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# Enzymatic Conversion of ε-Hexachlorocyclohexane And a Heptachlorocyclohexane Isomer, Two Neglected Components of Technical Hexachlorocyclohexane

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## ABSTRACT

α-, β, γ-, and δhexachlorocyclohexane (HCH), the four major isomers of technical HCH, are susceptible to biotic transformations, whereby only α- and γ-HCH undergo complete mineralization. Nevertheless, LinA and LinB catalyzing HCl elimination and hydrolytic dehalogenations, respectively, as initial steps in the mineralization also convert β- and δ-HCH to a variety of mainly hydroxylated metabolites. In this study, we describe the isolation of two minor components of technical HCH, ε-HCH and heptachlorocyclohexane (HeCH) and we present data on enzymatic transformations of both compounds by two dehydrochlorinases (LinA1 and LinA2) and a haloalkane dehalogenase (LinB) from *Sphingobium indicum* B90A. In contrast to reactions with α-, γ-, and δ-HCH, both LinA enzymes converted ε-HCH to a mixture of 1,2,4-, 1,2,3- and 1,3,5-trichlorobenzenes without the accumulation of pentachlorocyclohexene as intermediate. Furthermore, both LinA enzymes were able to convert HeCH to a mixture of 1,2,3,4- and 1,2,3,5-tetrachlorobenzene. LinB hydroxylated ε-HCH to pentachlorocyclohexanol and tetrachlorocyclohexane-1,4-diol, whereas hexachlorocyclohexanol was the sole product when HeCH was incubated with LinB. The data clearly indicate that various metabolites are formed from minor components of technical HCH mixtures. Such metabolites will contribute to the overall toxic potential of HCH contaminations and may constitute serious, yet unknown environmental risks and must not be neglected in proper risk assessments.



Keywords

HCH; LinA; LinB; dehydrochlorinase; pentachlorocyclohexanol; tetrachlorocyclohexane-1,4-diol; heptachlorocyclohexane; hexachlorocyclohexanol

## INTRODUCTION

Technical hexachlorocyclohexane (t-HCH) consists of the insecticidal γ-isomer (10-18%) and the non-insecticidal α- (53-70%), β- (3-14%), δ- (6-10%), and ε-isomers (1-5%), and also contains up to 5% hepta- and even smaller amounts of octachlorocyclohexanes (Figure S1).[1-4] Introduced in the 1940s, t-HCH was widely used in agriculture and insect control. In the 1970s, it was eventually banned in most industrialized countries and replaced by lindane, which consists of more than 99% of the insecticidal γ-isomer. However, HCH residues from former application of t-HCH and from lindane production continue to persist in the environment and to this date constitute a major environmental problem.[5] Due to environmental persistence and toxic potential, α-, β-, and γ-HCH, the three major components of t-HCH, were added to the list of persistent organic pollutants according to the Stockholm convention (POPs list) in 2009 (http://www.pops.int).[1, 6]

ε-HCH and heptachlorocyclohexane account for approximately 5% of t-HCH and, to our knowledge, they have never been rigorously characterized with respect to physico-chemical properties, toxicity, and degradability. In view of an estimated amount of 10 million tons of t-HCH used worldwide in the second half of the last century,[7] even minor components, such as ε-HCH and heptachlorocyclohexane, have led to total environmental loadings of up to 500,000 tons through direct application of t-HCH as insecticide or through improper disposal of HCH residues from γ-HCH production.[5] Therefore, such components may constitute serious, yet unknown environmental risks and must not be neglected.

Microbial degradation of α-, β-, γ-, and δ-HCH was investigated under aerobic and anaerobic conditions.[5, 8, 9] Dehydrochlorination, but not mineralization of all four isomers occurs under anaerobic conditions and mainly leads to chlorobenzenes and benzene.[8] Biochemical pathways for HCH transformation under anoxic conditions were proposed,[10-12] but responsible enzymes have not been identified yet. In contrast, several bacterial strains that

degrade HCH isomers under aerobic conditions were described in detail; they mainly belong to the family of *Sphingomonadaceae*.[13-15] Such isolates usually contain a set of genes (*lin* genes) coding for HCH transforming enzymes. A HCH dehydrochlorinase (LinA) and a haloalkane dehalogenase (LinB, E.C. 3.8.1.5) catalyze the first steps in the degradation pathway of γ-HCH. LinA is also responsible for dehydrochlorination of both enantiomers of the chiral α-HCH isomer and the more recalcitrant δ-HCH isomer, whereas LinB directly acts on δ-HCH and β-HCH.[16, 17] In general, more than 90% of the chlorine was released from γ-HCH after incubations with *Sphingobium indicum* B90A, *S. francense* Sp+, or *S. japonicum* UT26 indicating almost complete dechlorination.[18] Similar results were obtained for α-HCH, although only the first steps of the degradation pathway were described so far.[19] β-HCH is completely converted by LinB from *S. indicum* B90A, but partially hydroxylated metabolites seem to be terminal products of this biotransformation.[20] δ-HCH also serves as a direct substrate for LinA and LinB.[16, 19] This network of competing reactions in the degradative pathways yields a complex mixture of hydroxylated and dehydrochlorinated metabolites,[16, 20, 21] each of which may contribute to the overall toxic potential of HCH contaminations.

Since ε-HCH has so far not been commercially available in amounts suitable for transformation experiments, it was only hypothesized that ε-HCH should also be a direct substrate for LinA and LinB. Here, we conducted experiments to test this hypothesis and report on protocols for the enrichment of ε-HCH from t-HCH, on enzymatic transformations of ε-HCH and heptachlorocyclohexane, and on identifying emerging metabolites.

## EXPERIMENTAL PROCEDURES

### Plasmids and bacterial strains

LinA1, LinA2 and LinB from *Sphingobium indicum* were produced recombinantly in *E. coli* BL21 AI. The three genes *linA1*, *linA2,* and *linB* were codon optimized to allow higher expression levels of soluble protein and cloned into pDEST17 with an N-terminal His-tag

(plasmids were obtained from John Oakeshott, CSIRO, Canberra, Australia). For the expression of LinA1 and LinA2, the chaperone plasmid pGro7 (Takara Inc., Saint-Germain-en-Laye, France) was additionally transformed into the expression strains to enhance folding of the target proteins.

**Chemical and analytical reagents**

The analytical standard of ε-HCH (99.9% purity) was purchased from Dr. Ehrenstorfer GmbH, Germany. α-HCH (99.8%), β-HCH (99.2%), γ-HCH (99%), δ-HCH (99.7%), 1,2,3-, 1,2,4-, and 1,3,5-trichlorobenzene (TCBs), 1,2,3,4- 1,2,3,5-, 1,2,4,5-tetrachlorobenzene (TeCBs), pentachloronitrobenzene (PCNB), HPLC grade methanol, heptane, hexane, ethyl acetate and isopropanol were purchased from Sigma-Aldrich (Buchs, Switzerland). Technical HCH was obtained from India Pesticide Ltd. (Lucknow, Uttar Pradesh, India).

**Purification of ε-HCH from t-HCH**

ε-HCH was isolated by extraction of t-HCH with ice-cold methanol followed by preparative HPLC. The available batch of t-HCH contained approximately 1% ε-HCH (determined by GC-MS based on TIC). An enrichment up to 3.3% ε-HCH and about 50% δ-HCH (relative amounts determined by $^{1}$H NMR, see below) was obtained by a two-fold extraction of typically 1 g of t-HCH with 0.8 mL ice-cold methanol. The preparative HPLC system consisted of a model P680 HPLC pump (Dionex, Olten, Switzerland) fitted with a manual injection valve (Rheodyne 7125) and a refractive index (RI) detector (ERC-7512, Erma Cr Inc, Tokyo, Japan). Normal-phase separation was achieved on a pre-packed semi-preparative column (Nucleosil 100-10 CN, 10 µm, 250x10 mm, Macherey-Nagel AG, Düren, Germany) with 2.5 vol % isopropanol in heptane as the mobile phase (isocratic elution, flow rate 4 mL min$^{-1}$.) The dry residue of the methanolic extract of t-HCH was partially re-dissolved in 3 mL of the mobile phase and passed through a syringe filter (pore size, 45 µm;

Chromafil GF/PET-45/25, Macherey-Nagel AG, Düren, Germany). Approximately 2 mL of this solution was injected into the HPLC and we observed elution of α-HCH, γ-HCH, ε-HCH, δ-HCH, and β-HCH at 5.5, 6.0, 11.1, 14.5, and 16.0 min, respectively (Figure S2). The combined ε-HCH containing fractions of forty HPLC runs were recrystallized, from heptane : acetone 1:1 and washed with 1 mL of heptane. The purity of the enriched ε-HCH was evaluated by means of NMR and GC-MS.

**GC-MS analysis**

To follow the purification of ε-HCH from t-HCH and to monitor enzyme activities, we performed GC-MS analyses using a Finnigan Voyager quadrupole MS under electron impact ionization (EI, 70 eV, 200°C) and full-scan (m/z 35-435, 0.6 s/scan, nominal mass resolution) or selected ion monitoring (SIM) conditions. The GC conditions were as follows: split/splitless injection (250°C, 48 s splitless) on a 30-m BGB-5 fused silica column (0.25 mm i.D., 25 µm film, BGB Analytik, Adliswil, Switzerland) and a temperature program starting from 70°C, 2 min isothermal then increasing at 25°C/min to 120°C and at 5°C/min to 220°C and finally at 25°C/min to 280°C, followed by a 2-min isothermal hold. Helium was used as a carrier gas at constant pressure of 50 psi (3.4 bar).

**Expression and purification of 6X His-tagged LinA1, LinA2 and LinB**

*E. coli* BL 21 AI cells expressing 6X His-tagged LinA1 and LinB were grown in LB at 37°C.At an $OD_{600}$ of 0.6, the protein expression was induced by adding 2 g/L of L-(+)-arabinose. The strains expressing LinA1 and LinB were further incubated for 12 and 6 hours, respectively. Cells were harvested by centrifugation ,washed once with water and stored at -20°C.

A fed-batch fermentation was carried out to cultivate *E. coli* BL 21 AI cells expressing 6X His-tagged LinA2 according to Geueke et al.[22] The feed was started after 15 hours. The cells

were induced with 2 g/L of L-(+)-arabinose after 17 hours of cultivation and the temperature was reduced to 30°C. After a total fermentation time of 21 hours, 160 g of cells (wet weight) were harvested from 1.6 L, separated by centrifugation, frozen in liquid nitrogen and stored at -20°C.

For protein purification, the cells were suspended 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 7.5, lysed by ultrasonification or chemically by the addition of 1X BugBuster, 1 µl $mL^{-1}$ rlysozyme, and 2 µl $mL^{-1}$ benzonase (Novagen). After centrifugation, the supernatant was loaded onto an Ni-NTA Superflow cartridge (5 mL; Qiagen, Hombrechtikon, Switzerland) and the proteinwas eluted by increasing the imidazole concentration up to 300 mM. The proteins were stored in 50 mM sodium phosphate buffer, pH 7.5, at 4°C. We typically obtained 4-8 mg pure protein per L medium from the shaking flask cultivations with LinA1 and LinB. LinA2 was produced with a yield of 75 mg per L medium.

We used a NanoDrop ND-1000 device (ThermoScientific, Wilmington, US) for spectrophotometric quantification of purified protein. Novex 10% tricine gels (Invitrogen, Basel, Switzerland) were used to check the expression levels and purity of the proteins. LinA1 and LinA2 had a purity of about 70% according to digital image analysis of the Coomassie stained gels. LinA2 activity was assayed by measuring the degradation of 0.17 mM γ-HCH in 25 mM Tris / 192 mM glycine buffer (pH 8.3) in a total reaction volume of 1 ml at 22°C. LinB activity was analyzed under the same reaction conditions, but β-HCH was used as substrate. The reactions were stopped after defined time intervals by the addition of 2 ml ethylacetate and the extracts were analyzed by GC-MS. One Unit was defined as the amount of enzyme that degrades 1 µmol of substrate per minute. LinA2 and LinB exhibited specific activities of 5.4 and 0.004 U/mg protein, respectively.

**Conversion of ε-HCH by LinA1, LinA2 and LinB**

ε-HCH was incubated with purified LinA1, LinA2, and LinB from *S. indicum* B90A. Unless otherwise stated, the reaction mixtures contained 0.4, 0.08, and 0.5 mg $mL^{-1}$ of LinA1, LinA2, and LinB, respectively, in 25 mM Tris/192 mM glycine buffer (pH 8.3). For the time-dependent measurements with the two LinA enzymes, 0.1 mg of ε-HCH was dissolved in a total volume of 1 mL in 4-mL vials that were sealed with PTFE/rubber septa to prevent the loss of volatile compounds during incubations. 2 mL of ethyl acetate that contained 80 g of PCNB as internal standard[23] were added through the septum to stop and extract the reaction mixtures. For NMR experiments, 2.5 to 5 mg of ε-HCH were dissolved in a total volume of 5 mL, incubated with 2.5 to 5 mg of enzyme for 24 hours and extracted twice with equal volumes of ethyl acetate. The activities of the two LinA and the LinB enzymes were confirmed by incubation experiments with γ-HCH and β-HCH, respectively. To verify the persistence of ε-HCH under reaction conditions, a negative control was set up without the addition of enzyme. All vials were incubated at room temperature under shaking at 200 rpm for 24 hours.

**Chemical dehydrochlorination of ε-HCH**

For chemical dehydrochlorination, we incubated ε-HCH under alkaline conditions according to the procedure described by Trantírek et al.[24] for γ-HCH. Approximately 3 to 4 mg of ε-HCH were dissolved in 0.5 mL acetonitrile and the reaction was started by addition of 0.25 mL of 0.1 M NaOH. The reaction mixture was stirred for 20 min at 40°C, stopped by addition of 50 l of 12 M HCl, extracted twice with 1.5 mL of hexane, and analyzed by means of GC-MS and NMR.

**NMR analysis**

The $^{1}H$ and $^{13}C$ NMR spectra were recorded at 400.13 and 100.61 MHz, respectively, on a Bruker Avance-400 NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland). The

1D $^{1}H$ and $^{13}C$ NMR spectra and the $^{1}H$-$^{13}C$ and $^{1}H$-$^{1}H$ 2D correlation experiments were performed at 298 K using a 5 mm broadband inverse probe with z-gradient and 90° pulse lengths of 6.8 ($^{1}H$) and 14.5 s ($^{13}C$). All experiments were performed using the Bruker standard parameter sets and pulse programs and the $^{1}H/^{13}C$ chemical shifts were referenced internally using the resonances of acetone-$d_6$ at 2.05/29.8 ppm or $CDCl_3$ at 7.26/77.0 ppm. The relative amounts of metabolites were determined by integration of signals sufficiently separated from other resonances in the $^{1}H$ NMR spectra.

**Data analysis**

For parameter estimation by non-linear regression, we used the modelling software AQUASIM [25]. Degradation of parent and formation of metabolites were fitted to experimental data assuming a first order transformation reaction.

## RESULTS

### Isolation of ε-HCH and HeCH from t-HCH

Because neither commercial sources for mg-amounts nor procedures for the synthesis of ε-HCH were available, we first developed a protocol based on methanolic extraction and preparative HPLC for the isolation of ε-HCH from t-HCH. The purity of the ε-HCH preparation after separation by HPLC was evaluated by means of GC-MS and compared with the analytical standard for ε-HCH. Besides ε-HCH, it also contained approximately 30% of a heptachlorocyclohexane isomer (HeCH), which is a known minor component of t-HCH. Recrystallization from heptane/acetone significantly reduced the amount of the impurity. With this newly developed purification protocol we were able to prepare 100 mg of ε-HCH with a purity of 90%.

As outlined above, the purified ε-HCH fraction contained about 10% HeCH. This gave us the opportunity to not only investigate the enzymatic transformation of ε-HCH but also that of HeCH. HeCH had a GC retention time of 18.5 min (Figure 1A) and its highest mass ion

(*m/z* 287) in the mass spectrum corresponded to $(M\text{-}Cl)^+$. The compound was also characterized by NMR and from the relative signal intensities in the $^1H$ NMR spectrum (ratio of 2:2:1), we concluded that HeCH must be symmetric (mirror plane through positions 1 and 4 of the molecule). From the magnitudes of the $^1H,^1H$ coupling constants ($J_{23} \approx J_{34} \approx 10.5$ Hz), we inferred that the relative configurations of H-2 with respect to H-3 and of H-3 with respect to H-4 must both be *trans-diaxial*. Additionally, the chemical shift of 92.2 ppm found in the $^1H$-$^{13}C$ HMBC NMR spectrum was typical for a quarternary carbon bearing two chlorines (Table S3). These data clearly indicate that HeCH had the configuration depicted in Figure S1.

**Transformation of ε-HCH and HeCH by LinB**

Incubation experiments of ε-HCH with purified LinB indicated significant transformation of ε-HCH. After a reaction time of 24 hours, signals of two new metabolites, E1 and E2, were observed in the total ion chromatogram (Figure 1A). Based on mass spectra, they were identified as pentachlorocyclohexanol and tetrachlorocyclohexanediol (Figure 1C and 1D).

NMR data confirmed the formation of the two hydroxylated products from ε-HCH by LinB. The assigned $^1H$ and $^{13}C$ chemical shifts are summarized in Table S2 and S3 and they clearly show that the mono-hydroxylated product E1 had a mirror plane through positions 1 and 4 of the molecule (Figure 2). Positions 2/6 and 3/5 were equivalent with doubled signal intensities in the $^1H$ NMR spectrum. We determined $^1H,^1H$ coupling constants of $J_{12} = 9.5$; $J_{23} = 11.0$; $J_{34} = 2.9$ Hz and from the magnitude of $J_{12}$ it was evident that H-1 was in *trans-diaxial* position relative to H-2 (and also to H-6), whereas $J_{34} = 2.9$ Hz was typical for H-4 in equatorial position. E2 was identified as tetrachlorocylcohexane-1,4-diol having the two hydroxyl groups in 1,4-*trans-diequatorial* position (Figure 2). The bis-hydroxylated product E2 showed resonances at only two positions in the $^1H$ NMR spectra (intensity ratio of 2:1). As observed for the parent ε-HCH, the resonances of E2 were of higher order, but from line

shape simulations it was evident that $J_{12}$ was in the order of 10 Hz (2.9 Hz in the case of ε-HCH; coupling constants of similar magnitudes were obtained by line shape simulations of the $^1$H NMR resonances of E2 in benzene and in chloroform solutions, data not shown).

The incubation experiments with LinB revealed that also HeCH disappeared over time and that a hydroxylated metabolite (He1) with a retention time of 20.0 min concomitantly appeared (Figure 1A, chemical structure shown in Fig. 2). The mass spectrum of this metabolite could be rationalized in analogy to that of E1 as hexachlorocyclohexanol (Figure 1F; Table S1). In the $^1$H NMR spectra the magnitudes of $J_{12}$ changed from 10.5 Hz for HeCH to a value of 2.3 Hz for He1 (Table S3). Thus, we concluded that the configuration at position 1 in the molecule must have been inverted compared to the starting material.

**Transformation of ε-HCH and HeCH by LinA1 and LinA2**

Incubations of ε-HCH with LinA1 and LinA2 showed that ε-HCH served as substrate for both enzymes. The enzymes transformed ε-HCH mainly to 1,2,4-TCB and smaller amounts of 1,2,3- and 1,3,5-TCB (Figure S3A). We could not detect reaction intermediates derived from ε-HCH by means of GC-MS or NMR, although we specifically screened for the presence of pentachlorocyclohexene (PCCH) that would be the first expected intermediate. Attempts to prepare PCCH from ε-HCH chemically under alkaline conditions according to the same procedure as previously reported for α-HCH[19] were not successful. Even though ε-HCH reacted under these conditions, only the three TCB isomers and small amounts of tetrachlorocyclohexadiene (TCDN) were observed in such mixtures.

Both LinA enzymes also transformed HeCH to hexachlorocyclohexene (HCCH) and subsequently to 1,2,3,4- and 1,2,3,5-tetrachlorobenzene (TeCB) as confirmed by NMR and GC-MS analyses (Table S3, Figure S3A and S3B). Owing to C2 symmetry, HCCH has three pairs of equivalent positions and we concluded that it is generated from HeCH by *trans*-diaxial HCl elimination. The two aromatic metabolites 1,2,3,4- and 1,2,3,5-TeCB were

detected in ratios of 5.5 to 1 and 6.5 to 1 in incubation experiments with LinA1 and LinA2, respectively.

The transformation of ε-HCH and HeCH to the respective metabolites by the two LinA enzymes was monitored over a period of 20 hours. As indicated in Figure 3, analysis of parent compounds and metabolites revealed a well closed mass balances for all reactions. Furthermore, we evaluated rate constants for the transformation of ε-HCH and HeCH and for the formation of aromatic, chlorinated metabolites with the modeling software AQUASIM.[25] Measured concentrations were fitted to the first order reaction model scheme shown in Figure S7. Initial substrate concentration was also a fit parameter of this model (Table 1). Because PCCH was not detected and HCCH was only measured in traces, we concluded that for the transformation of ε-HCH and HeCH the first dehydrochlorination step was rate limiting and, therefore, the rate constants $k_{\text{-ε–HCH}}$ and $k_{\text{-HeCH}}$ corresponded to the sums of the single rate constants $k_{\text{TCB}}$ and $k_{\text{TeCB}}$, respectively (Table 1). The modelled progress curves are included in Figure 3. We also estimated the $k_{\text{cat}}/K_{\text{M}}$ values for the reactions. LinA2 showed about 20-fold higher degradation rates for ε-HCH and HeCH than LinA1 did and both enzymes converted HeCH faster than ε-HCH (Table 1).

## DISCUSSION

We previously reported that LinB from *S. indicum* B90A transformed δ- and β-HCH to pentachlorocyclohexanols and tetrachlorocyclohexanediols, respectively. Furthermore, it was described that LinB exhibits a high regioselectivity for α-, β-, and δ-HCH leading preferentially to tetrachloroclyclohexane-1,4-diols.[16, 20, 24] The formation of E1 and E2 from ε-HCH is consistent with these previous observations. HeCH, however, was dehalogenated exclusively at position 4, while no reaction occurred at C-1. The additional chlorine atom at C-1 presumably impeded nucleophilic attack mediated by LinB. Most likely, this is a result

of steric hindrance and is in line with accepted views on the effect of substituents on the rates of $S_N2$ reactions. As we previously discovered for LinB catalyzed degradation reactions of δ- and β-HCH, we exclusively observed reaction products formed by inversion of configuration.[20] A general preference of LinB for the selective substitution of *equatorial* or *axial* Cl-atoms was not found. Interestingly, the resulting molecule E2 carries all substituents in equatorial positions. Hence it shows structural similarities to β-HCH, which is the most recalcitrant HCH isomer. For thorough risk assessment of E2, more detailed information on environmental fate and formation and further transformation *in vivo* will be needed.

In contrast to what was observed previously in incubations of LinA with other HCH isomers [19, 21, 24], the two dehydrochlorinase variants LinA1 and LinA2 converted ε-HCH and HeCH directly to various chlorobenzene isomers without the transient accumulation of any dehydrochlorinated intermediates. Since also the chemical dehydrohalogenation of ε-HCH and HeCH led to the same metabolites, we concluded that the rate-limiting step in the transformation of ε-HCH and HeCH was the first dehydrochlorination reaction and that the putative ε-PCCH and HCCH intermediates, respectively, quickly underwent further dehydrochlorination steps in the course of the reaction. This view is supported by the fact that the first order reaction model depicted in Figure S7, except for transformation of HeCH by LinA1, well describes the transformation data (Figure 3). In case of LinA1 with HeCH as the substrate, it seems that after an initial exponential burst, the enzyme somehow switched to linear turnover. The values of the specificity constant $k_{cat}/K_M$ show that the HeCH is the better substrate than ε-HCH for both enzymes.

In Figure 4, we present a tentative reaction scheme for the degradation of ε-HCH by LinA enzymes. It has to be mentioned that ε-HCH is drawn in the energetically most preferred conformation with four equatorial Cl atoms and, therefore, four *trans*-diaxial HCl-arrangements. Due to a plane of symmetry, two of these arrangements, (5,4) and (6,1), and (3,4) and (2,1), respectively, are equivalent. Elimination of the two distinct *trans*-diaxial HCl-

pairs of ε-HCH (marked by bold lines) by LinA will yield (3*R*,4*S*,5*S*,6*S*)- and (3*S*,4*R*,5*R*,6*R*)-1,3,4,5,6-PCCH, respectively. In Figure 4, only the pathway starting with 5,4-*trans*-diaxial HCl-elimination is depicted. Elimination of 2,1-HCl is analogous except that the other PCCH enantiomer is formed as a tentative intermediate. The resulting PCCH enantiomer is stabilized in two half-chair conformations and both conformers still have at least one *trans*-diaxial HCl-pair accessible for elimination. Upon subsequent HCl elimination, one PCCH conformer yields (3*R*,6*S*)-1,3,4,6-tetrachlorocyclohexa-1,4-diene, while the other conformer yields (*E*)-1,3,5,6- tetrachlorocyclohexa-1,4-diene. These two TCDNs are susceptible to *syn*-1,4 elimination of HCl resulting in the formation of 1,2,4-TCB and 1,2,3- and 1,3,5-TCB, respectively.

The proposed pathway for transformation of HeCH by LinA is depicted in Figure 5. HeCH is dehydrochlorinated to HCCH by LinA. We postulate that HCCH is further converted via 1,2,3,5,6-pentachlorocyclohexa-1,3-diene to 1,2,3,4- and 1,2,3,5-TeCB. A similar elimination reaction was described for chorismate lyase releasing pyruvate from chorismate, which is also a cyclohexa-1,3-diene.[26] Control experiments have shown that for both pathways (ε-HCH and HeCH transformation) the first HCl elimination reaction is catalyzed by LinA, but at the moment it is not clear whether the second and third reaction steps are enzyme-catalyzed or occur spontaneously.

In general, our data support previous findings that the degradation of HCH isomers proceeds by a variety of competing reactions and leads to a complex mixture of chlorinated and hydroxylated metabolites. LinA1 and LinA2 catalyzed dehydrochlorination of ε-HCH and HeCH to different chlorinated benzenes, whereas LinB catalyzed regio- and stereoselective hydroxylation of ε-HCH and HeCH to pentachlorocyclohexanol (E1) and tetrachlorocyclohexane-1,4-diol (E2), and hexachlorocyclohexanol (He1), respectively. In a previous study, we demonstrate that metabolites generated by LinA and LinB persisted in

groundwater at a former HCH producton site. Especially the identification of hydroxylated metabolites originating form ε-HCH degradation will facilitate the specific analyses of pristine and contaminated soil and water samples and will allow the investigation of their metabolic and environmental fate. In view of the vast environmental loads of technical HCH, even minor components and their metabolites will contribute to the overall toxic potential of HCH contaminations and may constitute serious, yet unknown environmental risks and must not be neglected in proper risk assessments.

## ASSOCIATED CONTENT

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## Supporting Information Available

Structure of the HCH isomers; HPLC chromatogram; detailed analytical data: -EI-MS data, $^{1}H$-$^{13}C$ HMBC correlations used for NMR chemical shift assignment, $^{1}H$-$^{13}C$-HSQC data. This information is available free of charge via the Internet at http://pubs.acs.org/.

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**Table 1:** Modeled reaction rates and initial substrate concentrations for the formation of chlorinated benzenes from ε-HCH and HeCH.

| | | LinA1[a)] | LinA2[a)] |
|---|---|---|---|
| $c_{\varepsilon\text{–HCH},0}$ | [μM] | 262 ± 2 | 271 ± 3 |
| $k_{\text{-}\varepsilon\text{–HCH}}$ | [$10^{-3}$ $h^{-1}$] | 19.1 ± 0.8 | 73.8 ± 2.5 |
| $k_{1,2,4\text{-TCB}}$ | [$10^{-3}$ $h^{-1}$] | 17.2 ± 1.4 | 56.2 ± 3.4 |
| $k_{1,2,3\text{-TCB}}$ | [$10^{-3}$ $h^{-1}$] | 1.64 ± 0.70 | 9.9 ± 2.0 |
| $k_{1,3,5\text{-TCB}}$ | [$10^{-3}$ $h^{-1}$] | 0.29 ± 0.72 | 7.7 ± 1.9 |
| $k_{cat}/K_M$ | [$M^{-1}s^{-1}$] | 0.37 ± 0.02 | 7.32 ± 0.44 |
| | | | |
| $c_{HeCH,0}$ | [μM] | 14.6 ± 0.4 | 16.0 ± 0.4 |
| $k_{\text{-HeCH}}$ | [$10^{-3}$ $h^{-1}$] | 79.7 ± 6.8 | 325 ± 20 |
| $k_{1,2,3,4\text{-TeCB}}$ | [$10^{-3}$ $h^{-1}$] | 10.3 ± 4.5 | 47.8 ± 10.2 |
| $k_{1,2,3,5\text{-TeCB}}$ | [$10^{-3}$ $h^{-1}$] | 69.4 ± 10.1 | 277 ± 24 |
| $k_{cat}/K_M$ | [$M^{-1}s^{-1}$] | 1.56 ± 0.15 | 32.24 ± 2.55 |

**a)**Values are given ± standard deviation as determined by AQUASIM.[25] For derived quantities, proper error propagation was performed.

b)The $k_{cat}/K_M$ values were calculated according to the equation $k_{cat}/K_M = k_{-}/[E_0]$, where $[E_0]$ is the stoichiometric concentration of active centers and $k_{-}$ the rate constant for the degradation of the respective substrate. The coefficient of variation for the determination of $[E_0]$ was estimated to be 5%.

**Figure 1.** GC-MS total ion chromatogram of ethyl acetate extracts of incubation mixtures containing LinB and ε-HCH as the substrate (A) and corresponding EI mass spectra of ε-HCH (B), E1 (C), E2 (D), HeCH (E) and He1 (F). The highest mass ions correspond to $(M-Cl)^+$.

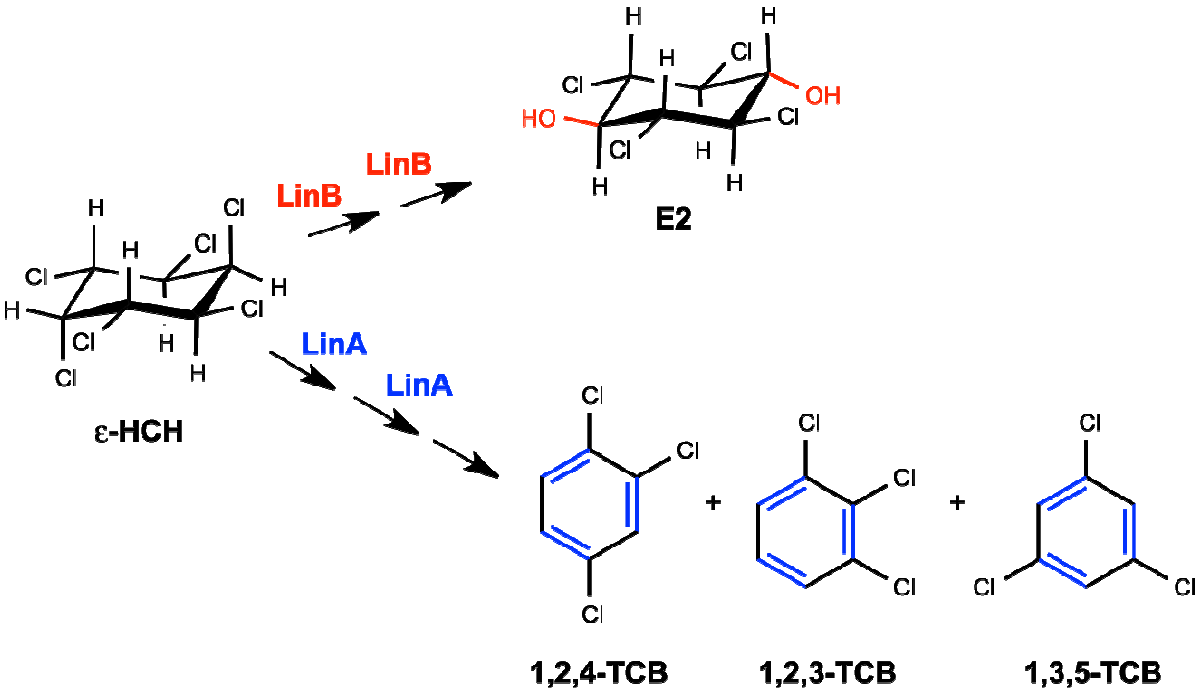
**Figure 2.** Proposed pathways for the transformation of ε-HCH and HeCH by LinB.

**Figure 3.** Progress curves of decrease of substrates and formation of metabolites for incubations of LinA1 and LinA2 with ε-HCH and HeCH as the substrates monitored by GC-MS. Incubations of LinA1 [0.4 mg $ml^{-1}$] with ε-HCH (A) and HeCH (C) and incubations of LinA2 [0.08 mg $mL^{-1}$] with ε-HCH (B) and HeCH (D). Measurements were done in duplicate. The symbols depict the substrates and metabolites as follows: ε-HCH (○), 1,2,4-TCB (▲), 1,2,3-TCB (△), 1,3,5-TCB (▼), HeCH (◇), 1,2,3,4-TeCB (■), 1,2,3,5-TeCB (□), sum of all reactants (●). Continuous lines represent the modeled progress curves.

**Figure 4.** Proposed pathway for the transformation of ε-HCH by LinA1 and LinA2. Depictions of conformations of chlorinated cyclohexenes and cyclohexadienes are based on the conformational analysis of core structures as given by Anet[27] and Rabideau.[28, 29]

**Figure 5.** Proposed pathway for the transformation of HeCH by LinA. Only the energetically most favored conformer of HeCH is considered. It should be noted that 6,1-HCl-elimmination in HeCH would lead to the other enantiomer of HCCH and pentachorocyclohexa-1,3-diene but to the same mixture of TeCBs.

504 **TOC ART**

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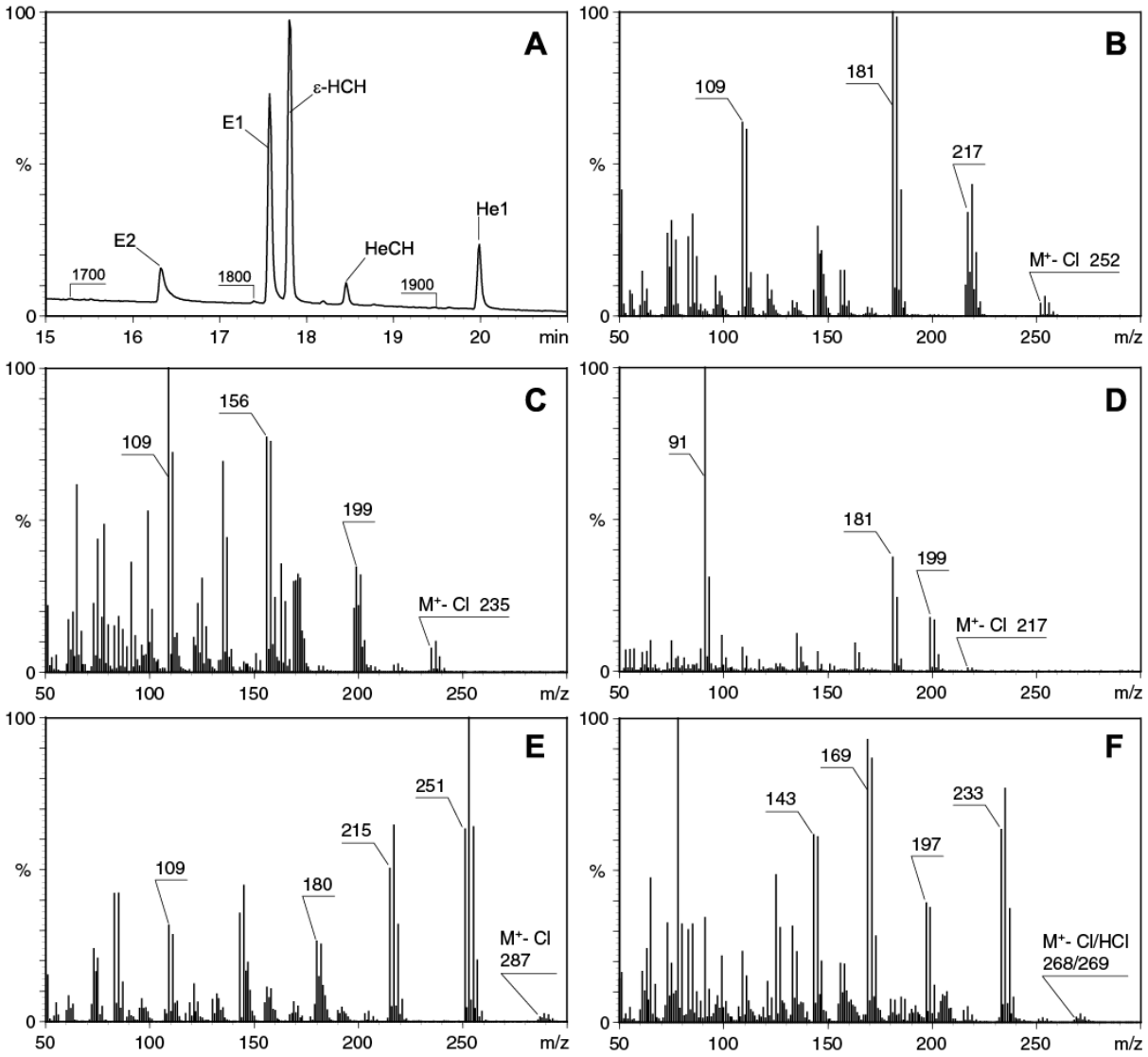


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509 Fig. 1

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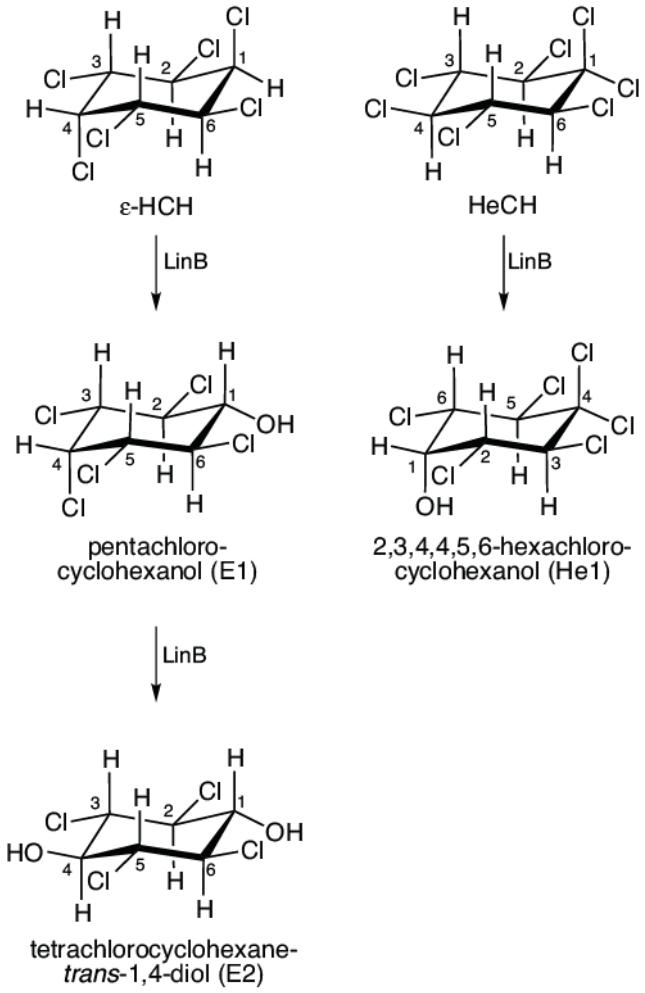


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512 Fig. 2

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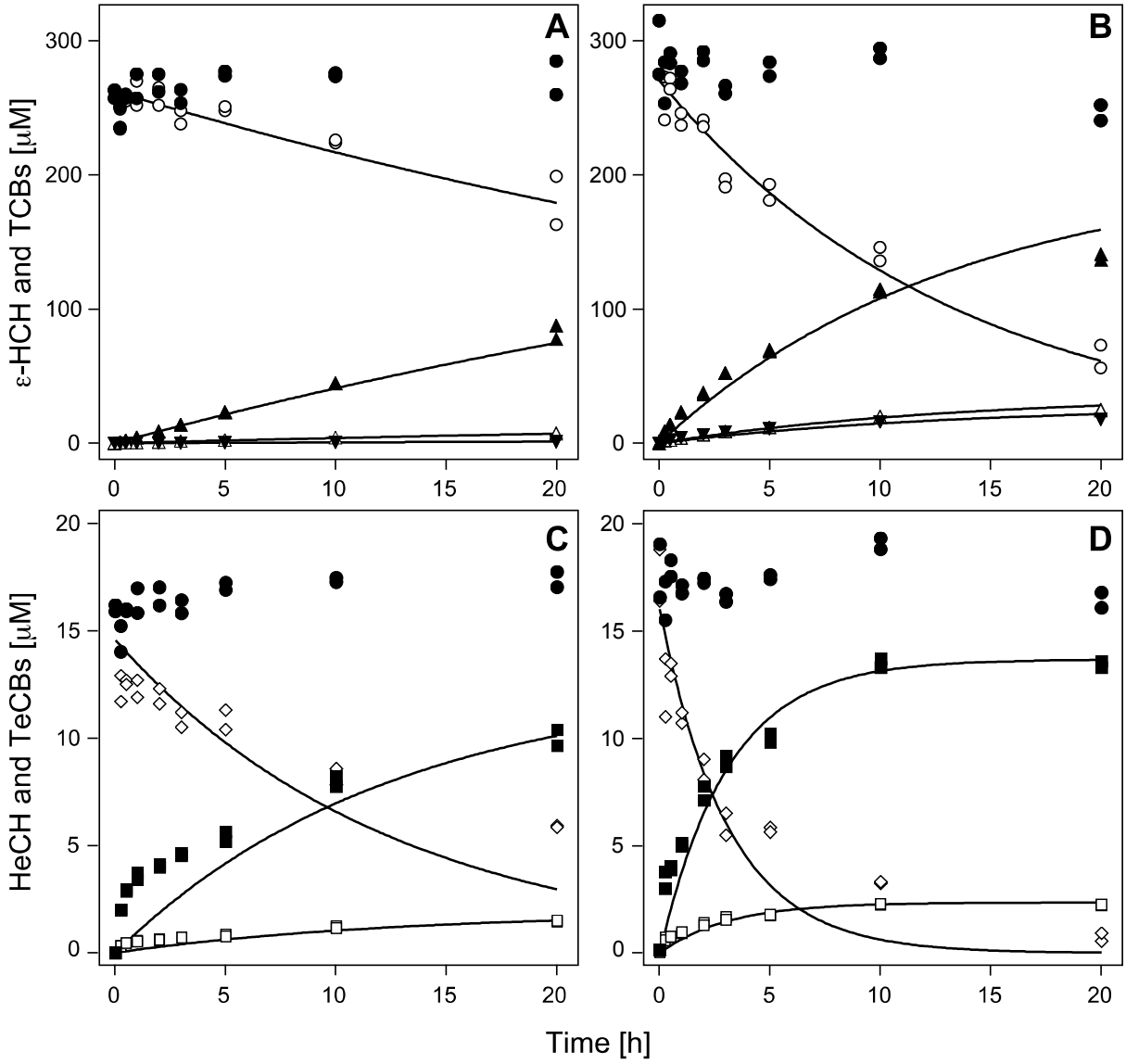


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515 Fig. 3

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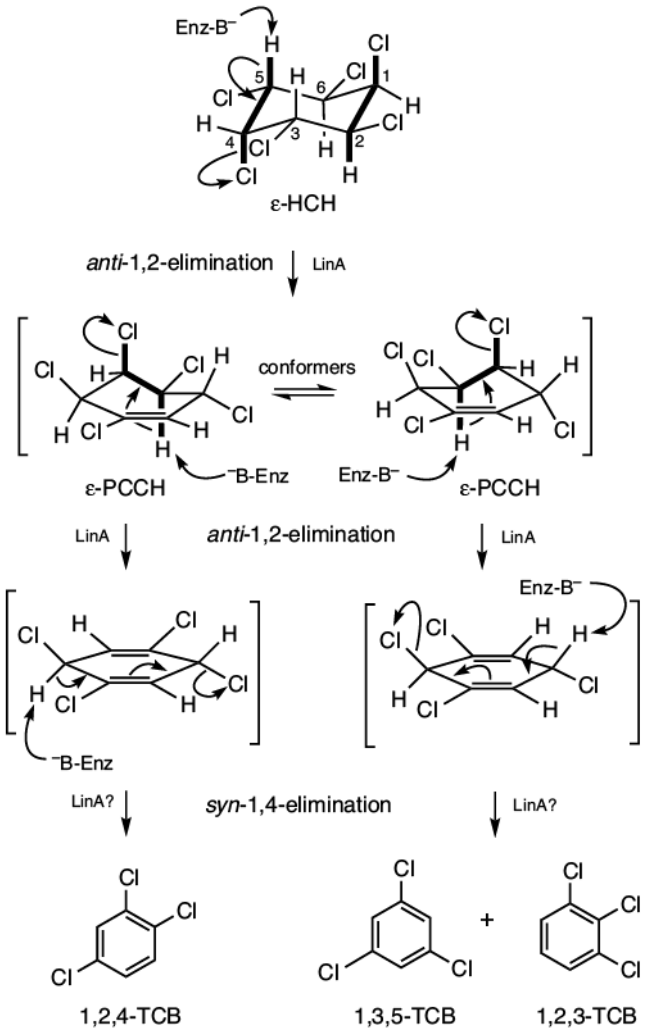


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518 Fig. 4

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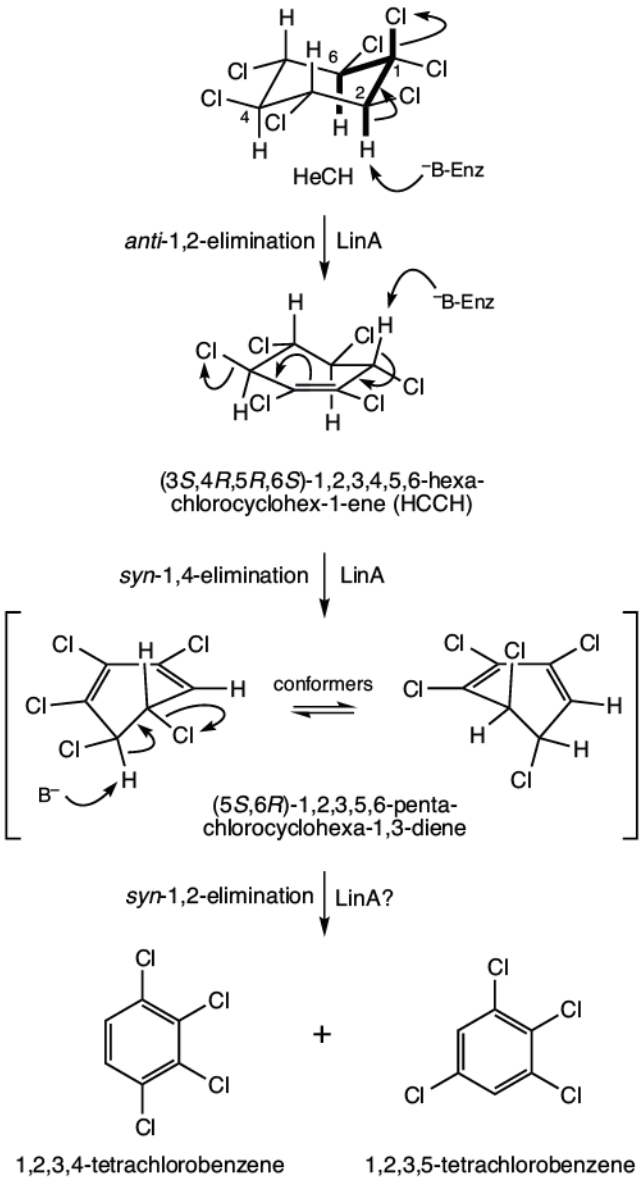


521 1,2,3,4-tetrachlorobenzene 1,2,3,5-tetrachlorobenzene

522 Fig. 5