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Metabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes eutrophus* JMP 134

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Abstract. 2-Chloro-4-methylphenoxyacetate is not a growth substrate for *Alcaligenes eutrophus* JMP 134 and JMP 134-1. It is, however, being transformed by enzymes of 2,4-dichlorophenoxyacetic acid metabolism to 2-chloro-4-methyl-*cis,cis*-muconate, which is converted by enzymatic 1,4-cycloisomerization to 4-carboxymethyl-2-chloro-4-methylmuconolactone as a dead end metabolite. Chemically, only 3,6-cycloisomerization occurs, giving rise to both diastereomers of 4-carboxychloromethyl-3-methylbut-2-en-4-olide. Those lactones harboring a chlorosubstituent on the 4-carboxymethyl side chain were surprisingly stable under physiological as well as acidic conditions.

Key words: 2-Chloro-4-methylphenoxyacetic acid – 2-Chloro-4-methyl-*cis,cis*-muconate – 4-Carboxymethyl-2-chloro-4-methylmuconolactone – 4-Carboxychloromethyl-3-methylbut-2-en-4-olide – *Alcaligenes eutrophus* JMP 134 – *Ortho*-cleavage – Cycloisomerization

Alcaligenes eutrophus JMP 134 can utilize a variety of chloro- and methylsubstituted aromatic compounds as sole source of carbon and energy. Catechols are central intermediates in these degradative pathways (Pieper et al. 1988). In addition to a *meta*-cleavage pathway, three distinct *ortho*-cleavage pathways can be induced by this strain. The first of these three affects the metabolism of 2,4-dichlorophenoxyacetate via 3,5-dichlorocatechol (Fig. 1A). The enzymes of this pathway comprise those responsible for the conversion of phenoxyacetates into catechols (2,4-D monooxygenase and chlorophenol hydroxylase) (Liu and Chapman 1984; Streber et al. 1987; Pieper et al. 1988) as well as those of the *ortho*-cleavage pathway modified for the degradation of chlorocatechols (catechol 1,2-dioxygenase II, dichloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase) (Pieper et al. 1988; Kukor et al. 1989; Vollmer et al. 1993).

The second *ortho*-cleavage pathway is responsible for the catabolism of benzoate (Kuhm et al. 1990; Schlömann et al. 1990c). The intermediate catechol is metabolized by enzymes of the 3-oxoadipate pathway (classical *ortho*-cleavage pathway) (Fig. 1B).

Neither of these first two *ortho*-cleavage pathways can deal with methylcatechols. 4-Carboxymethyl-4-methyl- or 4-carboxymethyl-2-methylbut-2-en-4-olide (4-methyl- or 2-methylmuconolactone) accumulate as dead-end products when methylcatechols are channeled into the *ortho*-cleavage route (Catelani et al. 1971; Knackmuss et al. 1976; Pieper et al. 1985).

In chlorocatechol degradation, muconolactones do not occur as intermediates, since cycloisomerization of chloromuconates gives rise to dienelactones with concomitant elimination of chloride (Schmidt and Knackmuss 1980). The dinelactones so formed are then subject to hydrolysis by dienelactone hydrolases (Schmidt and Knackmuss 1980). Analysis of the substrate specificity of the dienelactone hydrolases involved in chlorocatechol degradation have demonstrated an inability of such enzymes to turn over 3-oxoadipate enol-lactone the substrate of 3-oxoadipate enollactone hydrolase of the 3-oxoadipate pathway (Schmidt and Knackmuss 1980; Schlömann et al. 1990b, c). These dienelactone hydrolases are clearly restricted to hydrolysis of compounds possessing a dienelactone structure, which do not occur during the metabolism of methylcatechols.

The dead-end nature of methylmuconolactones in the 3-oxoadipate pathway can be attributed to one of two reasons: 1) In the case of 4-methylmuconolactone the muconolactone isomerase of the classical 3-oxoadipate pathway, due to the methylsubstitution, cannot act on this compound. 2) In the case of 2- and 3-methylmuconolactone, the muconolactone isomerase and/or the enollactone hydrolase of the 3-oxoadipate pathway obviously are of restricted substrate specificity and do not accept methylsubstituents (Pieper et al. 1992).

A third *ortho*-cleavage pathway, that can degrade methylaromatics via 4-methylcatechol was observed in *Alcaligenes eutrophus* JMP 134 (Pieper et al. 1985). This

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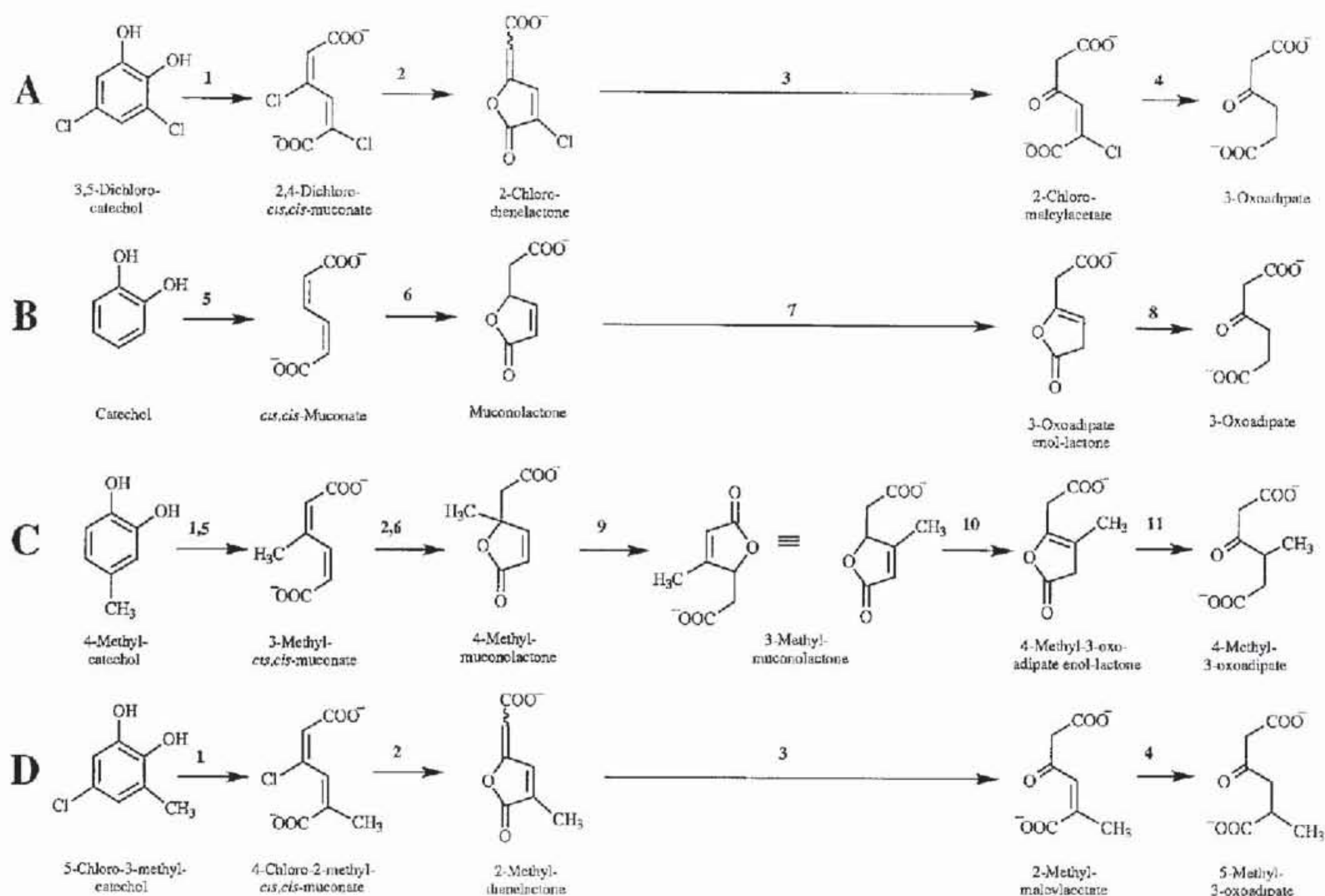


Fig. 1. Degradation of **A** 3,5-dichlorocatechol; **B** catechol; **C** 4-methylcatechol and **D** 5-chloro-3-methylcatechol by *Alcaligenes eutrophus* JMP 134 according to Pieper et al. (1985, 1988, 1991, 1992) and Vollmer et al. (1993). Enzymes involved are: *1*, catechol 1,2-dioxygenase type II; *2*, dichloromuconate cycloisomerase; *3*, dienelactone hydrolase; *4*, maleylacetate reductase; *5*, catechol 1,2-dioxygenase type I; *6*, muconate cycloisomerase; *7*, muconolactone isomerase; *8*, 3-oxoadipate enol-lactone hydrolase; *9*, 4-methylmuconolactone methyl-isomerase; *10*, muconolactone isomerase or methylmuconolactone isomerase, modified for the metabolism of methylmuconolactones; *11*, 3-oxoadipate enol-lactone hydrolase or isoenzyme modified for the metabolism of methyl-substituted analogs

pathway includes 4-methylmuconolactone methylisomerase (Pieper et al. 1990) as a key enzyme together with isoenzymes of the 3-oxoadipate pathway (Fig. 1C).

Because different *ortho*-cleavage routes are responsible for the metabolism of unsubstituted, chlorosubstituted and methylsubstituted catechols in JMP 134, we were interested in the metabolic routes of aromatic compounds carrying both methyl- and chlorosubstituents. It was previously demonstrated that 4-chloro-2-methylphenoxyacetic acid (MCPA) was metabolized by enzymes of the chlorocatechol pathway. The postulated reaction sequence involves cycloisomerization and dechlorination of 4-chloro-2-methylmuconate to form 2-methyldienelactone (Pieper et al. 1988, 1992) (Fig. 1D). Obviously, dienelactone hydrolase accepts this dienelactone as a substrate. Formation of a methylmuconolactone appears to be prevented by 1,4-cycloisomerization and dechlorination. Remarkably, JMP 134 could not use 2-chloro-4-methylphenoxyacetate as a growth substrate. We investigate here the metabolism of this compound in order to identify critical steps in the degradation of aromatic compounds carrying both methyl- and chlorosubstituents.

Materials and methods

Organisms

The 2,4-dichlorophenoxyacetate (2,4-D) degrading organism *Alcaligenes eutrophus* JMP 134 was isolated by Don and Pemberton (1981). *Alcaligenes eutrophus* JMP 134-1 is a spontaneous mutant of this strain, expressing the enzymes 2,4-D monooxygenase, chlorophenol hydroxylase, catechol 1,2-dioxygenase, dichloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase constitutively (Pieper et al. 1989).

Culture conditions

Growth in liquid culture was performed using mineral medium (Dorn et al. 1974) containing 5 mM of the respective substrate. Phosphate buffer was replaced with Tris/HCl buffer (50 mM, pH 7.4) when investigating the bioconversion of accumulated products by

dichloromuconate cycloisomerase. Cells were grown in fluted Erlenmeyer flasks incubated at 30 °C on a rotary shaker at 150 rpm. Growth was monitored photometrically. In the induction experiments, cultures were grown in 500 ml fluted Erlenmeyer flasks containing 50 ml of mineral medium and fructose (5 mM) as carbon source. During late exponential growth, the culture was transferred to a 3 l flask, containing 500 ml of medium supplemented with fructose (5 mM) and the respective inducer (2 mM). Cells were harvested after an incubation period of 15 h.

For bioconversion experiments with growing cells, cultures of JMP 134-1 grown on fructose (5 mM) were used to inoculate (1 : 10) fresh medium containing the growth substrate (5 mM) plus 2-chloro-4-methylphenoxyacetate (CMPA, 1 mM). Total conversion of CMPA was generally achieved inbetween 10–15 h. For transformation experiments with resting cells, JMP 134-1 was grown on fructose (5 mM). Cells were harvested at late exponential growth phase and resuspended to $A_{546nm} = 2–10$ in phosphate buffer (pH 7.4, 50 mM).

Large scale preparation of transformation products was done by inoculating two 7 l fermenters containing fructose (5 mM) with 1 l of JMP 134-1 grown on fructose. CMPA was added to a final concentration of 1 mM.

Preparation of cell extracts

Harvested cells were resuspended in Tris/HCl buffer (100 mM, pH 7.5) and disrupted using a French press (Aminco, Silver Spring, Md., USA). Cell debris were removed by centrifugation at 100,000 × *g* for 1 h at 4 °C.

Enzyme assays

Catechol 1,2-dioxygenase (C12O, EC 1.13.11.1.) was measured by the procedure of Dorn and Knackmuss (1978a, b). Dichloromuconate cycloisomerase was assayed by the method of Schmidt and Knackmuss (1980). 2,4-Dichloro-*cis,cis*-muconate was prepared in situ by incubation of 3,5-dichlorocatechol with partially purified catechol 1,2-dioxygenase of the type II, which was free of any cycloisomerase activity (Pieper et al. 1988). Dienelactone hydrolase as well as maleylacetate reductase were assayed as described by Pieper et al. (1988). Specific activities are expressed as µmol of substrate converted or product formed per minute per gram protein at 25 °C. Protein was determined by the Bradford procedure (Bradford 1976).

Activity measurements with whole cells

For measuring 2,4-D monooxygenase activity freshly harvested cells were resuspended in phosphate buffer (50 mM, pH 7.4) to an A_{546nm} of about 5 and incubated with 2,4-D (2 mM) in a waterbath at 30 °C. Substrate concentrations during incubation were measured by HPLC.

Chlorophenol hydroxylase activity was determined by measuring the rate of oxygen uptake polarographically using a Clark-type electrode (Pieper et al. 1988). Since the rate of oxygen uptake represents the combined activity of chlorophenol hydroxylase and catechol 1,2-dioxygenase, the determined activity was divided by two to yield respective activities. Protein was determined by the method of Schmidt et al. (1963).

Extraction and derivatization of metabolites

Cell free culture fluids were evaporated to a final volume of 1 l. For preparation of I and III, the culture fluid was acidified to pH 5 with phosphoric acid. After completely abolishing the absorption maximum at 265 nm (mainly through cyloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate to compound I), the culture fluid was acidified to pH 4 and extracted twice with 500 ml of ethylacetate. This resulted in the selective extraction of compound III. After acidification to pH 2, compound I and II were extracted from the culture fluid by 3 portions (500 ml each) of ethylacetate. The ethylacetate fractions were dried over $MgSO_4$ and evaporated. Compound III was obtained as a yellow oil which crystallized rapidly. Compound I and II were obtained in an 8 : 1 ratio.

For preparation of I, II and III, the concentrated culture fluid was rapidly acidified to pH 1.8 by the addition of HCl (32%). After the complete loss of the absorption maximum at 265 nm (cyloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate to compound I and II), the solution was adjusted to pH 4 by NaOH. Compound III was again selectively extracted by twice 500 ml of ethylacetate and crystallized from the evaporated extract. Compound I and II were extracted after reacidification to pH 2. They were obtained in a ratio of 2 : 1. This mixture was further purified by preparative HPLC. Compound I could be collected in high purity from the first separation. The yellow oil obtained after evaporation was used for further analysis. The fraction containing compound II, was still contaminated by I (20%). Only after a second purification by HPLC compound II could be obtained in high purity.

A portion of the purest fractions of I, II and III (20 mg each) were derivatized by diazomethan (De Boer and Bakker 1954).

Purification of enzymes

Dichloromuconate cycloisomerase and 4-methylmuconolactone methyl-isomerase from *Alcaligenes eutrophus* JMP 134 were purified by FPLC as previously described (Kuhm et al. 1990; Pieper et al. 1990).

Analytical methods

Bioconversion of CMPA and accumulation of metabolites were determined with the HPLC system described by Pieper et al. (1988) equipped with a SC125/Lichrospher 5 µm (Bischoff, Leonberg, Germany) column. The solvent system contained 1 ml of H_3PO_4 (85%) and 150–500 ml of methanol per liter of solvent. Column effluent was monitored at 210 and 270 nm. Retention volumes of metabolites are given in Table 1. Samples of culture fluid (2–10 µl) were injected after cells had been removed by centrifugation.

Chloride ion concentration was measured with an ion selective combination electrode (model 96/17, Orion Research, Cambridge, Mass., USA).

Spectrophotometric analysis was performed on a Uvikon 810 spectrophotometer (Kontron, Eching, Germany). ^{13}C and high resolution ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker CXP 300 (Bruker, Rheinstetten, Germany) with Aspect 2000 software using tetramethylsilane as internal standard and D_6-acetone as solvent. Mass spectra were recorded on a mass spectrometer MAT 711 from Varian (Palo Alto, Calif, USA). Optical activity was measured in aqueous solution with a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Norwalk, Conn., USA).

Melting points were measured with a heatable microscope (Reichert Thermovar, Nussloch, Germany).

Chemicals

Chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland) and Merck AG (Darmstadt, Germany) or prepared as described previously (Dorn et al. 1978b; Schmidt et al. 1980; Reineke and Knackmuss 1984; Pieper et al. 1985). 2-Chloro-4-methylphenol was supplied by Bayer AG (Leverkusen, Germany). 2-Chloro-4-methylphenoxyacetate was prepared from 2-chloro-4-methylphenol by the method of Winnacker and Küchler (1972). Its structure was confirmed by mass spectrometry.

Table 1. Retention volumes on reverse phase HPLC of metabolites formed from 2-chloro-4-methylphenoxyacetate

Metabolite	*Retention volume (ml)* Methanol concentrations of solvent (%)		
	40	30	15
2-Chloro-4-methyl-*cis*, *cis*-muconate (IV)	3.7	5.7	14.8
2-Chloro-4-methyl-muconolactone (III)	1.8	2.8	6.7
5-Chloro-3-methyl-muconolactone (I)	0.9	1.4	2.8
5-Chloro-3-methyl-muconolactone (II)	0.9	1.4	3.2

Results

2-Chloro-4-methylphenoxyacetate (CMPA) as inducer of enzymes of the chloroaromatic pathway

Alcaligenes eutrophus JMP 134 cannot use 2-chloro-4-methylphenoxyacetate as sole source of carbon and energy, even after long term adaptation. However, CMPA induced the enzymes of the chloroaromatic pathway (2,4-D monooxygenase, chlorophenol hydroxylase, catechol 1,2-dioxygenase type II, dichloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase) (Table 2). It thus appears that CMPA can induce enzymes for its own catabolism, which, however, is unproductive in JMP 134 or its constitutive derivative JMP 134-1.

Cometabolism of 2-chloro-4-methylphenoxyacetate and identification of compound IV as 2-chloro-4-methyl-cis,cis-muconate

To analyze the critical steps of CMPA metabolism, this compound (1 mM) was cometabolized by JMP 134-1 growing on fructose. During cooxidation, four metabolites I–IV were detected (see Table 1). Since no chloride was found in the culture fluid, all four metabolites apparently still contain bound chlorine. UV-Spectra measured in situ by HPLC diode array measurements showed that only metabolite IV exhibited an absorption maximum (273 nm) outside of the far UV region (210–220 nm). Spectrophotometric analysis of the cell free supernatant (pH 7.4) revealed an absorption maximum at 265 nm. When acidified top H 5, pH 3 or pH 1.5 by the addition H_3PO_4 (40%), this absorption maximum shifted to 273 nm before disappearing completely. HPLC analysis revealed that upon acidification, metabolite IV yielded varying amounts of two compounds that had already been observed during cooxidation (metabolite I and II). The formation of I predominated under mild acidic conditions ($c_I:c_{II} = 7-10:1$ at pH 5), whereas at lower pH-values, higher amounts of II were produced ($c_I:c_{II} = 2-3:1$ at pH 3 and $1-2:1$ at pH 1.5).

A variety of differently substituted muconic acids have been reported to exhibit absorption maxima at 260–280 nm (Schmidt et al. 1980). Because of the known instability of these compounds under acidic conditions, compound IV was assumed to be 2-chloro-4-methyl-*cis,cis*-muconate. To test this assumption, the activity of homogenous dichloromuconate cycloisomerase (Kuhm et al. 1990) with compound IV was analyzed. Because the enzyme has been reported to be inhibited by phosphate, cometabolism of CMPA was carried out with cells of JMP 134-1 growing on fructose in Tris/HCl buffer (50 mM, pH 7.4). The same 4 metabolites as described above accumulated in the culture fluid. Spectrophotometric analysis clearly demonstrated that addition of purified dichloromuconate cycloisomerase to the cell-free culture fluid abolished the absorption maximum at 265 nm. HPLC analysis revealed that compound IV was completely converted to metabolite III, with no formation of I or II.

Identification of compound III as 4-carboxymethyl-2-chloro-4-methylbut-2-en-4-olide (2-chloro-4-methylmuconolactone)

The ^{1}H-NMR-data of metabolite III (Table 3) are very similar to those of 4-carboxymethyl-4-methyl- and 4-carboxymethyl-2,4-dimethylbut-2-en-4-olide (Knack-

Table 2. Specific activities of catabolic enzymes from cells of *Alcaligenes eutrophus* JMP 134 induced with 2,4-dichlorophenoxyacetic acid (2,4-D) or 2-chloro-4-methylphenoxyacetic acid (CMPA)

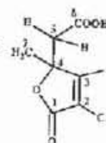
Enzyme activity	Assay substrate	Sp. act. (U/g protein) after induction with[a]		
		2,4-D	CMPA	Fructose
2,4-D monooxygenase	2,4-D	32	14	6
Chlorophenol hydroxylase	2,4-Dichlorophenol	130	40	12
Catechol 1,2-dioxygenase	3-Chlorocatechol	190	410	10
Dichloromuconate cycloisomerase	2,4-Dichloro-*cis*, *cis-muconate*	240	130	<5
Dienelactone hydrolase	*trans*-4-Carboxymethylene-but-2-en-4-olide	760	1300	55
Maleylacetate reductase	Maleylacetate	500	550	15

[a] Cells were harvested after 15 h of induction with 2,4-D (2 mM) or CMPA (2 mM). Enzyme activities of 2,4-D monooxygenase and chlorophenol hydroxylase were determined with whole cells. All other activities were determined in cell-free extracts

Table 3. ^1H and ^{13}C NMR data of compound III[a]

Nucleus	Chemical shift parts/10^6	Coupling assignment	Coupling constant (Hz)	Nucleus	Chemical shift parts/10^6
3-H	7.80 (7.60)[c]			C-1	170.5[b]
5-H_A	3.02 (3.00)	$^2J(5\text{-}H_A, 5\text{-}H_B)$	16.2 (16.0)[c]	C-2	124.6
5-H_B	2.93 (2.69)	$^2J(5\text{-}H_A, 5\text{-}H_B)$	16.2 (16.0)	C-3	153.1
7-H	1.62 (1.61)			C-4	85.1
				C-5	42.9
				C-6	167.4[b]
				C-7	24.8

[a] III has the following structure



[b] Assignments to C-1 and C-6 are tentative

[c] Values in parentheses confer to chemical shifts and coupling constants of the methylester. The chemical shift of the protons of the methoxysubstituent was 3.71

muss et al. 1976; Hartmann et al. 1978; Pieper et al. 1990) and demonstrate that metabolite III is 4-carboxymethyl-2-chloro-4-methylbut-2-en-4-olide (2-chloro-4-methylmuconolactone). The formation of this compound can be readily explained by the 1,4-cycloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate.

The ^{1}H-NMR data of the methylester are similar to those of the unmethylated product except for the presence of a singlet centered at $\delta = 3.71$ parts/10^6 due to the three protons of the methylester group. This confirms the presence of a single carboxylic group in the original metabolite.

The ^{13}C NMR data correspond to those observed for 4-fluoromuconolactone (Schlömann et al. 1990a) and provide additional evidence for the postulated structure (Table 3).

Mass spectrometric analysis (Table 4) showed molecular ions of m/z 192 and 190 and of m/z 206 and 204 of the derivatized product, demonstrating that the metabolite contains chloride. The fragments M^+-m/z 41 and M^+-m/z 42 of the derivatized product are typical for a methylester. Although chloride elimination was observed from both the free acid and the methylester, a variety of fragments still contained chloride. Prominent signals at m/z 144 and 146, m/z 131 and 133 and m/z 103 and 105 in both spectra were due to M^+-HCOOH or M^+-$HCOOCH_3$, M^+-CH_2COOH or M^+-CH_2COOCH_3 and to elimination of CO from the latter. Similar fragmentation patterns have been observed for 4-carboxymethyl-4-methyl- and 4-carboxymethyl-2,4-dimethylbut-2-en-4-olide (4-methyl- and 2,4-dimethylmuconolactone) (Knackmuss et al. 1976; Hartmann et al. 1978; Pieper et al. 1990). Consequently the identity of III with 2-chloro-4-methylmuconolactone (2C4MML) can be assumed. This metabolite, formed biologically from 2-chloro-4-methyl-*cis,cis*-muconate by dichloromuconate cycloisomerase, was shown to be optically active with $[\alpha]_{D25} = +6.9$ (9.4 mg/ml). The melting point was 90 °C.

Table 4. Mass spectrometric data of methylated compounds I, II and III

m/z	Intensity % of basepeak		m/z	Intensity % of basepeak
	Compound I	Compound II		Compound III
206	0.4	0.9	206	3.1
204	1.4	2.7	204	9.4
169	15.3	9.6	175	1.6
168	4.4	6.2	174	1.8
138	4.5	2.9	173	4.6
137	54.1	36.3	172	4.4
136	8.0	8.9	169	9.3
98	11.2	7.6	163	2.6
97	100.0	100.0	161	8.5
69	61.0	52.2	149	4.2
			147	15.5
			146	11.2
			144	32.3
			133	46.9
			131	100.0
			105	6.7
			103	17.5

Characterization of compounds I and II as the diastereomers of 4-carboxychloromethyl-3-methylbut-2-en-4-olide (5-chloro-3-methylmuconolactone)

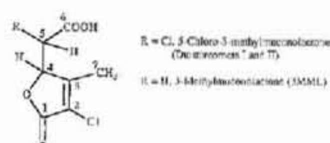
Mass spectra of methylated metabolite I and II were nearly identical and showed molecular ions at m/z 204 and 206. The same ions were observed for the methylated metabolite III. Evidently, both I and II contained the organic chloride of CMPA. Signals at m/z 168 and 169 were due to the elimination of Cl and HCl, respectively. In contrast to compound III, no major chloride containing fragments were observed. Elimination of Cl/HCl as well as OCH_3/$HOCH_3$ gave fragments m/z = 138, 137 and 136. The basepeak at m/z = 97 is due to elimination of $CHClCOOCH_3$. This fragmentation pattern suggests that I and II were diastereomers of 5-chloro-3-methylmuconolactone (5C3MML I and II).

The ^{1}H-NMR-spectra of compounds I and II were very similar and were in accordance with the postulated structure (Table 5). In contrast to NMR data of pre-

Table 5. [1]H data of compound I and II and ^{13}C NMR data of compound I and 3-methylmuconolactone (3MML)[a]

Nucleus	Compound	Chemical shift parts/10^6	Coupling assignment	Coupling constant (Hz)	Nucleus	Compund	Chemical shift parts/10^6
2-H	I	6.03 (6.01)	^{4}J(2-H,4-H)	1.5 (1.5)	C-1	I	171.9[b]
			^{4}J(2-H,7-H)	1.5 (1.5)		3MML	172.1[b]
	II	6.04 (6.02)	^{4}J(2-H,4-H)	1.5 (1.5)			
			^{4}J(2-H,7-H)	1.5 (1.5)	C-2	I	119.6
						3MML	117.1
4-H	I	5.60 (5.56)	^{3}J(4-H,5-H)	3.7 (3.6)	C-3	I	166.3
			^{4}J(2-H,4-H)	1.5 (1.5)		3MML	161.9
			^{4}J(4-H,7-H)	0.7 (0.8)			
	II	5.72 (5.67)	^{3}J(4-H,5-H)	2.2 (2.1)	C-4	I	84.2
			^{4}J(2-H,4-H)	1.5 (1.5)		3MML	82.1
			^{4}J(4-H,7-H)	0.7 (0.8)			
					C-5	I	57.9
5-H	I	5.20 (5.19)[c]	^{3}J(4-H,5-H)	3.7 (3.6)[c]		3MML	37.4
	II	5.24 (5.25)	^{3}J(4-H,5-H)	2.2 (2.1)			
					C-6	I	167.2[b]
7-H	I	2.14 (2.08)	^{4}J(4-H,7-H)	0.7 (0.8)		3MML	170.7[b]
			^{4}J(2-H,7-H)	1.5 (1.5)			
	II	2.19 (2.14)	^{4}J(4-H,7-H)	0.7 (0.8)	C-7	I	14.1
			^{4}J(2-H,7-H)	1.5 (1.5)		3MML	13.8

[a] The compounds are of the following structure



[b] Assignments to C-1 and C-6 are tentative

[c] Values in parentheses confer to the chemical shifts and coupling constants of the methylester. The chemical shift of the protons of the methoxysubstituent were 3.81 (I) and 3.83 (II), respectively

viously characterized muconolactones (Catelani et al. 1971; Knackmuss et al. 1976; Hartmann et al. 1978; Miller 1981; Pieper et al. 1985; Cain et al. 1989; Pieper et al. 1990; Schlömann et al. 1990a), the typical AB spectrum of two diastereotopic methylene protons is missing in the spectra of I and II, indicating the presence of a substituent on the 4-carboxymethyl side chain. In accordance with a chlorine substituted 4-carboxymethyl side chain, single protons with chemical shifts of $\delta =$ 5.20 (compound I) and 5.24 parts/10^6 (compound II) were observed. The vicinal coupling constants (3.7 Hz for compound I, 2.2 Hz for compound II) were significantly lower than those observed for 3-methylmuconolactone (4 and 8 Hz, respectively; Miller 1981; Pieper et al. 1985; Cain et al. 1989) indicating the presence of an electronegative substituent.

Comparison of ^{13}C NMR data of I with that of authentic 3-methylmuconolactone (Table 5) gives further evidence for its identity with a diastereomer of 5-chloro-3-methylmuconolactone. Chemical shifts were very similar with the exception of the chlorosubstituted C-5 atom.

Biological activity of 2-chloro-4-methyl-cis,cis-muconate

Compound IV (2-chloro-4-methyl-*cis,cis*-muconate) was converted by purified dichloromuconate cycloisomerase to 2C4MML. Because the muconate was too unstable to be extracted under acidic conditions, kinetic data were determined with the metabolite prepared in situ and therefore yielded only approximate values. A solution of 2-chloro-4-methyl-*cis,cis*-muconate in Tris/HCl buffer pH 8, 50 mM was prepared via cooxidation of CMPA (1 mM) by JMP 134-1. The concentration of the muconate was determined by following the increase in product concentration (only 2C4MML was formed during turnover by dichloromuconate cycloisomerase). Aliquots of the solution (0.05–0.27 mM) were used as substrate in the dichloromuconate cycloisomerase reaction. Using this substrate preparation, a K_m value of about 60 µM was observed. The K_{cat} was about 55% of that found for 2,4-dichloro-*cis,cis*-muconate.

Quantification of metabolites

When CMPA (1 mM) was cometabolized by cells growing with fructose considerable amounts of 2C4MML (30–50%) were formed. 2-Chloro-4-methyl-*cis,cis*-muconate accumulated to levels of 40% of the introduced CMPA. High amounts of 5C3MML I (up to 25%) occurred due to the spontaneous cycloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate under the incubation conditions. The concentration of 5C3MML II was always less than 10% of that of 5C3MML I. Consequently it can be assumed that half of the CMPA was actually excreted as 2-chloro-4-methyl-*cis,cis*-muconate.

Transformation of CMPA by resting cells ($A_{546\,nm}$ = 2–10), however, led to the formation of about 80% of 2C4MML under these conditions. 2-Chloro-4-methyl-

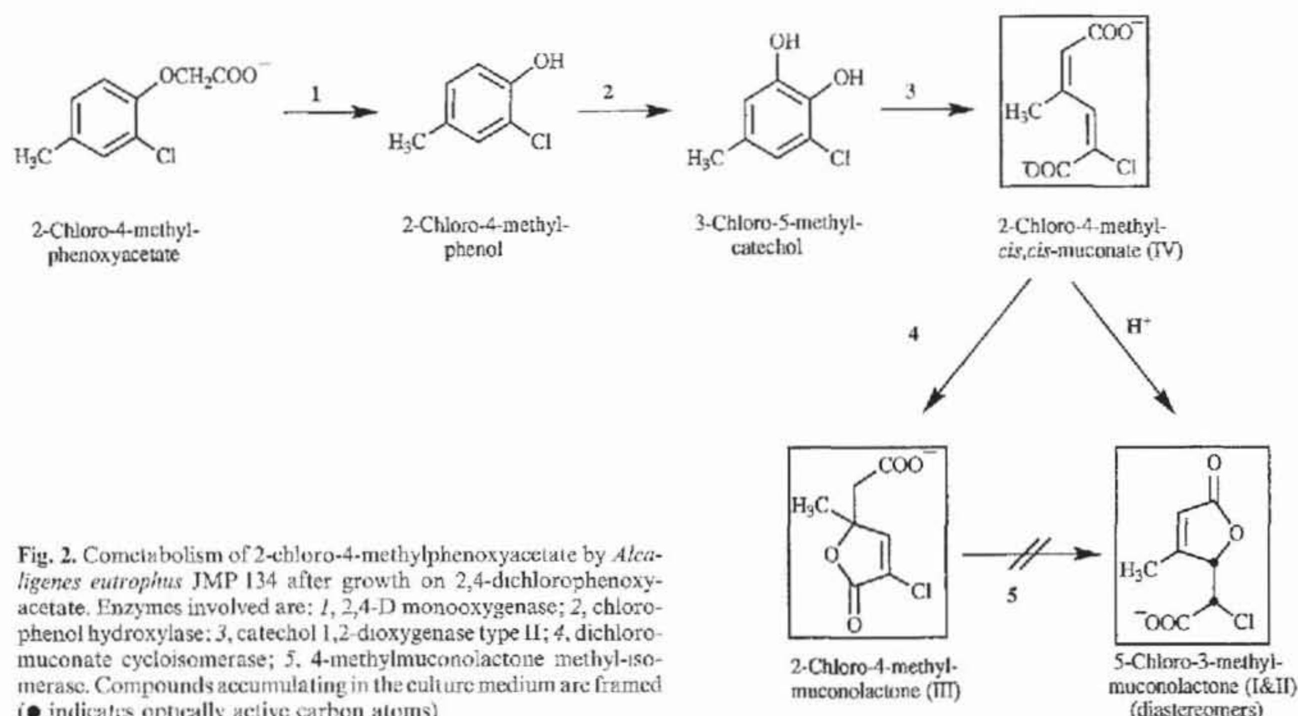


Fig. 2. Cometabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes eutrophus* JMP 134 after growth on 2,4-dichlorophenoxyacetate. Enzymes involved are: *1*, 2,4-D monooxygenase; *2*, chlorophenol hydroxylase; *3*, catechol 1,2-dioxygenase type II; *4*, dichloromuconate cycloisomerase; *5*, 4-methylmuconolactone methyl-isomerase. Compounds accumulating in the culture medium are framed (● indicates optically active carbon atoms)

cis,cis-muconate was present in amounts of about 20% only. Due to the fast bioconversion (CMPA was totally converted within 2 h), only minor amounts of 5C3MML I and II were formed.

Further metabolism of chloromethylmuconolactones

2-Chloro-4-methylmuconolactone was not further converted by resting cells or cell-free extracts of fructose grown cells of JMP 134-1 or benzoate as well as 2,4-D grown cells of JMP 134. Purified 4-methylmuconolactone methyl-isomerase, which has previously been shown to catalyze the isomerization of 4-carboxymethyl-4-methylbut-2-en-4-olide (4-methylmuconolactone) to 4-carboxymethyl-3-methylbut-2-en-4-olide (3-methylmuconolactone) (Pieper et al. 1990), exhibited no activity towards 2C4MML. This compound thus appears to be a dead-end metabolite in JMP 134 and JMP 134-1. Resting cells and cell-free extracts of both fructose grown JMP 134-1 and 2,4-D grown JMP 134 were also inactive towards both diastereomers of 5C3MML. However, cell free extracts of benzoate grown cells were active, especially towards diastereomer I. Analysis of the enzymatic activity responsible for the further metabolism of 5C3MML, and of the reaction products, is in progress.

Stability of 5-chloro-3-methylmuconolactone diastereomers

5-Chloromuconolactone has been postulated to be a highly unstable intermediate of the cycloisomerization of 2-chloro-*cis,cis*-muconate and spontaneously lose chloride (Schmidt and Knackmuss 1980). However, the 3-methylsubstituted homologues of 5-chloromuconolactone were found to be stable under both physiological and acidic conditions. Addition of NaOH results in the spontaneous and quantitative elimination of chloride. Under different alkaline conditions, a 100 µM solution of compound I showed the following half-lifes: 4 h in 100 mM Tris/HCl pH 9; 18 min in 100 mM Na_2CO_3/$NaHCO_3$, pH 10; 170 s in 100 mM Na_2HPO_4/NaOH, pH 11 and 10 s in Na_2HPO_4/NaOH, pH 12. Products formed during this reaction are currently being characterized.

Discussion

Although not a growth substrate for *Alcaligenes eutrophus* JMP 134, 2-chloro-4-methylphenoxyacetate (CMPA) is transformed by this organism, yielding four major metabolites. Of these, 2-chloro-4-methyl-*cis,cis*-muconate and 2-chloro-4-methylmuconolactone (2C4MML) were identified as true metabolites. Although 2-chloro-4-methyl-*cis,cis*-muconate is a substrate for dichloromuconate cycloisomerase, significant amounts of this metabolite accumulated in the culture fluid. Enzyme catalyzed cycloisomerization gives rise to 2C4MML only. This compound is a dead-end metabolite in JMP 134. Both diastereomers of 5-chloro-3-methylmuconolactone (5C3MML) are formed by chemical cycloisomerization (Fig. 2).

The enzymatic 1,4-cycloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate is analogous to the bacterial cycloisomerization of 3-methyl-*cis,cis*-muconate (Catelani et al. 1971; Knackmuss et al. 1976; Pieper et al. 1985; Bruce and Cain 1988) but differs from the mode of cycloisomerization of 2-chloro-*cis,cis*-muconate (Schmidt and Knackmuss 1980). Evidently, the enzymatic cy-

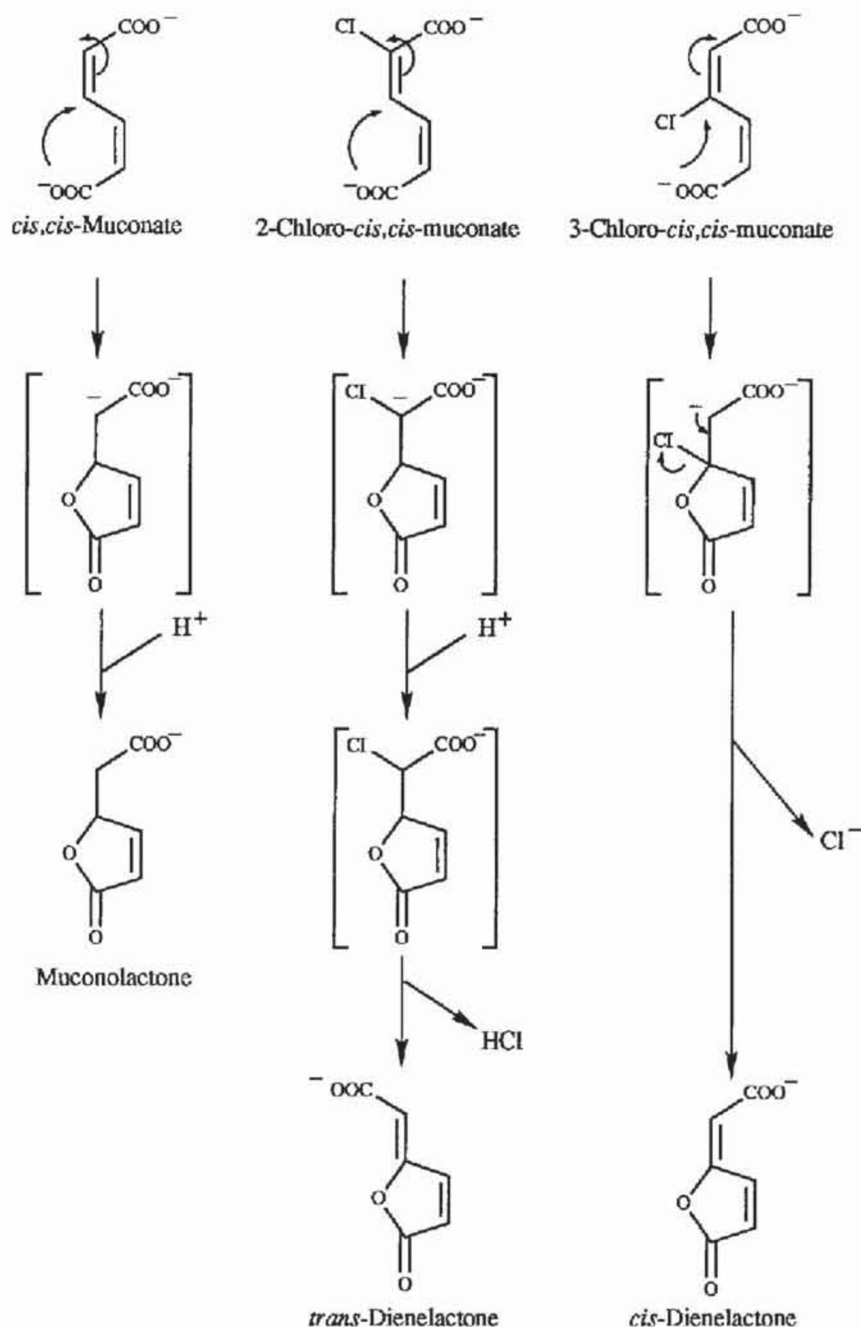


Fig. 3. Metabolism of halosubstituted *cis,cis*-muconates via carbanionic intermediates in analogy to the mechanism proposed for cycloisomerization of *cis,cis*-muconate by muconate cycloisomerase (Ngai and Kallen 1983). The cycloisomerization mechanism of 3-chloro-*cis,cis*-muconate has been proposed by Schlömann et al. (1990a)

cloisomerization of 2-chloro-4-methyl-*cis,cis*-muconate is governed by the methyl rather than the chlorosubstituent.

Chemically, only 3,6-cycloisomerization occurred, a process that gives rise to a mixture of diastereomers of 5C3MML. Analogously, Cain et al. (1989) showed formation of 3-methylmuconolactone by acid catalyzed 3,6-cycloisomerization of 3-methyl-*cis,cis*-muconate. The varying amounts of diastereomer I and II observed to be formed from 2-chloro-4-methyl-*cis,cis*-muconate at different pH-values are due to an initial *cis,trans* isomerization preceeding cycloisomerization at very low pH values (data not shown) (Cain et al. 1989).

The observed difference in the mode of cycloisomerization of enzyme and acid catalyzed reactions must be due to different reaction mechanisms. Whereas the enzyme catalyzed cycloisomerization has been shown to proceed via a carbanionic intermediate (Ngai and Kallen 1983), chemical cycloisomerization appears to be initiated by the addition of a proton.

Both diastereomers of 5C3MML were found to be highly stable under physiological as well as acidic conditions. The corresponding 5-chloromuconolactone has been postulated to be the primary cycloisomerization product of 2-chloro-*cis,cis*-muconate (Schmidt and Knackmuss 1980) and to be highly unstable.

Since the methylsubstituent at the 3-position is unlikely to significantly stabilize the 5-chloromuconolactone structure, 5-chloromuconolactone should also be stable under physiological conditions. However, *trans*-dienelactone rather than 5-chloromuconolactone was

reported to be the only product of the enzyme catalyzed cycloisomerization of 2-chloro-*cis,cis*-muconate (Schmidt and Knackmuss 1980).

The occurrence of an unstable chloromuconolactone as an intermediate has also been suggested for the enzyme catalyzed cycloisomerization of 3-halomuconates (Schmidt and Knackmuss 1980; Schreiber et al. 1980). *cis*-Dienelactone was shown to be the product of cycloisomerization of 3-chloro-*cis,cis*-muconate. Recent studies have shown, however, that 4-fluoromuconolactone is a relatively stable metabolite of 4-fluorocatechol metabolism (Schlömann et al. 1990a). Based on the model of Ngai and Kallen (1983) and Kozarich et al. (1986) of syn-addition to a double bond via a carbanionic intermediate, Schlömann et al. (1990a) suggested that the cycloisomerization reaction of 3-chloro-*cis,cis*-muconate is completed by the elimination of chloride rather than the protonation of the carbanionic intermediate. 4-Chloromuconolactone does not occur as an intermediate in this reaction cycle (Fig. 3). However, an analogous elimination of chloride from the carbanionic intermediate is not possible during the cycloisomerization of 2-chloro-*cis,cis*-muconate. In this case, the intermediate nature of a chloromuconolactone still has to be assumed. The elimination of chloride appears to be a fortuitous side reaction of the cycloisomerase enzyme.

Fungal muconate cycloisomerases have been shown to catalyze 1,4-cycloisomerization of 3-methyl-*cis,cis*-muconate (Powlowski and Dagley 1985; Cain et al. 1989) as opposed to 3,6-cycloisomerization. According to this, if active at all on 2-chloro-4-methyl-*cis,cis*-muconate a direct cycloisomerization to 5-chloro-3-methylmuconolactone can be assumed. The analysis of the elimination of chloride by these enzymes would be interesting. The considerable differences in both the reaction mechanisms and the enzyme structures between fungal and bacterial enzymes suggest that findings based on the fungal enzymes can not be used to understand the bacterial enzymes. Detailed analysis of bacterial cycloisomerases of chloride elimination from 2-chloro-*cis,cis*-muconates will therefore be necessary. We are currently engaged in the purification of such enyzmes in order to analyze the product formation and in crystallization of the enzymes in order to analyze the substrate binding pocket and amino acids involved in catalysis.

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