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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

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

KEYWORDS: HCH; LinA; LinB; dehydrochlorinase; halidohydrolase; *Sphingobium indicum* B90A; haloalkane dehalogenase; dehydrohalogenation; hydroxylation; pentachlorocyclohexene; PCCH; tetrachlorcyclohexenol; trichlorocyclohexenediol

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 1940s 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 because of 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

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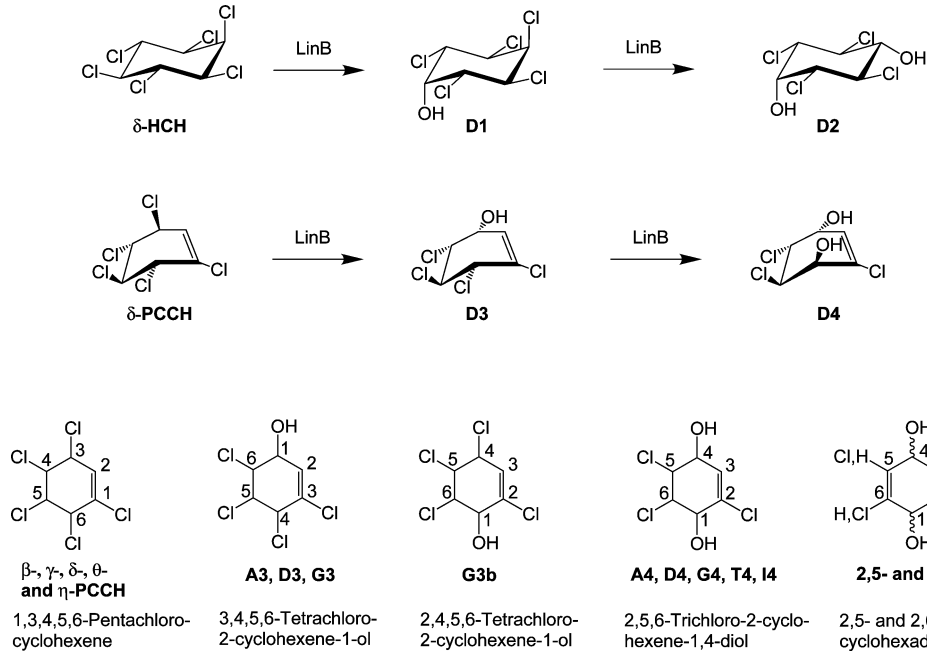
Scheme 1. (Top) Degradation Pathway of δ-HCH and δ-PCCH with the Formation of Hydroxylated Metabolites by LinB (Absolute Stereochemistry of δ-PCCH and the Following Products D3 and D4 Is Shown Arbitrarily) and (Bottom) Structures and Numbering of Carbon Atoms Used for NMR Signal Assignments of PCCHs and Hydroxylated Metabolites



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 (*Sphingobium japonicum* UT26), India (*Sphingobium indicum* B90A), and France (*Sphingobium 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, because of 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*). Because 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, identify the reaction products, and propose a modification of the established γ-HCH degradation pathway.

MATERIALS AND METHODS

Chemicals and Reference Compounds. Pure α-, γ- and δ-HCH (98−99%, respectively) were obtained from Riedel-de-Haën (Seelze, Germany). γ- and δ-PCCH were chemically synthesized from γ- and δ-HCH by alkaline dehydrochlorination according to published procedures (*14*), β-PCCH was synthesized from α-HCH according to ref *1*. Typically, reaction products were percolated through silica gel (silica gel 60, 230−400 mesh; Merck, Darmstadt, Germany; glass column; 25 cm × 2 cm i.d.) with gradient elution of hexane/diethyl ether mixtures of 100:0 (100 mL), 40:1 (100 mL), and 20:1 (100 mL). The combined extracts were washed with equal volumes of dilute HCl, 5% $NaHCO_3$ solution, and water and finally dried over a column of anhydrous Na_2SO_4. The purities of the PCCHs were established by gas chromatography−mass spectrometry (GC−MS), and the relative stereochemistries were established by 1D and 2D nuclear magnetic resonance (NMR) spectrometry (see below). Whereas both, γ- and δ-PCCH were pure (>99%), the synthesized β-PCCH contained additional PCCHs and remaining starting material (β-PCCH mixture 1; structures and typical sample composition, see the Results). For some resting cell assays (see below), this crude reaction product was further purified by flash chromatography (see the Supporting Information), resulting in a mixture of β-PCCH and θ-PCCH (~80:20) (β-PCCH mixture 2). PCCHs are chiral, and all of the chemically synthesized material was used as racemate.

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

Resting Cell Assay with Various Clones and Extraction of Metabolites. A total of 500 mL of LB medium was inoculated with an overnight seed culture (1% v/v) of *Escherichia coli* BL21-pET-3c

Table 1. ^{1}H and ^{13}C Chemical Shifts and ^{1}H,^{1}H Coupling Constants of β-, γ-, δ-, θ-, and η-PCCH in Benzene-d_6

	δ (^{1}H) (ppm) and J (^{1}H,^{1}H) (Hz)					δ (^{13}C) (ppm)				
position[a]	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
	$J_{23} = 6.3$	$J_{23} = 3.5$	$J_{23} = 2.3$	$J_{23} = 3.1$	$J_{23} = 5.7$					
	$J_{26} = 1.3$		$J_{26} = 1.6$							
	$J_{34} = 3.7$	$J_{34} = 7.6$	$J_{34} = 8.4$	$J_{34} = 8.2$	$J_{34} = 3.9$					
	$J_{36} = 0.8$		$J_{36} = 2.8$							
	$J_{45} = 10.8$	$J_{45} = 2.6$	$J_{45} = 11.1$	$J_{45} = 11.5$	$J_{45} = 11.5$					
	$J_{56} = 7.4$	$J_{56} = 4.0$	$J_{56} = 8.0$	$J_{56} = 3.6$	$J_{56} = 3.9$					

[a] For atom numbers, see **Scheme 1**. [b] The three pairs of ^{1}H/^{13}C signals are assigned to the starting material α-HCH: 3.57/62.3, 4.21/59.0, and 3.90/64.1. [c] In DMSO-d_6 solution. [d] θ- and η-PCCH correspond to the compounds mentioned as X1 and X2 in ref *16*.

(containing *linB*). The culture was incubated at 37 °C and 200 rpm until it reached an $OD_{600} \sim 0.6$–0.8. At this point, the culture was induced with isopropyl-β-D-thiogalactopyranosid (IPTG) at a final concentration of 0.5 mM and further incubated 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 resuspended in the same amount (500 mL) of buffer. To this suspension, β-, γ-, or δ-PCCH was added separately and incubated at 30 °C 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 Na_2SO_4 (Fluka, Buchs, Switzerland) and 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 ref *10*.

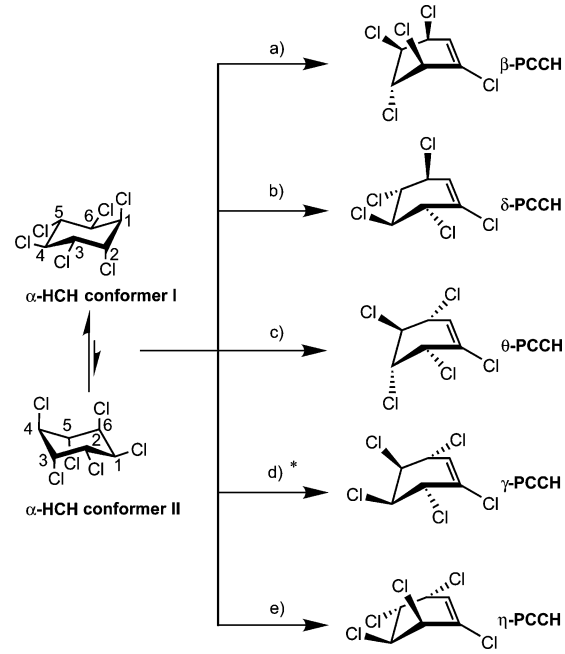
GC−MS Analysis. The samples were analyzed on a VG Tribrid double-focusing magnetic sector hybrid mass spectrometer (VG Analytical, Manchester, U.K.) operated under electron ionization conditions (EI; 50 eV; ion source, 180 °C), using a 30 m BGB-5 capillary column (0.32 mm i.d.; film thickness, 0.25 μm; 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, and 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 (RIs; e.g., RI = 1800 for *n*-octadecane), using linear interpolation in the temperature-programmed runs. Some samples were re-analysed after acetylation, which was carried out as reported earlier (*10*).

NMR Analysis. Stereochemical information was obtained from ^{1}H 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 ^{1}H and ^{13}C NMR spectra and the ^{1}H,^{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 μs (^{1}H) and 14.9 μs (^{13}C). All spectra were recorded with the Bruker standard pulse programs and parameter sets, and the ^{1}H/^{13}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.

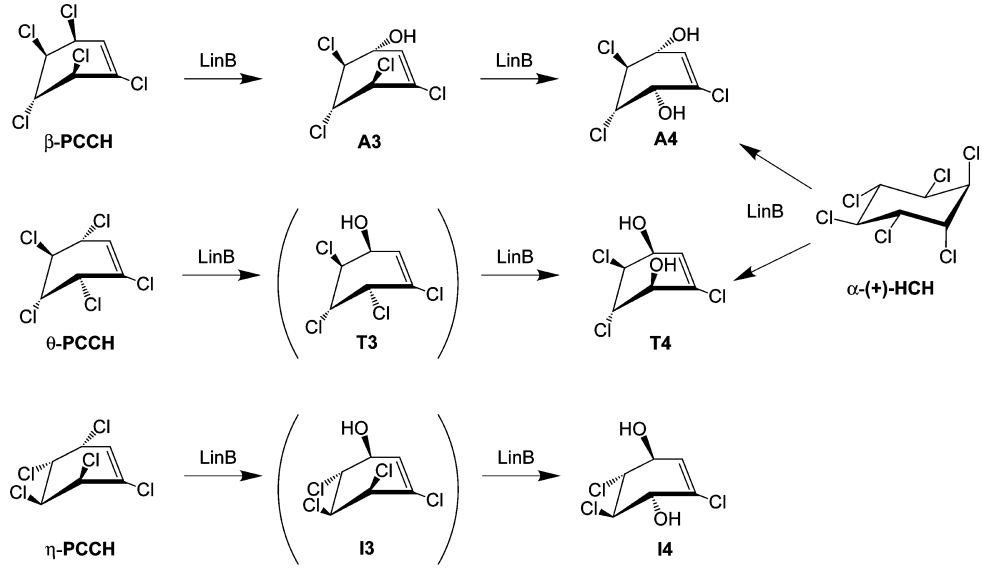
RESULTS

Stereochemical and Conformational Analysis of Pentachlorocyclohexenes (PCCHs) Produced from Different 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 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

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, Corresponding 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 (∗, Second Reaction Results in the Other Enantiomer of γ-PCCH), and (e) *cis* Elimination of H-4_a/Cl-5_e



Scheme 3. Degradation Pathway of α-HCH and β-, θ-, and η-PCCH (Reaction Scheme Arbitrarily Shown for (+)-α-HCH and Its PCCHs)[a]



[a] Compounds in parentheses were not isolated or characterized.

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 we abbreviate 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*).

Dependent upon the solvent, the overlap of resonances was observed (^{1}H and/or ^{13}C NMR spectra), inhibiting appropriate signal assignment and/or exact determination of ^{1}H,^{1}H 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 ^{1}H NMR signals were significantly broadened and incomplete resolution of the coupling pattern in the ^{1}H 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, a nuclear Overhauser enhancement (NOE) 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 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 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, and $J_{56} = 8.0$ 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** and **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-, δ-, θ-, and 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 a 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 six 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. The formation of γ- 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 analogue 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 the 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 RIs for the A metabolites. When samples were acetylated and re-analyzed, the corresponding mono- and diacetates were observed with

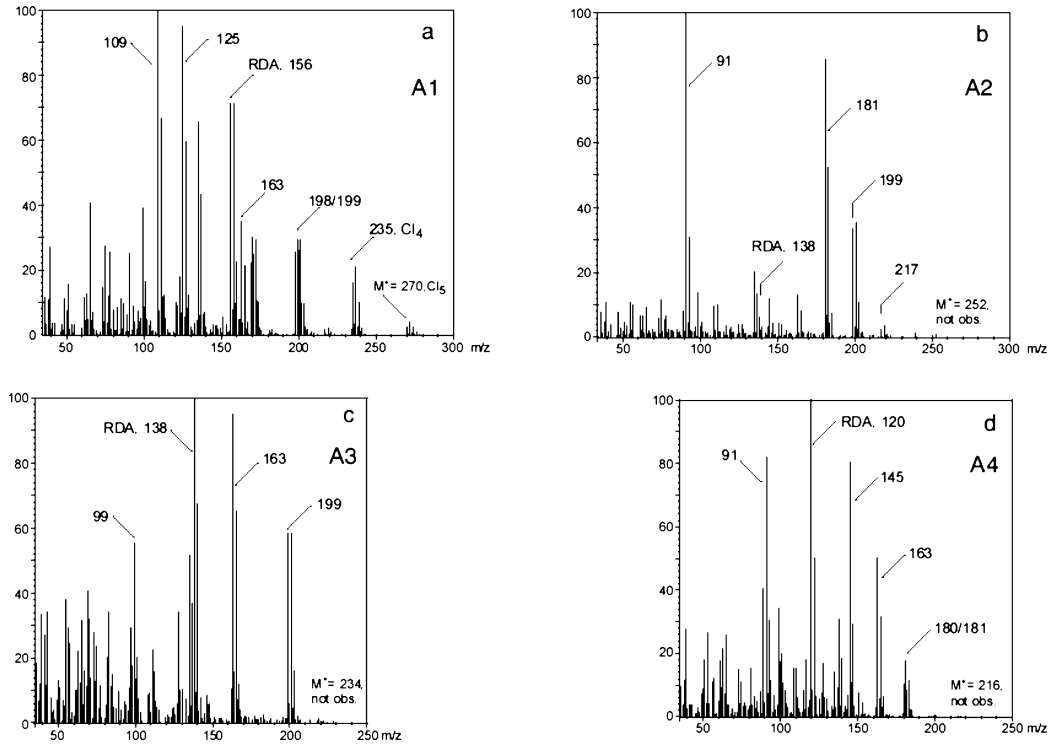


Figure 1. EI mass spectra of metabolites A1−A4.

Table 2. Retention and EI MS Data of (a) α-HCH/β-PCCH and (b) γ-HCH/γ-PCCH Metabolites

compound[a]	type of compound	RI	EI MS data[b]
	(a) Retention and EI MS Data of α-HCH/β-PCCH Metabolites		
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 **Figure 1a**
A2	tetrachlorocyclohexanediol	1804	(252); 217, 199, 181, 138 (RDA, weak), 91; see **Figure 1b**
A3	tetrachlorocyclohexenol	1535	(234); 199, 163, 138 (RDA); see **Figure 1c**
A4	trichlorocyclohexenediol	1564	(216); 180/181, 163, 145, 120 (RDA), 91; see **Figure 1d**
T4 and I4	trichlorocyclohexenediols	1553	(216); 180/181; 163; 145; 120 (RDA); 91
	(b) Retention and EI MS Data of γ-HCH/γ-PCCH Metabolites		
γ-HCH	hexachlorocyclohexane	1758	288
γ-PCCH	pentachlorocyclohexene	1460	252
2,5-DDOL	dichlorocyclohexadienediol	1429	180, 178, 162, 145; see **Figure 2d**
2,6-DDOL	dichlorocyclohexadienediol	1454	180, 178, 162, 145
G3	tetrachlorocyclohexenol	1532	(234); 199, 163, 138 (RDA); see **Figure 2a**
G4	trichlorocyclohexenediol	1628	(216); 180/181, 163, 145, 120 (RDA), see **Figure 2c**
G3b	tetrachlorocyclohexenol	1558	234, Cl_4; 199, 163, 138 (RDA); see **Figure 2b**

[a] For structures, see **Schemes 1–4**. [b] EI MS data are reported as follows: all ions, monoisotopic, first number, molecular ion (not observed if in parentheses), followed by important fragment ions.

appropriate shifts in retention times and masses (data not shown). Further, traces of a dichlorophenol ($M^+ = 162$, Cl_2) and a trichlorobenzene ($M^+ = 180$, Cl_3) 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, H-6, H-5, and H-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

Scheme 4. Degradation Pathway of γ-HCH and γ-PCCH (Absolute Stereochemistry Shown Arbitrarily)

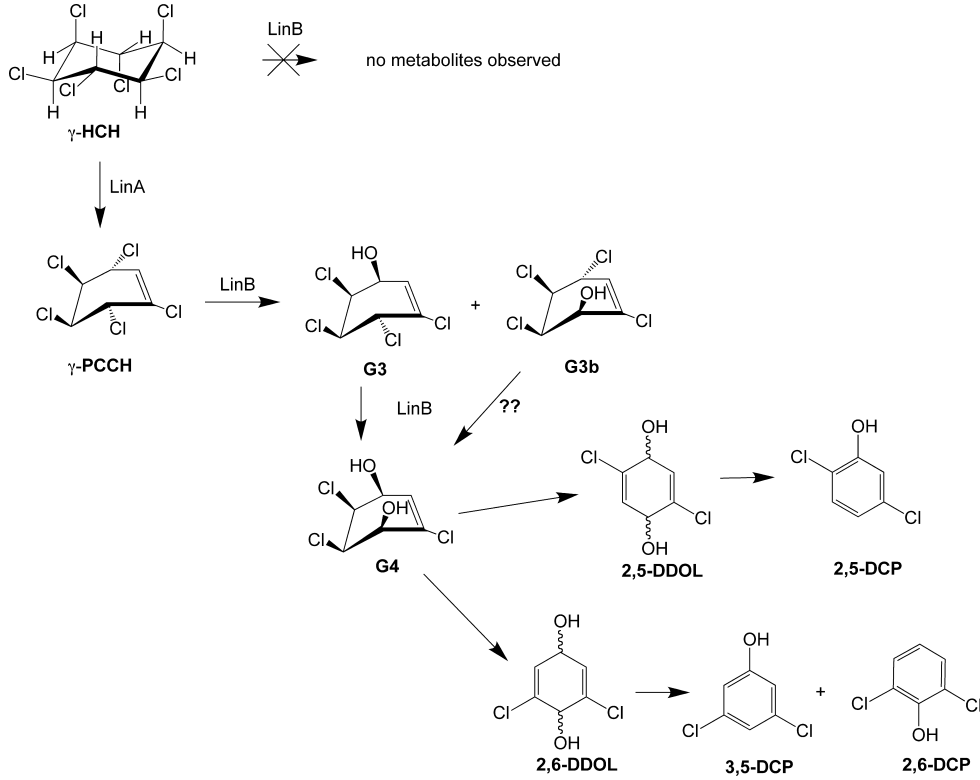


Table 3. ^{1}H and ^{13}C Chemical Shifts and ^{1}H,^{1}H Coupling Constants of Tetrachlorocyclohexeneols and Trichlorocyclohexenenediols in Benzene-d_6

	δ (^{1}H) (ppm) and J (^{1}H,^{1}H) (Hz)							δ (^{13}C) (ppm)						
position[a]	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	nd	134.2	136.1	nd
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		$J_{1\text{-OH}}$ = 10.2	J_{13} = 1.4	$J_{1\text{-OH}}$ = 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} = nd	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 $CDCl_3$ and G4 measured in acetone-d_6 solution. [c] All other OH resonances were broad (and not assignable) or not detected.

scalar coupling constants (*10*)]. In the ^{1}H NMR spectra, the signals of at least one additional compound could be detected. Although the correlations observed in the ^{1}H,^{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 PCCHs. Besides remaining α-HCH, these reaction mixtures contained A4, D4, T4, and I4 (typical composition 58:10:3:2). Furthermore, detailed analysis of the aromatic region of the ^{1}H NMR spectra revealed the presence of small amounts of 1,2,4-trichlorobenzene and 2,5-dichlorophenol. These data also clearly show that PCCHs were more rapidly converted than α-HCH. Incubation of pure α-HCH with *E. coli* expressing LinB mainly led to the formation of A4 and T4 (see the Discussion).

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

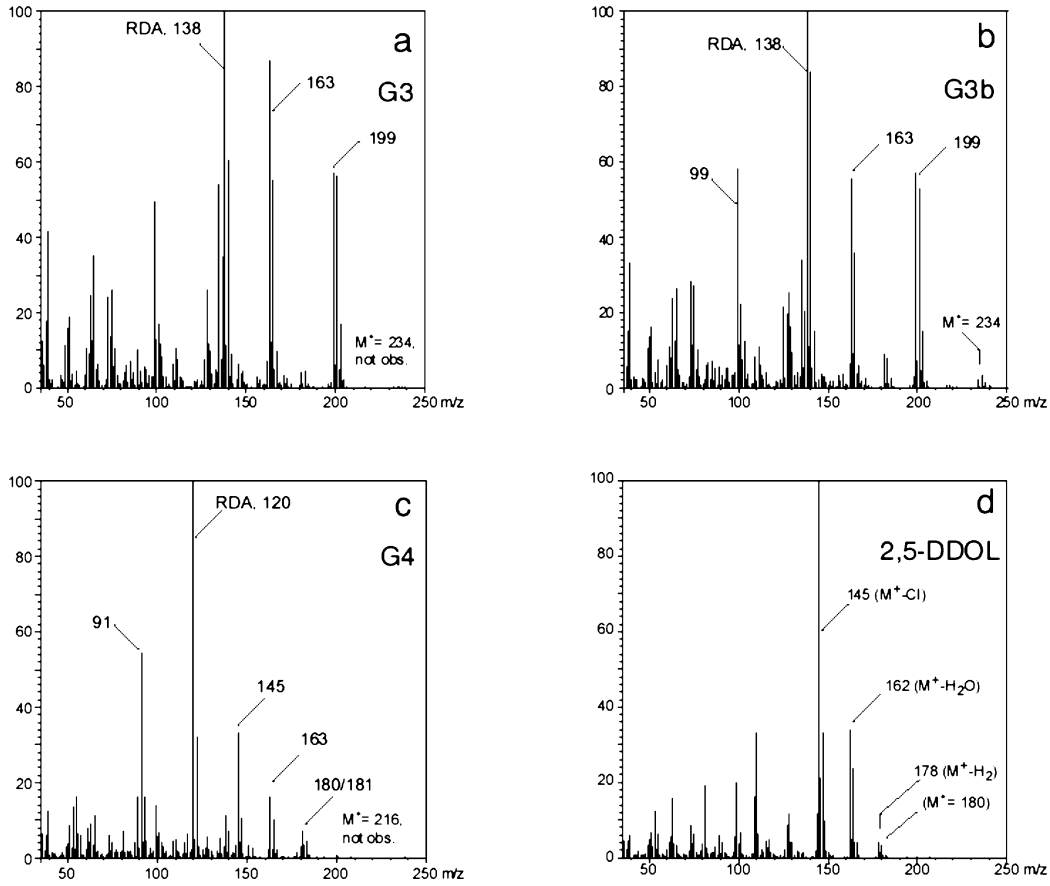


Figure 2. EI mass spectra of γ-PCCH metabolites.

The identification of 2,5- and 2,6-dichloro-2,5-cyclohexadiene-1,4-diol (2,5- and 2,6-DDOL) by GC−MS was not straightforward (see spectrum for 2,5-DDOL in **Figure 2d**) because molecular ions (M^+) at *m/z* 180 are not easily recognized. However, the fragment ions (monoisotopic) at *m/z* 178, 162, and 145, interpreted as M^+-2H, M^+-H_2O, and M^+-Cl, respectively, and the formation of a diacetate eventually confirmed this identification (data not reported). In the ^1H NMR spectra of samples from γ-PCCH incubation experiments with *E. coli* expressing LinB, the resonances of five unsaturated metabolites were observed. These metabolites were identified as G3, G3b, G4, 2,5-DDOL, and 2,6-DDOL (**Scheme 4**).

In the case of G3, it is evident from the $^1H,^1H$ coupling constants that H-4 and H-5 are in axial and pseudo-axial positions ($J_{45} = 6.5$ Hz, **Table 3**), whereas from $J_{56} = 2.3$ Hz and $J_{16} = 3.7$ Hz, we conclude that the relative stereochemistry indicated in **Scheme 4** must be correct (for atom numbering, see **Scheme 1**). In benzene solution, NOEs were observed from H-5 → H-1 and from H-1 → H-2, H-5, and H-6. This proved that H-1 and H-5 are on the same side of the cyclohexane ring plane and H-4 is on the opposite side. Again, the data indicate that the Cl/OH substitution reaction from γ-PCCH to G3 proceeded in a S_N2-type reaction under inversion of the configuration at C-3.

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

Table 4. ^1H and ^{13}C Chemical Shifts and $^1H,^1H$ Coupling Constants of Dichlorocyclohexadienediols (DDOL) in Benzene-d_6

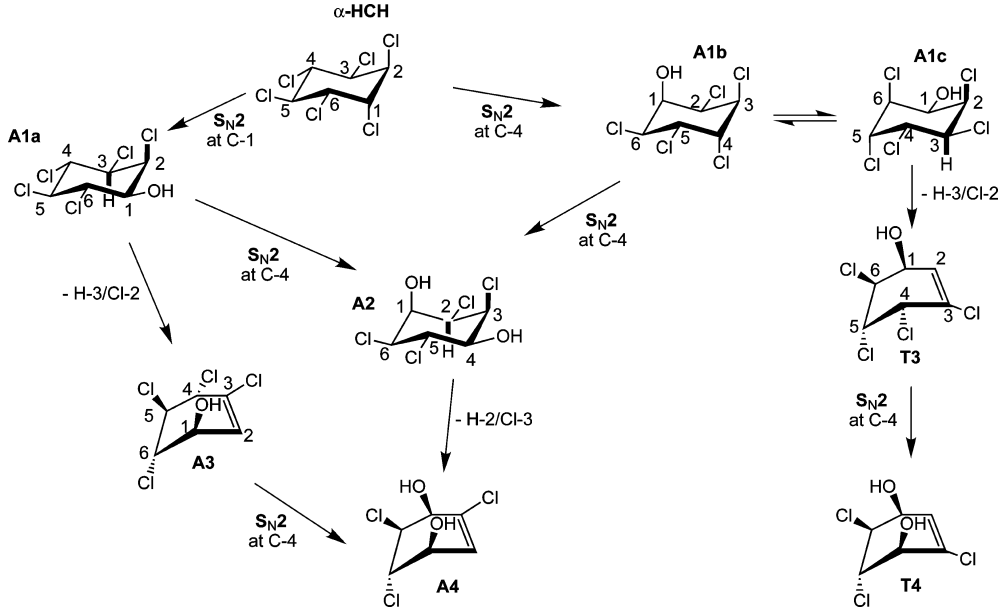
	δ (^1H) (ppm) and J ($^1H,^1H$) (Hz)		δ (^{13}C) (ppm)	
position[a]	2,5-DDOL[b]	2,6-DDOL	2,5-DDOL	2,6-DDOL
1	3.85	3.85	66.7	68.4
2			135.2	133.6
3	5.63	5.67	126.2	127.0
4		3.9		64.2
	$J_{16} = 3.8$	$J_{34} = 4.0$		

[a] For atom numbers, see **Scheme 1**. [b] δ (^1H) of 4.56 and 6.14 ppm observed in $CDCl_3$ solution corresponds to earlier findings (*17*).

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 the Supporting Information). From the NMR data, it is evident that H-5 and H-6 have a dihedral angle close to 90° ($J_{56} = 2.0$ 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

Scheme 5. Proposed Reaction Mechanism for the Formation of Metabolites A4 and T4 from α-HCH by LinB



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 $^{1}H,^{1}H$ coupling constants. The signals of H-4 and H-5 were both 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 the absence of biological active media, 1.6% of 2,6-DCP was also 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 and D1) and tetrachlorocyclohexanediols (B2 and 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 were both formed by S_N2-type reactions, with replacements of equatorial chlorines in the parent HCHs.

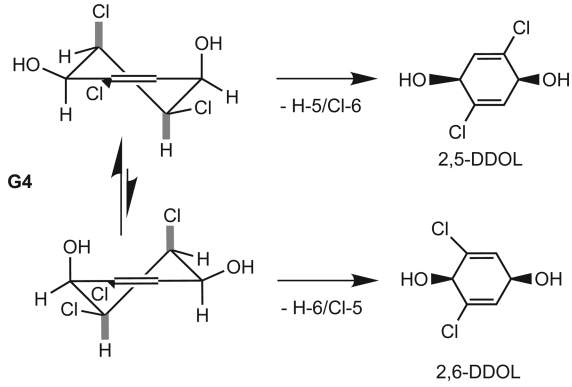
In the present study, we show that LinB from *S. indicum* B90A readily metabolizes β- and γ-PCCH. The tetrachlorocyclohexenols (A3 and G3) as well as the trichlorocyclohexenediols (A4 and G4) were isolated and characterized; both pairs are constitutional isomers of D3 and D4, respectively. Each metabolite 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 of 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.

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

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

Scheme 6. Formation of 2,5- and 2,6-DDOL by *trans*-Diaxial HCl Eliminations of Two Conformers of γ-HCH Metabolite G4



tabolites. 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 the 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, because at the neighboring carbons, a chlorine and a proton are always 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. On the basis of 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 dehydratation reactions occur non-enzymatically 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 thus far neither 1,4-TCDN nor 2,4,5-DNOL was 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. On the basis of our data, we suggest an additional branch of the pathway for the degradation of γ-HCH in *S. indicum* B90A (**Scheme 4**) with hydroxylation reactions catalyzed by LinB to yield G3 and G4, with 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 metabolites. More detailed experiments will be needed to exactly evaluate the formation and further metabolism of such metabolites *in vivo*.

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Supporting Information Available: $^{1}H,^{13}C$ HMBC correlations used for NMR shift assignments (Table S1), characterization of aromatic compounds, ^{1}H NMR spectra of PCCH isomers in benzene-d_6 (Figure S1), ^{1}H NMR spectra of metabolites from β-PCCH incubated with LinB (B90A) measured in C_6D_6 (Figure S2), ^{1}H NMR spectra of metabolites from γ-PCCH incubated with LinB (B90A) in $CDCl_3$ (Figure S3), and samples separated by flash chromatography, elution conditions, and compositions of fractions of interest (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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