configuration of G4 was obtained from the 2D NMR experiments of a purified fraction containing mainly DDOLs and G4 (flash chromatography, see Appendix). From the NMR data it is evident that H-5 and H-6 have a dihedral angle close to 90 degrees ($J_{56} = 2.0$ Hz), and $J_{16} = 4.9$ Hz also pointed to a *cis* configuration of H-1 with respect to H-6. Unfortunately, J_{45} was not resolved and only weak correlations over 2-3 bonds were observed also with longer mixing times in the HSQC-TOCSY spectrum. Assuming the relative stereochemistry for G4 as shown in Scheme 4 (no H atom is *trans* to the next one), the absence of correlation signals in the HSQC-TOCSY is explained by the small ^1H , ^1H coupling constants. The signals of H-4 and H-5 both were rather broad without any resolved coupling indicating that G4 exists in more than one stable conformation.

From a typical incubation experiment of γ -PCCH with Lin B, the isolated product contained 62% G3, 0.9% G3b, 1.4% G4, 17% 2,5-DDOL, 3.7% 2,6-DDOL, 13% 2,5-DCP, 0.7% 3,5-DCP, 0.7% 3,4-DCP, and trace amounts of other aromatic compounds. After extended storage of the metabolite mixture in absence of biological active media, also 1.6% of 2,6-DCP was observed. Concomitant to the formation of 2,6-DCP, the amounts of 2,6-DDOL decreased to 0.5%.

Surprisingly and in contrast to the observations for α -, β - and δ -HCH, no metabolites were formed when pure γ -HCH was incubated with *E. coli* expressing LinB.

DISCUSSION

In our previous studies we showed that LinB from *S. indicum* B90A converted β - and δ -HCH into pentachlorocyclohexanols (B1, D1) and tetrachlorocyclohexanediols (B2, D2) (7). LinB also converted δ -PCCH to the tetrachlorocyclohexenol D3 and the trichlorocyclohexenediol D4 (see Scheme 1 for the products of δ -HCH and δ -PCCH) (10). The pentachlorocyclohexanols B1 and D1 both were formed by S_N2 type reactions with replacements of equatorial chlorines in the parent HCHs.

In the present study, we show that LinB from *S. indicum* B90A readily metabolizes β - and γ -PCCH. The tetrachlorocyclohexenols (A3 and G3) as well as the trichlorocyclohexenediols (A4 and G4) were isolated and characterized; both pairs are constitutional isomers of D3 and D4, respectively. Each metabolite shows a well-defined stereochemistry that agrees with formation by S_N2 type mechanisms as was observed with δ -PCCH (10). Apparently, the Cl/OH replacement takes place mainly at C-3 of a PCCH, irrespective whether the chlorine in allylic position is in *pseudo equatorial* (δ -PCCH) or *pseudo axial* (β -PCCH) position. Only in the case of γ -PCCH some Cl/OH exchange at the alternative allylic position C-6 was observed (metabolite G3b) but, notably, still following a S_N2 mechanism.

1 The formation of D4, T4 and I4 in our experiments with β -PCCH mixture 1 can be
2 explained by sequential S_N2 type Cl/OH exchange reactions of LinB with the additional PCCHs
3 (δ -, θ - and η -PCCH) present (Schemes 1 and 3). The small amounts of 1,2,4-TCB observed are
4 possibly formed by an alternative pathway with 1,4,5,6-tetrachlorocyclohexa-1,3-diene as
5 intermediate product after elimination of Cl-3/H-4 of β -PCCH in *trans-diaxial* positions. The
6 release of either Cl-5 or Cl-6 followed by re-aromatization yields 1,2,4-TCB as single product.
7 Finally, the traces of 2,5-dichlorophenol detected may be formed from the low amount of γ -
8 PCCH present in this mixture as discussed below.

9
10 NMR analysis of incubations of α -HCH with LinB from *S. indicum* B90A revealed the
11 presence of A4 and T4. Other metabolites were not detected by NMR. These observations are
12 surprising, because we did not observe HCl elimination products (cyclohexenols) in incubations
13 of β - and δ -HCH with *E. coli* expressing LinB (10) and it was clearly established that D3 and D4
14 were the products of hydroxylations of δ -PCCH and not of HCl eliminations of D1 and D2,
15 respectively. As shown in Scheme 5, we propose that A4 and T4 are formed by HCl elimination
16 of hydroxylated metabolites of α -HCH. In contrast to the metabolites D1, D2, B1, and B2
17 described earlier (10), the putative metabolites A1a, A1c, and A2 have *trans diaxial* H/Cl
18 arrangements in α,β -position relative to the hydroxy group and therefore *trans diaxial* HCl
19 elimination is a reasonable and perhaps favorable reaction for these compounds. Although not
20 detected by NMR, trace amounts of A1 and A2 (see Table 2a and Figs. 1a and b) were detected
21 by GC-MS when α -HCH was incubated with *E. coli* expressing LinB. This further supports the
22 mechanism proposed in Scheme 5. However, presently it is not known whether the proposed HCl
23 eliminations occur spontaneously or whether they are enzyme catalyzed.

Our data clearly show that γ -PCCH served as a direct substrate for LinB from *S. indicum* B90A forming the hydroxylated metabolites G3 and G4. Further metabolites, such as 2,5-DDOL, 2,6-DDOL, and 2,5-, 2,6- and 3,5-dichlorophenols were also detected. Scheme 4 shows a reaction scheme that explains the formation of these metabolites. It is assumed that both 2,5- and 2,6-DDOL are formed from G4: elimination of H-5/Cl-6 of G4 will lead to 2,5-DDOL, and elimination of H-6/Cl-5 to 2,6-DDOL (see Scheme 6). Preferably, the HCl elimination reaction is favored when both atoms are in pseudo axial positions. Owing to the neighboring effect of Cl-2, OH-1 probably is forced into *pseudo equatorial* position. Possibly, the conformation of G4 with H-5 and Cl-6 in *pseudo axial* position is preferred and hence, 2,5-DDOL is the main product. Considering the stereochemistry at C-5 and C-6 of G4, obviously HCl is readily eliminated, since at the neighboring carbons always a chlorine and a proton are *in trans diaxial* position. Therefore, elimination reactions are expected to occur rapidly explaining why only small amounts of G4 were detected. We were not able to determine the relative configurations of the hydroxyl groups in 2,5- and 2,6-DDOL. Based on the stereochemistry at G4, *cis* configuration of the OH groups is expected (see Scheme 6). Similar elimination reactions are not expected for A4 or D4, because *trans diaxial* HCl eliminations are not possible (*equatorial* chlorine atoms at positions C-5 and C-6). Indeed, incubations of *E. coli* expressing LinB with α -HCH, β -PCCH, or δ -PCCH gave rise neither to DDOLs nor to dichlorophenols. We can therefore attribute the stereoselective Cl/OH substitution reactions to LinB. However, the question whether some of the subsequent dehydrochlorination or dehydration reactions occur nonenzymatically remains unanswered.

At the moment, it is widely agreed that LinA transforms γ -PCCH to an unstable 1,4-TCDN that then undergoes a two step hydrolytic dehalogenation mediated by LinB to form 2,4,5-DNOL and 2,5-DDOL. It is suggested that the metabolites 1,2,4-TCB and 2,5-DCP are formed non-enzymatically from 1,4-TCDN and 2,4,5-DNOL, respectively (5), and a similar pathway is assumed for the metabolism of α -HCH. However, it should be emphasized that so far neither 1,4-TCDN nor 2,4,5-DNOL were actually detected. Their role in the metabolism of HCH is solely based on circumstantial evidence (18). Here, we show that γ -PCCH as well as β -PCCH are direct substrates of LinB and we were able to isolate and characterize novel metabolites (G3 and G4). We have to conclude that besides the established reactions, LinB also catalyzes hydroxylations of γ -PCCH and G3. Based on our data we suggest an additional branch of the pathway for the degradation of γ -HCH in *Sphingobium indicum* B90A (Scheme 4) with hydroxylation reactions catalyzed by LinB to yield G3 and G4, the latter subsequently undergoing dehydrochlorination to 2,5-DDOL and small amounts of 2,6-DDOL. Although the LinB enzyme of strain B90A differs from those of strain Sp⁺ and strain UT26 by six and seven amino acids, respectively, and the LinB enzymes of strain Sp⁺ and strain UT26 differ from each other by three amino acids (7), this branch might well be operative in other HCH-degrading strains, because resting cell incubations of wild type strains B90A, UT26, and Sp⁺ also showed degradation of PCCHs to hydroxylated metabolites (data not shown).

Our data imply that LinB will compete with LinA or LinA1/A2 for HCHs as well as for PCCHs as substrates in HCH-degrading bacteria. They further indicate that degradation of HCH isomers is probably not channeled along a well-defined pathway but rather ramifies into a network of competing reactions that possibly lead to a range of chlorinated and hydroxylated

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metabolites. More detailed experiments will be needed to exactly evaluate formation and further metabolism of such metabolites *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by grants under the Indo Swiss Collaboration in Biotechnology (ISCB) from the Swiss Agency for Development and Co-operation (SDC) (Berne, Switzerland) and from the Department of Biotechnology (DBT) (Ministry of Science & Technology, Government of India). We would like to thank Simon Huber (Empa) for help with flash-chromatography.

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1 Table 1. ¹H and ¹³C chemical shifts and ¹H, ¹H coupling constants of β-, γ-, δ-, θ- and η-PCCH in benzene-d₆.

Position ^{a)}	δ(¹ H) / ppm and J(¹ H, ¹ H) / Hz					δ (¹³ C) / ppm				
	β-PCCH ^{b)}	γ-PCCH ^{c)}	δ-PCCH	θ-PCCH ^{b, d)}	η-PCCH ^{c, d)}	β-PCCH ^{b)}	γ-PCCH ^{c)}	δ-PCCH	θ-PCCH ^{b, d)}	η-PCCH ^{c, d)}
1						133.6	130.7	131.4	132.3	132.6
2	5.28	6.34	5.33	5.19	6.43	127.0	128.0	128.1	127.2	127.9
3	3.68	5.07	3.59	3.80	5.21	55.8	57.7	58.5	60.1	58.6
4	3.02	4.83	3.32	4.20	4.86	59.4	59.7	63.8	62.0	57.8
5	4.38	5.04	3.56	2.98	4.95	62.8	62.3	64.8	60.1	58.5
6	4.04	5.18	3.87	3.83	5.19	63.3	59.8	63.0	62.1	63.6
	J ₂₃ = 6.3	J ₂₃ = 3.5	J ₂₃ = 2.3	J ₂₃ = 3.1	J ₂₃ = 5.7					
	J ₂₆ = 1.3		J ₂₆ = 1.6							
	J ₃₄ = 3.7	J ₃₄ = 7.6	J ₃₄ = 8.4	J ₃₄ = 8.2	J ₃₄ = 3.9					
	J ₃₆ = 0.8		J ₃₆ = 2.8							
	J ₄₅ = 10.8	J ₄₅ = 2.6	J ₄₅ = 11.1	J ₄₅ = 11.5	J ₄₅ = 11.5					
	J ₅₆ = 7.4	J ₅₆ = 4.0	J ₅₆ = 8.0	J ₅₆ = 3.6	J ₅₆ = 3.9					

2 ^{a)} For atom numbers, see Scheme 1.
3 ^{b)} The 3 pairs of ¹H/¹³C signals are assigned to the starting material α-HCH: 3.57/62.3; 4.21/59.0; 3.90/64.1.
4 ^{c)} In DMSO-d₆ solution.
5 ^{d)} θ-PCCH and η-PCCH correspond to the compounds mentioned as X1 and X2 in (16).

Table 2a. Retention and EI MS data of α -HCH/ β -PCCH metabolites.

Compound ^{a)}	Type of compound	RI	EI MS data ^{b)}
α -HCH	hexachlorocyclohexane	1696	288
β -PCCH	pentachlorocyclohexene	1591	252
θ -PCCH	pentachlorocyclohexene	1565	252
η -PCCH	pentachlorocyclohexene	1607	252
δ -PCCH	pentachlorocyclohexene	1533	252
γ -PCCH	pentachlorocyclohexene	1460	252
A1	pentachlorocyclohexanol	1753	270, Cl ₅ ; 235, 156 (RDA), 125, 109; see Fig. 1a
A2	tetrachlorocyclohexanediol	1804	(252); 217, 199, 181, 138 (RDA, weak), 91; see Fig. 1b
A3	tetrachlorocyclohexenol	1535	(234); 199, 163, 138 (RDA); see Fig. 1c
A4	trichlorocyclohexenediol	1564	(216); 180/181, 163, 145, 120 (RDA), 91; see Fig. 1d
T4, I4	trichlorocyclohexenediols	1553	(216); 180/181; 163; 145; 120 (RDA); 91

Table 2b. Retention and EI MS data of γ -HCH/ γ -PCCH metabolites.

Compound ^{a)}	Type of compound	RI	EI MS data
γ -HCH	hexachlorocyclohexane	1758	288
γ -PCCH	pentachlorocyclohexene	1460	252
2,5-DDOL	dichlorocyclohexadienediol	1429	180, 178, 162, 145; see Fig. 2d
2,6-DDOL	dichlorocyclohexadienediol	1454	180, 178, 162, 145
G3	tetrachlorocyclohexenol	1532	(234); 199, 163, 138 (RDA); see Fig. 2a
G4	trichlorocyclohexenediol	1628	(216); 180/181, 163, 145, 120 (RDA), see Fig. 2c
G3b	tetrachlorocyclohexenol	1558	234, Cl ₄ ; 199, 163, 138 (RDA); see Fig. 2b

a) For structures, see Schemes 1 to 4.

b) EI MS data is reported as follows: all ions, monoisotopic, first number, molecular ion (not observed if in parentheses) followed by important fragment ions.

Table 3. ¹H and ¹³C chemical shifts and ¹H, ¹H coupling constants of tetrachlorocyclohexeneols and trichlorocyclohexenediols in benzene-d₆.

Pos. ^{a)}	$\delta(^1\text{H}) / \text{ppm}$ and $J(^1\text{H}, ^1\text{H}) / \text{Hz}$							$\delta(^{13}\text{C}) / \text{ppm}$						
	A3	A4	G3	G3b ^{b)}	G4 ^{b)}	T4	I4	A3	A4	G3	G3b ^{b)}	G4 ^{b)}	T4	I4
1	3.38	3.56	3.48	4.45	4.30	3.68	3.60	71.1	73.2	66.7	69.5	70.4	74.9	74.7
2	5.46		5.41					130.2	132.1	129.6	n.d.	134.2	136.1	n.d.
3		5.49		6.06	5.92	5.44	5.45	130.3	129.0	130.3	124.4	128.6	125.6	127.7
4	4.04	3.56	4.34	4.82	4.59	3.56	3.50	64.0	71.6	61.7	57.3	68.8	66.4	72.0
5	3.70	3.90	3.68	4.41	4.61	3.26	3.35	64.5	62.5	62.2	61.3	63.5	66.4	65.5
6	3.16	3.11	3.85	4.77	4.69	4.08	3.53	65.2	62.4	62.6	62.2	60.8	62.0	64.6
OH-1 ^{c)}		1.97	1.85		4.49	2.00								
OH-4 ^{c)}		1.58				1.65								
	J ₂₄ = 1.4		J _{1-OH} = 10.2	J ₁₃ = 1.4	J _{1-OH} = 8.6									
	J ₁₄ = 2.7		J ₁₄ = 1.8	J ₁₄ = 2.0	J ₁₃ = 0.9	J ₁₃ = 1.4	J ₁₃ = 1.7							
	J ₁₂ = 2.2	J ₃₄ = 2.6	J ₁₂ = 2.9	J ₃₄ = 3.7	J ₃₄ = 3.1	J ₃₄ = 5.3	J ₃₄ = 1.7							
	J ₁₆ = 8.2	J ₄₅ = 7.7	J ₁₆ = 3.7	J ₄₅ = 6.3	J ₄₅ = n.d	J ₄₅ = 3.3	J ₄₅ = 8.1							
	J ₅₆ = 11.5	J ₅₆ = 11.5	J ₅₆ = 2.3	J ₅₆ = 2.4	J ₅₆ = 2.0	J ₅₆ = 10.1	J ₅₆ = 11.6							
	J ₄₅ = 8.0	J ₁₆ = 3.4	J ₄₅ = 6.5	J ₁₆ = 4.4	J ₁₆ = 4.9	J ₁₆ = 6.6	J ₁₆ = 8.1							

- a) For atom numbers, see Scheme 1.
b) G3b measured in CDCl₃ and G4 in acetone-d₆ solution.
c) All other OH resonances were broad (and not assignable) or not detected.

Table 4. ^1H and ^{13}C chemical shifts and ^1H , ^1H coupling constants of dichlorocyclohexadienediols (DDOL) in benzene- d_6 .

Position ^{a)}	$\delta(^1\text{H})$ / ppm and $J(^1\text{H}, ^1\text{H})$ / Hz		$\delta(^{13}\text{C})$ / ppm	
	2,5-DDOL ^{b)}	2,6-DDOL	2,5-DDOL	2,6-DDOL
1	3.85	3.85	66.7	68.4
2			135.2	133.6
3	5.63	5.67	126.2	127.0
4		3.9		64.2
	$J_{16} = 3.8$	$J_{34} = 4.0$		

^{a)} For atom numbers, see Scheme 1.

^{b)} $\delta(^1\text{H})$ of 4.56 and 6.14 ppm observed in CDCl_3 solution corresponds to earlier findings (17).

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1 Scheme 1. A) Degradation pathway of δ -HCH and δ -PCCH with formation of
2 hydroxylated metabolites by LinB. The absolute stereochemistry of δ -PCCH and the
3 following products D3 and D4 is shown arbitrarily. B) Structures and numbering of
4 carbon atoms used for NMR signal assignments of PCCHs and hydroxylated
5 metabolites.

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Scheme 2. Structures of PCCHs formed from α -HCH by different 1,2 HCl elimination reactions (absolute stereochemistry shown arbitrarily; the reactions were carried out with racemic α -HCH). a) *trans diaxial* elimination of H-3_a / Cl-2_a; b) *cis* elimination of H-2_e / Cl-1_a; c) *trans* elimination of H-2_e / Cl-3_e, this corresponds to *trans diaxial* elimination of H-2_a / Cl-3_a for the inverted α -HCH conformer II in equilibrium; d) *cis* elimination of H-4_a / Cl-3_e or H-3_a / Cl-4_e (*: the 2nd reaction results in the other enantiomer of γ -PCCH); e) *cis* elimination of H-4_a / Cl-5_e

1 Scheme 3. Degradation pathway of α -HCH, β -, θ - and η -PCCH (reaction scheme
2 arbitrarily shown for (+)- α -HCH and its PCCHs). Compounds in parentheses were
3 not isolated or characterized.

1 Scheme 4. Degradation pathway of γ -HCH and γ -PCCH (absolute stereochemistry
2 shown arbitrarily).

1 Scheme 5. Proposed reaction mechanism for the formation of metabolites A4 and T4
2 from α -HCH by LinB.
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1 Scheme 6. Formation of 2,5- and 2,6-DDOL by *trans diaxial* HCl eliminations of two
2 conformers of γ -HCH metabolite G4.
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Figure 1. EI mass spectra of metabolites A1 to A4.

Figure 2. EI mass spectra of γ -PCCH metabolites.