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Chloromethylmuconolactones as Critical Metabolites in the Degradation of Chloromethylcatechols: Recalcitrance of 2-Chlorotoluene

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To elucidate possible reasons for the recalcitrance of 2-chlorotoluene, the metabolism of chloromethylcatechols, formed after dioxygenation and dehydrogenation by *Ralstonia* sp. strain PS12 tetrachlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase, was monitored using chlorocatechol dioxygenases and chloromuconate cycloisomerases partly purified from *Ralstonia* sp. strain PS12 and *Wautersia eutropha* JMP134. Two chloromethylcatechols, 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol, were formed from 2-chlorotoluene. 3-Chloro-4-methylcatechol was transformed into 5-chloro-4-methylmuconolactone and 2-chloro-3-methylmuconolactone. For mechanistic reasons neither of these cycloisomerization products can be dehalogenated by chloromuconate cycloisomerases, with the result that 3-chloro-4-methylcatechol cannot be mineralized by reaction sequences related to catechol *ortho*-cleavage pathways known thus far. 4-Chloro-3-methylcatechol is only poorly dehalogenated during enzymatic processing due to the kinetic properties of the chloromuconate cycloisomerases. Thus, degradation of 2-chlorotoluene via a dioxygenolytic pathway is evidently problematic. In contrast, 5-chloro-3-methylcatechol, the major dioxygenation product formed from 3-chlorotoluene, is subject to quantitative dehalogenation after successive transformation by chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase, resulting in the formation of 2-methyldienelactone. 3-Chloro-5-methylcatechol is transformed to 2-chloro-4-methylmuconolactone.

Contamination of the environment with halogenated organic compounds is a widespread problem arising from the extensive use of pesticides in agriculture or from industrial chemical waste. Chlorobenzenes and chlorotoluenes are important intermediate products of the chemical industry and are widely used as precursors in dye synthesis, in the pharmaceutical industry, and as solvents, pesticides, and additives (6, 7, 48).

In recent years an overwhelming number of microorganisms capable of mineralizing various chlorinated aromatics have been described (10). As an example, bacterial isolates are available for the degradation of all mono- and dichlorobenzoates, with the exception of 2,6-dichlorobenzoate (15–17, 24). Also, for the majority of chlorobenzene congeners (chlorobenzene, all dichlorobenzenes, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, and 1,2,3,4-tetrachlorobenzene) (37, 41, 43) and chlorotoluene congeners (3-chlorotoluene and 4-chlorotoluene as well as 2,4-dichlorotoluene, 2,5-dichlorotoluene, 3,4-dichlorotoluene, and 3,5-dichlorotoluene) (2, 13), isolates capable of using these compounds as a sole carbon and energy source have been isolated. It is thus surprising that despite many attempts to isolate bacteria with new metabolic properties and despite the broad capabilities and plasticity of microorganisms, some simple chloroaromatics such as 2-chlorotoluene obviously are highly recalcitrant, and no organisms capable of mineralizing them have been isolated to date. This raises the issue of whether there are inherent problems with 2-chlorotoluene such that it cannot be mineralized by pathways evolved for the degradation of chloroaromatics.

The degradation of chloroaromatics is usually initiated by peripheral enzyme reactions, which activate the aromatic ring and a special chlorocatechol pathway (40). Two distinct peripheral pathways have been described for the metabolism of chlorotoluenes (Fig. 1). Degradation can be initiated by dioxygenation, such that the methyl substituent stays intact and the degradation occurs via the respective chloromethylsubstituted catechols (13). Alternatively, degradation can be initiated via oxidation of the methyl substituent which is subsequently eliminated during further processing reactions, resulting in chlorocatechols as intermediates (2). Pathways via monooxygenation of the side chain of chlorotoluenes have been reported to be functional for 3-chlorotoluene and 4-chlorotoluene as well as 3,5-dichlorotoluene in strains harboring the TOL plasmid-encoded upper pathway for the transformation of the substrates into the respective benzoates and catechols and harboring a chlorocatechol pathway for degradation of chlorocatechols (2). However, due to the restricted substrate specificity of xylene monooxygenase (2) and toluate dioxygenase (11), 2-chlorotoluene (and probably other 2-chlorosubstituted toluenes also) cannot be degraded by such strains, and engineering using a monooxygenolytic pathway has failed thus far (14).

The alternative dioxygenolytic pathway has been shown to be productive for the mineralization of 4-chlorotoluene (2, 13) as well as of 2,4-, 2,5-, and 3,4-dichlorotoluene (34). The failure of the 2,4-, 2,5-, and 3,4-dichlorotoluene-degrading strain *Ral-*

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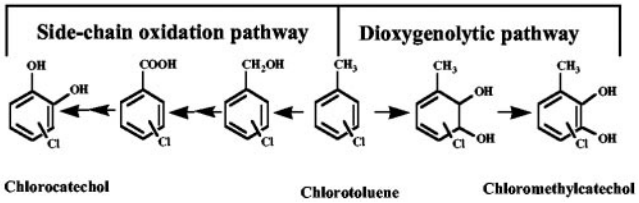

FIG. 1. Metabolism of chlorotoluenes via the side-chain oxidation pathway with chlorocatechols as metabolites and via a dioxygenolytic pathway with chloromethylcatechols as intermediates.

stonia sp. strain PS12 to grow with 2-chlorotoluene or 3-chlorotoluene or with 2,3-dichlorotoluene, 2,6-dichlorotoluene, and 3,5-dichlorotoluene was due to the fact that tetrachlorobenzene dioxygenase catalyzed predominantly a monooxygenation of the methyl side chain that results in the misrouting of these substrates into a dead-end pathway (33). Similarly, toluene dioxygenase of *P. putida* F1 was observed to catalyze predominantly monooxygenation of the side chain of 2- and 3-chlorotoluene (22). The optimization of chlorotoluene degradation by rational engineering of the regioselectivity of toluene dioxygenases to avoid monooxygenation was therefore suggested (35).

The degradation of chlorotoluenes via a dioxygenolytic pathway, however, necessitates ready degradability of the chloromethylcatechol intermediates. Commonly, the degradation of chlorocatechols requires the elimination of a chlorosubstituent from the chloromuconates (formed after intradiol cleavage) by chloromuconate cycloisomerase and the formation of (substituted) dienelactones. Dehalogenation can occur either directly, as is the case with 3-chloromuconate, resulting in the formation of *cis*-dienelactone (Fig. 2), or via a chloromuconolactone intermediate, as is the case with 2-chloromuconate transformation, with dehalogenation occurring after abstraction of a proton from the intermediate 5-chloromuconolactone to give *trans*-dienelactone (Fig. 2) (19, 44, 45). Dehalogenation thus requires cycloisomerization either toward a chlorosubstituted carbon atom or toward an unsubstituted carbon atom, allowing proton abstraction and dehalogenation. As cycloisomerization of asymmetrically substituted muconates can occur in two different directions (1,4- as well as 3,6-cycloisomerization), the cycloisomerization direction can critically determine whether dehalogenation is possible. The importance of proper cycloisomerization for degradation has been previously shown (29, 30) for the metabolism of chloro- and methylsubstituted phenoxyacetates. 4-Chloro-2-methylphenoxyacetate is mineralized by *Wautersia eutropha* JMP134 (29), and cycloisomerization of intermediary 4-chloro-2-methylmuconate can be assumed, by analogy with the transformation of 3-chloromuconate by this strain, to result in the formation of 2-methyl-*cis*-dienelactone, which should be a substrate for

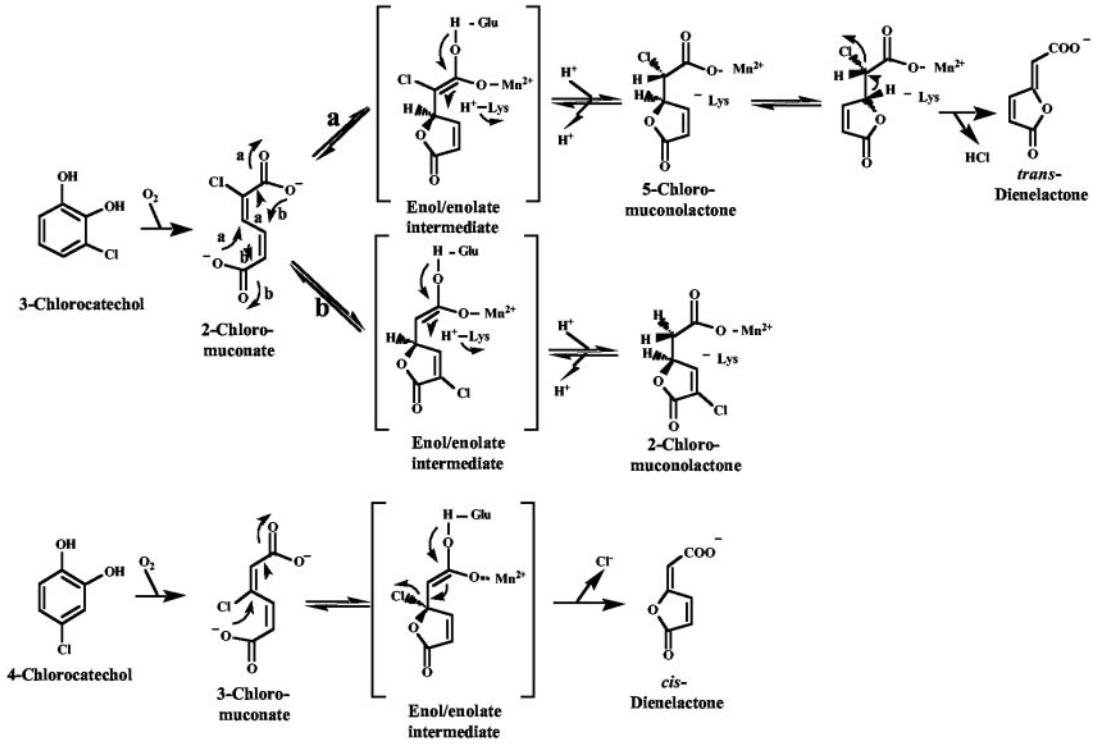

FIG. 2. Mechanisms of dehalogenation of 3-chloromuconate (the ring cleavage product of 4-chlorocatechol) and of 2-chloromuconate (the ring cleavage product of 3-chlorocatechol) by proteobacterial chloromuconate cycloisomerases. Hypothetical intermediates are shown in brackets, with important active-site residues and Mn^{2+} included. Dehalogenation of 3-chloromuconate is proposed to occur from a metal-stabilized enol-enolate intermediate (12). 2-Chloromuconate is subject to both 3,6-cycloisomerization to form 5-chloromuconolactone (reaction a) and 1,4-cycloisomerization to form 2-chloromuconolactone (reaction b) (53, 54). The protonation of the enol-enolate intermediate as well as deprotonation of 5-chloromuconolactone are catalyzed by the same active site lysine (19, 44). Therefore, chloride elimination from 5-chloromuconolactone to give *trans*-dienelactone is proposed to occur only after rotation of the lactone ring (44).

dienelactone hydrolase. 2-Chloro-4-methylphenoxyacetate is not mineralized but is converted into 2-chloro-4-methylmuconolactone as a dead-end product, indicating that mineralization can only be achieved when cycloisomerization proceeds in a way that allows dehalogenation during cycloisomerization (30). By analogy with problems encountered during the metabolism of chloromethylphenoxyacetates, it can be assumed that chloromethylcatechols formed during the metabolism of chlorotoluenes are transformed into dead-end products by enzymes of chlorocatechol pathways.

In the present study, we analyzed the metabolism of chloromethylcatechols formed from 2- and 3-chlorotoluene by tetrachlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase of *Ralstonia* sp. strain PS12 by the use of chlorocatechol enzymes of this strain as well as chlorocatechol pathway enzymes of one of best-characterized chloroaromatic degraders, *Wautersia eutropha* JMP134.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Wautersia eutropha* JMP 222 (pBBR1 M-I) (32) and *Ralstonia* sp. strain PS12 (DSMZ 8910) were grown in mineral salts medium (3) containing 50 mM phosphate buffer (pH 7.4) supplemented with 5 mM 3-chlorobenzoate or 1,2,4,5-tetrachlorobenzene as a sole carbon source as fine mortar-ground crystals corresponding to a concentration of 5 mM. Baffled Erlenmeyer flasks (1 to 3 liter) were filled with 100 to 300 ml of medium, sealed with Teflon-coated screw caps, and incubated at 30°C on a rotary shaker (150 rpm).

Escherichia coli DH5α (pSTE44) containing the tetrachlorobenzene dioxygenase *tecA1A2A3A4* and chlorobenzene dihydrodiol dehydrogenase *tecB* open reading frames (33) originating from *Ralstonia* sp. strain PS12 was grown at 37°C in Luria broth medium (42) containing 1 mM IPTG (isopropyl-ā-D-thiogalactopyranoside) and 0.1 mg of ampicillin/ml.

Enzyme assays and partial enzyme purification. Levels of chlorocatechol 1,2-dioxygenases, chloromuconate cycloisomerases, and dienelactone hydrolases were measured as previously described (5, 45). The activities of partially purified chloromuconate cycloisomerases were determined in the presence of an excess of dienelactone hydrolase partially purified by anionic exchange chromatography and free of any interfering enzyme activity. Activities against chloromethylcatechol mixtures and chloromethylmuconate mixtures were estimated on the basis of an assumption of an extinction coefficient of $\varepsilon_{260\ nm} = 12{,}000\ M^{-1}\ cm^{-1}$ of chloromethylmuconates produced (all mono-, di-, and trisubstituted muconates being methyl and/or chlorosubstituted have been shown to have extinction coefficients of $\varepsilon_{260\ nm} = 9{,}200$ to $18{,}000\ M^{-1}\ cm^{-1}$) (4, 34). Protein concentrations in the cell extracts were determined by the Bradford procedure (1). One enzyme unit is defined as the amount of enzyme that catalyzes the transformation of 1 μmol of substrate per min. In the case of chlorocatechol dioxygenases, the activities with 0.1 mM 4-chlorocatechol are given; in the case of chloromuconate cycloisomerases, the activities with 0.1 mM 3-chloromuconate are given. Chlorocatechol 1,2-dioxygenases, chloromuconate cycloisomerases, and dienelactone hydrolases were partially purified from *Wautersia eutropha* JMP 222 (pBBR1 M-I) (32), a derivative of *Wautersia eutropha* JMP 134, devoid of plasmid pJP4 but containing the well-described *tfdR-C$_I$D$_I$E$_I$F$_I$* open reading frames on a medium-copy plasmid (25), and from *Ralstonia* sp. strain PS12 (34) by anion exchange chromatography on a MonoQ HR 5/5 column as described previously (34). By this purification procedure, chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase activities were clearly separated and the respective enzyme fractions were devoid of possibly interfering catechol 1,2-dioxygenase and muconate cycloisomerase activity.

Transformation of substrates. For transformation by partially purified enzymes, aqueous solutions of chloromethylcatechol mixtures (0.1 ml, corresponding to concentrations of approximately 0.2 to 0.4 mM) were supplemented with an equal volume of Tris-HCl (pH 7.5) (100 mM) and 2 mM $MnCl_2$. Samples were then supplemented with chlorocatechol 1,2-dioxygenase (1 to 20 mU) and further supplemented with the chloromuconate cycloisomerase (5 to 20 mU). The single reactions were monitored by high-pressure liquid chromatography (HPLC) analyses. For larger-scale preparation of metabolites, 20 ml of a solution containing approximately 0.5 mM chloromethylcatechols in 50 mM Tris-HCl (pH 7.5)–2 mM $MnCl_2$ was supplemented with 3 U of JMP 222 (pBBR1 M-I)-derived chlorocatechol 1,2-dioxygenase and 2 U of JMP 222 (pBBR1 M-I)-derived chloromuconate cycloisomerase.

For transformation by whole cells, PS12 bacteria were harvested in late exponential growth by centrifugation and resuspended to an A_{546} of 5 in 10 ml of phosphate buffer (50 mM, pH 7.4). 2-Chlorotoluene was added from a 100 mM stock solution in dimethyl sulfoxide to give a final concentration of 0.3 mM. Flasks were incubated at 30°C on a rotary shaker. For quantification of intermediates, cell-free supernatant fluids were directly analyzed by HPLC analysis and substrate depletion and product accumulation followed for a time period of 2 h.

HPLC analyses. Metabolites were analyzed by injection of 10-μl samples. Product formation was analyzed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, and FCV-10AL solvent mixer) equipped with a SC125/Lichrospher column (Bischoff, Leonberg, Germany) (5 μm). For analyzing substrate transformation, the aqueous solvent system (flow rate, 1 ml/min) contained 0.01% (vol/vol) H_3PO_4 (87%) and 50% (vol/vol) methanol as previously described (35). For analysis of metabolites, the methanol concentration was reduced to 25%. Where possible, the metabolites were identified by comparison of retention volume and UV absorption spectra with those of authentic standards.

^{1}H nuclear magnetic resonance (NMR) spectroscopic analyses. The transformation of 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol by chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase was monitored by HPLC analyses. After complete transformation of chloromethylcatechols and partial transformation of intermediate chloromethylcatechols, 80% of the reaction mixture was extracted at pH 6.5 (to eliminate 2-chlorobenzylalcohol), pH 3.8, and pH 2, twice at each pH, with equal volumes of ethyl acetate. The rest of the reaction mixture was extracted after complete transformation of chloromethylmuconates as follows: the reaction mixture was extracted twice after acidification to pH 6.5 to eliminate residual 2-chlorobenzylalcohol. Then, the reaction products were extracted after acidification of the reaction mixture to pH 2. All extracts were dried over $MgSO_4$ and evaporated under vacuum on a rotary evaporator. The residues were each dissolved in 0.7 ml of acetone-d_6, and diluted aliquots were subjected to HPLC analyses.

^{1}H NMR analysis was performed on all three extracts comprising different amounts of intermediates with acetone-d_6 as the solvent. The composition and identity of the product mixtures were analyzed using one-dimensional (^{1}H and nuclear Overhauser enhancement difference spectra with 10-s delays between pulses) and 2-dimensional (correlation spectroscopy) NMR spectra recorded on an AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten, Germany).

Quantification of metabolites. The relative amounts of metabolites formed from 3-chloro-4-methyl- and 4-chloro-3-methylcatechol were calculated by ^{1}H-NMR through comparison of the well-separated resonance lines at $\delta = 5.34$ ppm (proton of 3-chloro-2-methylmuconolactone), $\delta = 5.40$ ppm (proton of 2-chloro-3-methylmuconolactone), $\delta = 4.77$ ppm (proton of 5-chloro-4-methylmuconolactone), $\delta = 6.36$ ppm (proton of 3-chloro-2-methylmuconate), and $\delta = 6.52$ ppm (proton of 5-methyl-*cis*-dienelactone). The respective product mixtures were analyzed by HPLC and compared with an authentic standard of 5-methyl-*cis*-dienelactone, allowing quantification of all metabolite concentrations. The defined product mixtures served as standards for all further quantifications.

Chemicals. Mixtures of 3-chloro-4-methyl- and 4-chloro-3-methylcatechol and of 3-chloro-5-methyl- and 5-chloro-3-methylcatechol were prepared from 2-chlorotoluene and 3-chlorotoluene, respectively, by the use of *E. coli* (pSTE44) as previously described (35).

3-Chloro-, 2-methyl-, 3-methyl-, 2,4-dichloro-, and dichloromethylmuconates as substrates for chloromuconate cycloisomerase were prepared in situ from 4-chloro-, 3-methyl-, 4-methyl-, 3,5-dichloro-, and dichloromethylcatechols by the use of partially purified chlorocatechol 1,2-dioxygenase which was free of any (chloro)muconate cycloisomerase activity. 2-Chloro-4-methylmuconolactone and 5-chloro-3-methylmuconolactones as well as 3-methyl-*cis*- and 3-methyl-*trans*-dienelactone were available from previous preparations (30, 39), whereas 2-methyl-*cis*- as well as 5-methyl-*cis*-dienelactone (synthesized as previously described) (18, 34) were kindly supplied by Walter Reineke (Chemical Microbiology, Bergische University Wuppertal, Wuppertal, Germany) and Stefan Kaschabek (Interdisziplinäres Ökologisches Zentrum, TU Bergakademie Freiberg, Freiburg, Germany).

2-Methyl-*trans*-dienelactone was produced by the irradiation of an aqueous solution (2 ml [0.2 mM in a 10-ml beaker]) by the use of an UV lamp and irradiation with light ($\lambda = 254$ nm) at a distance of 6 cm for 2 h as described for the preparation of 2-chloro-*trans*-dienelactone from 2-chloro-*cis*-dienelactone (19). Reactions were monitored by HPLC. The *E*210/*E*260 ratio and the absorption maximum of the product ($\lambda_{max} = 291$ nm), which was identical to that of the substrate, confirmed the identity of the expected isomer. All other chemicals

were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany).

RESULTS

Transformation of chloromethylcatechols by chlorocatechol 1,2-dioxygenases and chloromuconate cycloisomerases. To elucidate whether chloromethylcatechols can be transformed by chlorocatechol pathway enzymes and, specifically, whether intermediary substituted muconates can be subjected to dehalogenation, a mixture of 3-chloro-4-methylcatechol (8%), 4-chloro-3-methylcatechol (29%), and 2-chlorobenzylalcohol (63%) was prepared from 2-chlorotoluene and a mixture of 3-chloro-5-methylcatechol (2%), 5-chloro-3-methylcatechol (18%), and 3-chlorobenzylalcohol (80%) was prepared from 3-chlorotoluene by the use of *E. coli* DH5α (pSTE44) containing the tetrachlorobenzene dioxygenase *tecA1A2A3A4* and chlorobenzene dihydrodiol dehydrogenase *tecB* open reading frames (33) originating from *Ralstonia* sp. strain PS12 as previously described (35) (Fig. 1).

Upon addition of chlorocatechol 1,2-dioxygenase (50 mU/ml) from either strain JMP222 (pBBR1 M-I) or strain PS12 to solutions containing 20 μM 3-chloro-4-methylcatechol plus 75 μM 4-chloro-3-methylcatechol both chloromethylcatechols were rapidly transformed to concentrations < 1 μM in less than 10 min whereas 2-chlorobenzylalcohol remained unchanged. No significant differences were observed between the two chlorocatechol 1,2-dioxygenases, with the overall transformation rate being similar to the rate of 4-chlorocatechol transformation. As evidenced by HPLC analyses, both catechols were transformed simultaneously and the 3-chloro-4-methylcatechol/4-chloro-3-methylcatechol ratio did not change significantly during transformation, indicative of similar specificity constants (k_{cat}/K_m values) for 3-chloro-4-methylcatechol- and 4-chloro-3-methylcatechol.

Similar results were obtained with solutions of 3-chloro-5-methylcatechol (10 μM) plus 5-chloro-3-methylcatechol (90 μM). Both chlorocatechol 1,2-dioxygenases tested transformed these chloromethylcatechols simultaneously, with overall rates three to fourfold greater than that of 4-chlorocatechol, whereas 3-chlorobenzylalcohol remained unchanged. As the 3-chloro-5-methylcatechol/5-chloro-3-methylcatechol ratio did not change significantly during transformation, similar specificity constants (k_{cat}/K_m values) for these substrates were indicated.

Addition of chloromuconate cycloisomerase (50 mU/ml) of either strain JMP222 (pBBR1 M-I) or strain PS12 to solutions after complete turnover of 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol or 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol resulted in a decrease in absorbance at 260 to 280 nm indicative of further transformation of intermediate chloromuconates. Initial transformation rates were always approximately 30 to 50% of the rate observed with 3-chloromuconate as the substrate.

The products formed from 10 μM 3-chloro-5-methylcatechol plus 90 μM 5-chloro-3-methylcatechol after successive ring cleavage (50 mU of chlorocatechol 1,2-dioxygenase/ml) and cycloisomerization (50 mU of chloromuconate cycloisomerase/ml) could easily be characterized by comparison with authentic standards (Fig. 3). No significant differences in the product compositions formed were observed between experiments using JMP222 (pBBR1 M-I)-derived or PS12-derived enzymes. 2-Methyl-*cis*-dienelactone (λ_{max} = 291 nm; $RV_{25\%\ MeOH}$ = 5.6 ml) dominated the product mixture (80 ± 5 μM), and 2-methyl-*trans*-dienelactone (λ_{max} = 291 nm; $RV_{25\%\ MeOH}$ = 2.3) was formed in amounts equivalent to 10% ± 2% of the *cis*-isomer. Both these compounds evidently originated from 5-chloro-3-methylcatechol and are assumed to be formed by 1,4-cycloisomerization of 4-chloro-2-methylmuconate. 2-Chloro-4-methylmuconolactone (λ_{max} = 220 nm; $RV_{25\%\ MeOH}$ = 2.6), which should originate from 3-chloro-5-methylcatechol, was formed in minor amounts (7 ± 2 μM). 5-Chloro-3-methylmuconolactone or 3-methyldienelactones were not formed, as evidenced by HPLC using authentic standards (detection limit, 1 μM).

Two new products, M1 (λ_{max} = 273 nm; $RV_{50\%\ MeOH}$ = 4.2 ml; $RV_{25\%\ MeOH}$ = 27.6 ml) and M2 (λ_{max} = 278 nm; $RV_{50\%\ MeOH}$ = 2.1 ml; $RV_{25\%\ MeOH}$ = 10.7), probably chloromethylmuconates, were formed by chlorocatechol 1,2-dioxygenase from 40 μM 3-chloro-4-methylcatechol plus 150 μM 4-chloro-3-methylcatechol. Addition of chloromuconate cycloisomerase resulted in the formation of one predominant product, L1 (λ_{max} = 223 nm; $RV_{25\%\ MeOH}$ = 3.9 ml), as well as three minor products, L2 (λ_{max} = 291 nm; $RV_{25\%\ MeOH}$ = 6.2 ml), L3 (λ_{max} = 223 nm; $RV_{25\%\ MeOH}$ = 2.5 ml), and L4 (λ_{max} = 206 nm; $RV_{25\%\ MeOH}$ = 1.7 ml). The absorption spectra indicated that L1, L3, and L4 have a muconolactone structure. L2 was identified as 5-methyl-*cis*-dienelactone by comparison with an authentic standard (Fig. 4). The concentration of this product was only 8 ± 2 μM. Similar results were obtained in experiments using *Ralstonia* sp. strain PS12- instead of JMP222 (pBBR1 M-I)-derived enzymes. Neither extended incubation (up to 4 h) nor further addition of chloromuconate cycloisomerase (200 mU/ml) changed the final product composition (L1:L2:L3:L4).

Elucidation of the structure of the products formed from 3-chloro-4-methyl- and 4-chloro-3-methylcatechol. To elucidate the structure of the cycloisomerization products formed from 3-chloro-4-methyl- and 4-chloro-3-methylcatechol, the mixture was extracted after complete transformation of the chloromethylcatechols and M2 but only partial transformation of M1. 2-Chlorobenzylalcohol was eliminated from the reaction mixture by extraction with ethyl acetate at pH 6.5. The extract obtained by extracting at pH 3.8 (extract 1), as measured by HPLC, contained L1 and L3 in a ratio similar to that seen with the reaction mixture, as well as a slightly elevated amount of L2 (5-methyl-*cis*-dienelactone). The residue obtained by extracting at pH 2 (extract 2) contained L1 and L3 in a ratio similar to that seen with the reaction mixture, as well as a slightly reduced amount of L2. Only this extract contained M1 as well as L4. A third extract, obtained after complete transformation of M1, elimination of 2-chlorobenzylalcohol by extraction at pH 6.5, and extraction at pH 2, contained L1 through L4 in ratios similar to those seen with the reaction mixture.

^{1}H NMR analysis was then performed for all three extracts comprising different amounts of intermediates. Signals arising from 5-methyl-*cis*-dienelactone were identified by comparison with an authentic standard (Fig. 4). Two olefinic protons resonate at 6.52 and 8.41 ppm, respectively, and the small coupling constant of 5.6 Hz indicates the presence of the olefinic

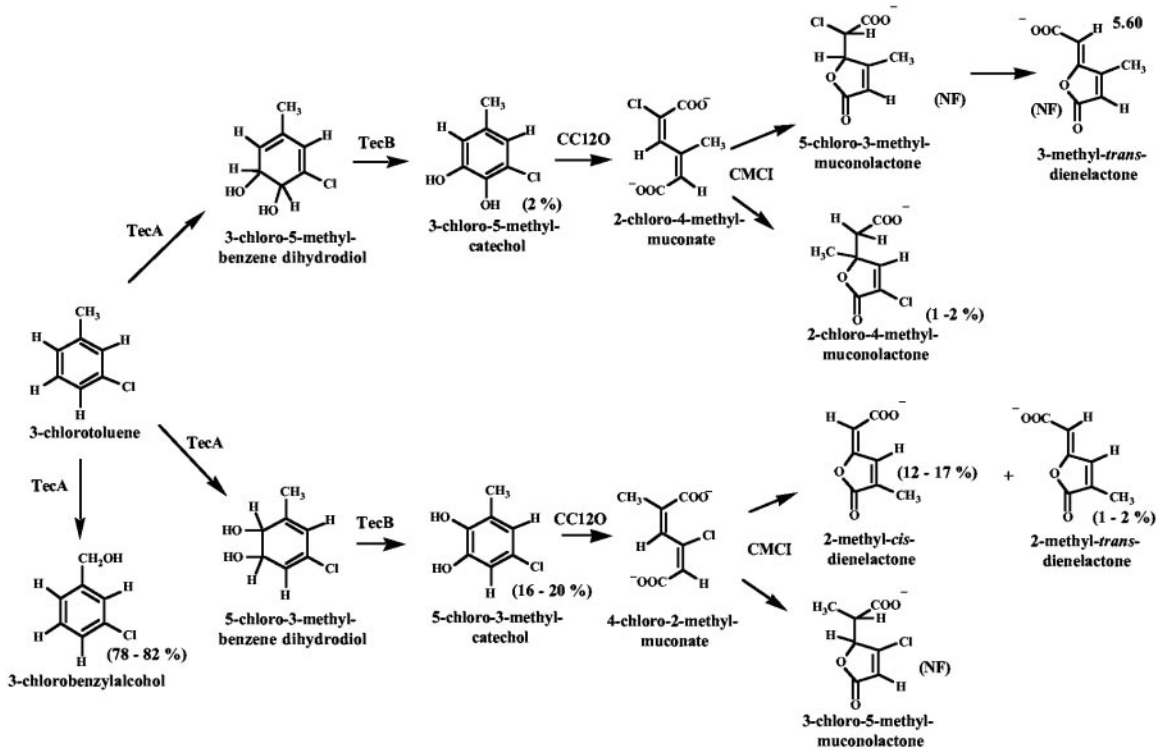

FIG. 3. Metabolic fate of 3-chlorotoluene with an in vitro-constituted pathway comprising TecA tetrachlorobenzene dioxygenase and TecB chlorobenzene dihydrodiol dehydrogenase (expressed by *E. coli* DH5α) (pSTE44) (35), chlorocatechol 1,2-dioxygenase (CC12O) (both $TfdC_I$ chlorocatechol dioxygenase, derived from *Wautersia eutropha* JMP222 [pBBR1 M-I] [32], and chlorocatechol dioxygenase, derived from *Ralstonia* sp. strain PS12 [34], were used) and chloromuconate cycloisomerase (CMCI) (both $TfdD_I$ chloromuconate cycloisomerase, derived from *Wautersia eutropha* JMP222 [(pBBR1 M-I] [32] and chloromuconate cycloisomerase, derived from *Ralstonia* sp. strain PS12 [34], were used). The relative amounts of 3-chlorobenzylalcohol and chloromethylcatechols formed by *E. coli* DH5α (pSTE44) (35) are given in parentheses. The relative amounts of lactones formed were calculated on the basis of their abundance after transformation by chlorocatechol dioxygenase and chloromuconate cycloisomerase, taking into account 3-chlorobenzylalcohol formation. NF, not formed.

system in a closed five-membered ring system (26). Protons of the methyl group resonate at 2.09 ppm. This compound could be estimated to comprise 4 to 5% of the products present in the different extracts.

The product dominating in all extracts showed an NMR spectrum similar to the spectra previously reported for 2-methylmuconolactone (20), 3-methylmuconolactone (27), and 2,3-dimethylmuconolactone (47) and evidently contains a CH_2-CH coupled system. Two protons of a methylene group resonate at 2.66 and 3.07 ppm, respectively. The signals were split into doublets of doublets due to a geminal coupling of 16.7 Hz and vicinal couplings of 8.2 and 3.6 Hz. A proton resonating at 5.34 ppm showed the respective vicinal coupling constants of 8.2 and 3.6 Hz, in addition to a homoallylic coupling of 1.8 Hz with a methyl substituent (1.85 ppm). Such a homoallylic coupling can be expected to occur only in 3-chloro-2-methylmuconolactone. Moreover, the chemical shift is similar to those of 2-methylsubstituted muconolactones (20, 31, 47) but is significantly lower than those of 3-methylsubstituted muconolactones (27, 31, 47), suggesting that the structure of the dominant cycloisomerization product is 3-chloro-2-methylmuconolactone and not 2-chloro-3-methylmuconolactone. This was confirmed by low-power irradiation of the methyl signal, which did not afford any nOe. 3-Chloro-2-methylmuconolactone was thus the major product, comprising 73% ± 5% of all three product mixtures, and is thus identical to the product previously designated L1 (Fig. 4).

A third product showed a ^1H NMR spectrum very similar to that of 3-chloro-2-methylmuconolactone in comprising an ABX system. It differed from the major metabolite by the chemical shift of the methyl protons (2.17 ppm) and the lower coupling of the methyl protons of 0.9 Hz. Such behavior is compatible with its structure being 2-chloro-3-methylmuconolactone. In accordance with the postulated structure, low-power irradiation of the methyl signal afforded a nOe to the signal at 5.40 ppm. 2-chloro-3-methylmuconolactone comprised 18% ± 3% of all three product mixtures and is thus identical to the product previously designated L3 (Fig. 4).

A fourth product (L4) was present in extracts 2 and 3 only. Its NMR characteristics were similar to those previously reported for 5-chloromuconolactone and 5-chloro-3-methylmuconolactones (30, 54) by the absence of the typical AB spectrum of two diastereotopic methylene protons. In accordance with the presence of a chlorine-substituted 4-carboxymethyl side chain, a single proton with a chemical shift of 4.77 ppm was observed. Two olefinic protons resonate at 6.22 and 7.77 ppm with a small coupling constant of 5.7 Hz indicative of the presence of the olefinic system in a closed five-membered ring system. In accordance with the postulated structure (5-chloro-4-methylmuconolactone), low-power irradiation of the methyl

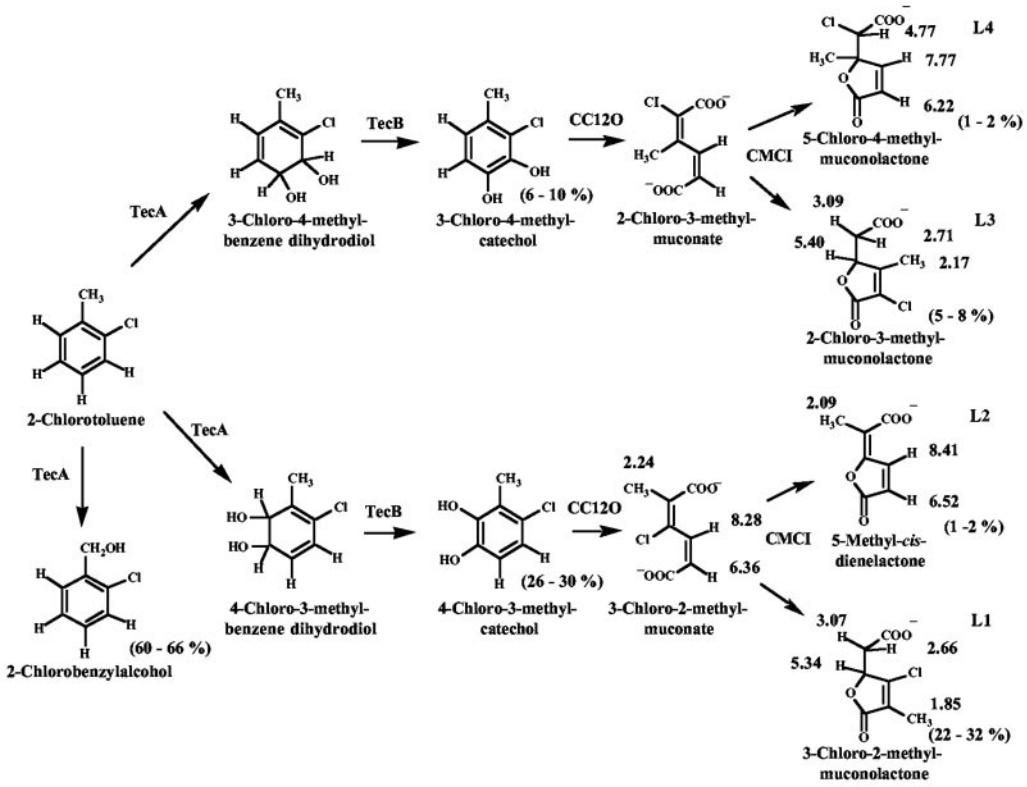

FIG. 4. Metabolic fate of 2-chlorotoluene with an in vitro-constituted pathway comprising TecA tetrachlorobenzene dioxygenase and TecB chlorobenzene dihydrodiol dehydrogenase (expressed by *E. coli* DH5α) (pSTE44) (35), chlorocatechol 1,2-dioxygenase (CC12O) (both $TfdC_I$ chlorocatechol dioxygenase, derived from *Wautersia eutropha* JMP222 [pBBR1 M-I] [32], and chlorocatechol dioxygenase, derived from *Ralstonia* sp. strain PS12 [34], were used), and chloromuconate cycloisomerase (CMCI) (both $TfdD_I$ chloromuconate cycloisomerase, derived from *Wautersia eutropha* JMP222 [pBBR1 M-I] [32], and chloromuconate cycloisomerase, derived from *Ralstonia* sp. strain PS12 [34], were used). Chemical shifts of protons from the ^{1}H-NMR analyses are given. The relative amounts of 2-chlorobenzylalcohol and chloromethylcatechols formed by *E. coli* DH5α (pSTE44) (35) are given in parentheses. The relative amounts of lactones formed were calculated on the basis of their abundance after transformation by chlorocatechol dioxygenase and chloromuconate cycloisomerase, taking into account 2-chlorobenzylalcohol formation.

signal afforded nOes at the signals at 4.77 and 7.77 ppm. No further signals with chemical shifts similar to 4.77, 6.22, and 7.77 were observed, excluding the presence of a diastereomeric mixture of 5-chloro-4-methylmuconolactone. Thus, this compound was obviously produced by enzymatic action. Extract 3 was shown to contain about 5% of this metabolite.

A fifth product (M1) was observed in extract 2 only. Two olefinic protons resonate at 6.36 and 8.28 ppm, respectively, and the large coupling constant of 15 Hz indicates that they are located in an open-chain configuration, as present in muconates (46). The absence of any nOe upon low-power irradiation of the methyl signal at 2.24 ppm indicates that M1 is 3-chloro-2-methylmuconate.

Transformation of 2-chlorotoluene by *Ralstonia* sp. strain PS12. From the above-described experiments, it can be seen that the amounts of metabolites formed by successive transformation by TecA tetrachlorobenzene dioxygenase, TecB chlorobenzene dihydrodiol dehydrogenase, chlorocatechol 1,2-dioxygenase, and chloromuconate cycloisomerase can be calculated. According to previous results, 8% ± 2% of applied 2-chlorotoluene is transformed into 3-chloro-4-methyl- and 29% ± 2% is transformed into 4-chloro-3-methylcatechols by TecAB (35). 3-Chloro-4-methylcatechol is converted into 2-chloro-3-methyl- and 5-chloro-4-methylmuconolactone in a 3- to 4:1 ratio, indicating that >1% to 2% and 5% to 8%, respectively, of applied 2-chlorotoluene are converted to these products. 4-Chloro-3-methylcatechol is converted into 3-chloro-2-methylmuconolactone and 5-methyl-*cis*-dienelactone in a 13- to 20:1 ratio, indicating the 1% to 2% and 25% to 30%, respectively, of applied 2-chlorotoluene is converted to these products.

To establish whether the same products were formed in similar ratios by wild-type *Ralstonia* sp. strain PS12, 2-chlorotoluene (300 μM) was transformed with 1,2,4,5-tetrachlorobenzene-grown cells of this organism. As previously described (22), 2-chlorobenzylalcohol accumulated as an intermediate and was further transformed slowly into 2-chlorobenzoate as a dead-end product. 2-Chloro-3-methyl-, 5-chloro-4-methyl-, and 3-chloro-2-methylmuconolactone accumulated in amounts corresponding to 5 to 8%, <2%, and 20 to 24%, respectively, amounts similar to those expected from experiments with overexpressed and partially purified enzymes. 5-Methyl-*cis*-dienelactone accumulated in concentrations ≤ 2 μM, indicating that this compound is further transformed by dienelactone hydrolase and possibly mineralized. However, according to the above-presented data, only about 1 to 2% of the applied

2-chlorotoluene is transformed into 5-methyl-*cis*-dienelactone whereas the rest is transformed into dead-end products.

DISCUSSION

We have previously reported that 2-chlorotoluene is predominantly monooxygenated by tetrachlorobenzene dioxygenase to form 2-chlorobenzylalcohol (22), although more than 30% of the substrate is subject to dioxygenation (35). Similarly, a significant amount of 3-chlorotoluene (approximately 20%) is subject to dioxygenation, resulting mainly in 5-chloro-3-methylcatechol (35). Also toluene dioxygenase of *Pseudomonas putida* F1 was shown to catalyze both mono- and dioxygenation of 2- and 3-chlorotoluene, indicating that multiple orientations of these substrates in the active site of toluene-chlorobenzene dioxygenases are possible. Usually, chlorosubstituted catechols arising as intermediates after dioxygenation of chloroaromatics are easily degraded by enzymes of chlorocatechol pathways (28, 34, 36, 51). However, as shown here, the presence of an additional methyl function can severely disturb degradation and out of four chloromethylcatechols analyzed only one can be easily degraded and dehalogenated by chlorocatechol pathway enzymes (Fig. 3 and 4).

As suggested previously for the degradation of 4-chloro-2-methylphenoxyacetate, the ring cleavage of 5-chloro-3-methylcatechol (4-chloro-2-methylmuconate) is exclusively subject to 1,4-cycloisomerization to yield 2-methyl-*cis*-dienelactone as the predominant cycloisomerization product (29, 30). This can be subsequently degraded by dienelactone hydrolase (data not shown). Hence, mineralization of 5-chloro-3-methylcatechol, in contrast to that of 3-chloro-5-methylcatechol, should be rather easily achieved. However, neither 3-chloro-4-methylcatechol nor 4-chloro-3-methylcatechol, as metabolites of 2-chlorotoluene, can be mineralized by enzymes of the chlorocatechol pathways described here and chloromuconate cycloisomerization was determined to constitute the pathway bottleneck. This is clearly evident for 3-chloro-4-methylcatechol metabolism. 3-Chloro-4-methylcatechol is transformed (via 2-chloro-3-methylmuconate) into a mixture of 2-chloro-3-methyl- and 5-chloro-4-methylmuconolactone, indicating that dehalogenation does not occur during 1,4- or 3,6-cycloisomerization. This is not unexpected, as neither of the two dehalogenating mechanisms described for chloromuconate dehalogenation (19, 44, 53) can occur (Fig. 2). It can thus be postulated that the incapability to dehalogenate during 2-chloro-3-methylmuconate cycloisomerization is a feature shared by chloromuconate cycloisomerases.

It has previously been observed that, in addition to chloromuconate cycloisomerases, muconolactone isomerases are capable of abstracting the C-4 proton of 5-chlorosubstituted muconolactones (38) and can be integrated into a functional pathway for 3-chlorocatechol degradation (23). Also, transformation of 2-chloromuconolactone into protoanemonin by this enzyme has been described previously (49). However, even if muconolactone isomerases can be assumed to exhibit some activities toward 2-chloro-3-methylmuconolactone, no pathways for the degradation of protoanemonin derivatives or even protoanemonin itself have been described thus far. Thus, degradation of 2-chloro-3-methylcatechol is not possible by any of the known metabolic pathways for chloroaromatic degradation after intradiol cleavage.

4-Chloro-3-methylcatechol is transformed into a mixture of 3-chloro-2-methylmuconolactone and 5-methyl-*cis*-dienelactone (Fig. 4). Evidently, dehalogenation occurs during 3,6-cycloisomerization, analogous to the dehalogenation of 3-chloromuconate. Previously, Vollmer et al. (54) showed that muconate cycloisomerase catalyzes the formation of an equilibrium between 2-chloromuconate and 2-chloro- as well as 5-chloromuconolactone and that all three compounds are substrates for the cycloisomerase (Fig. 2). Transformation of 2-chloromuconolactone by chloromuconate cycloisomerase was described to proceed via 2-chloromuconate and 5-chloromuconolactone into *trans*-dienelactone (53). As such, chloromuconate cycloisomerase is capable of correcting for a "wrong" cycloisomerization reaction, as the cycloisomerization reaction is reversible and the "right" cycloisomerization product is taken out of the equilibrium mixture by dechlorination. Such a metabolic route has previously been suggested to occur in the degradation of 3,6-dichloro-4-methylcatechol (34). However, as the ratio of 5-methyl-*cis*-dienelactone versus 3-chloro-2-methylmuconolactone did not change upon extended incubation with chloromuconate cycloisomerase, 3-chloro-2-methylmuconolactone must be a poor substrate for chloromuconate cycloisomerases.

Thus, it is clear that dioxygenation of 2-chlorotoluene results in the formation of chloromethylcatechols which are difficult to degrade. 4-Chloro-3-methylcatechol degradation seems to be restricted by the kinetic properties of chloromuconate cycloisomerases, which favor 3-chloro-2-methylmuconolactone formation. These prevent its transformation into 5-methyl-*cis*-dienelactone, which would be a reasonably good substrate for dienelactone hydrolases. However, whereas the general mechanism of chloromuconate turnover and dehalogenation is probably shared among chloromuconate cycloisomerases, significant differences have been observed in their catalytic properties (23, 32, 50, 52) and whether 3-chloro-2-methylmuconate dehalogenation is generally problematic remains to be elucidated. Chloromuconate cycloisomerases of proteobacterial origin described thus far share >60% of amino acid identity and rapidly form *trans*- and *cis*-dienelactone from 2-chloro- and 3-chloromuconate, respectively; however, some differences in their catalytic activities were previously observed (52). The only exception to this is TfdDII chloromuconate cycloisomerase of *Wautersia eutropha* JMP134, which shares only 30 to 34% amino acid identity with other chloromuconate cycloisomerases (21) and is very ineffective at 2-chloromuconate cycloisomerization (32). In addition to the closely related chloromuconate cycloisomerases of proteobacterial origin, chloromuconate cycloisomerases, which differed in their cycloisomerization reaction for 2-chloromuconate, have been observed in *Rhodococcus* isolates (8, 9, 50). Whether these enzymes, which share only 36 to 40% amino acid identity with their proteobacterial counterparts, are capable of 3-chloro-2-methylmuconate cycloisomerization remains to be analyzed. Currently, however, none of the enzymes investigated that are involved in aromatic degradation via the *ortho*-cleavage pathway can be assumed to be capable of degradation of lactones formed from 3-chloro-4-methylcatechol.

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