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# A *meta* Cleavage Pathway for 4-Chlorobenzoate, an Intermediate in the Metabolism of 4-Chlorobiphenyl by *Pseudomonas cepacia* P166

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**Bacterial degradation of biphenyl and polychlorinated biphenyls proceeds by a well-studied pathway which produces benzoate and 2-hydroxypent-2,4-dienoate (or, in the case of polychlorinated biphenyls, the chlorinated derivatives of these compounds). *Pseudomonas cepacia* P166 utilizes 4-chlorobiphenyl for growth and produces 4-chlorobenzoate as a central intermediate. In this study we found that strain P166 further transforms 4-chlorobenzoate to 4-chlorocatechol, which is mineralized by a *meta* cleavage pathway. Key metabolites which we identified include the *meta* cleavage product (5-chloro-2-hydroxymuconic semialdehyde), 5-chloro-2-hydroxymuconate, 5-chloro-2-oxopent-4-enoate, 5-chloro-4-hydroxy-2-oxopentanoate, and chloroacetate. Chloroacetate accumulated transiently, and slow but stoichiometric dehalogenation was observed.**

Biphenyl is an aromatic hydrocarbon which is metabolized by a wide variety of bacterial isolates. A pathway for the metabolism of biphenyl to benzoate has been described (8, 12, 18). The first step involves the transformation of biphenyl to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (biphenyl dihydrodiol) by biphenyl dioxygenase. The dihydrodiol is reduced by a dihydrodiol dehydrogenase to 2,3-dihydroxybiphenyl, which undergoes extradiol (*meta*) cleavage that is catalyzed by 2,3-dihydroxybiphenyl dioxygenase. The *meta* cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, is cleaved by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase to produce benzoate and 2-hydroxypent-2,4-dienoate. It has recently been reported that a pseudomonad degrades benzoate, which is produced from biphenyl, by a *meta* cleavage pathway (17). There apparently have been no other studies of the fate of benzoate or 2-hydroxypent-2,4-dienoate in relation to biphenyl metabolism, but pathways have been suggested by analogy to other compounds.

Polychlorinated biphenyls (PCBs) are chlorinated derivatives of biphenyl which sometimes can be metabolized by biphenyl degraders. The bacterial metabolism of polychlorinated biphenyls is mediated by the same enzymes that are used in the metabolism of biphenyl (2, 10, 11). It has often been reported that chlorobenzoates accumulate as dead-end products. Strains which utilize monochlorobiphenyls derive carbon and energy for growth from 2-hydroxypent-2,4-dienoate, which is produced from the nonchlorinated ring.

Chlorobenzoates are not always dead-end metabolites of PCB metabolism. *Alcaligenes* and *Acinetobacter* isolates have been reported to mineralize 4-chlorobiphenyl (4-CBP) via dehalogenation of 4-chlorobenzoate to form 4-hydroxybenzoate (16, 22). Barton and Crawford (5) have described a 4-CBP degrader which also utilizes 4-chlorobenzoate, although the pathway was not determined. There have been other reports of chlorobenzoate transformation by biphenyl-degrading bacteria (6, 19, 23), but no complete pathway has been described. We previously reported that *Pseudomonas cepacia* P166 utilizes all three monochlorobiphenyls as growth substrates, producing chlorobenzoate intermediates which are further metabolized to chlorocatechols (3). 2- and 3-Chlorobiphenyls were both metabolized to 3-chlorocatechol, which was *meta* cleaved to produce a toxic acyl halide. 4-CBP, however, was transformed to 4-chlorobenzoate and then to 4-chlorocatechol, which was mineralized, as shown by chloride release. In this paper we describe the pathway for 4-chlorobenzoate metabolism during utilization of 4-CBP by strain P166.

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## MATERIALS AND METHODS

**Bacterial strain and culture conditions.** The isolation and identification of *P. cepacia* P166 have been described previously (3). Strain P166 was grown in a mineral salts medium (1) supplemented with biphenyl or 4-CBP as previously described (3).

**Chemicals.** Biphenyl, 4-chlorobenzoate, and chloroacetaldehyde were purchased from Aldrich Chemical Co., Milwaukee, Wis. 4-CBP was purchased from Pfaltz & Bauer, Inc., Waterbury, Conn. 4-Chlorocatechol was obtained from Helix Biotech Corp., Richmond, British Columbia, Canada. Methanol and diethyl ether were purchased from Fisher Scientific Co., Fair Lawn, N.J. Chloroacetate was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was recrystallized twice from a methylene chloride solution before it was used. Acetonitrile was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich.

Diazomethane was generated as follows. *N*-nitroso-*N*-methylurea (Sigma Chemical Co., St. Louis, Mo.) was dissolved in water, and 10 ml of the solution was slowly stirred in a small Erlenmeyer flask on ice. An equal volume of diethyl ether was added to form a second phase. A pellet of KOH was then added to the flask. The KOH immediately reacted with the *N*-nitroso-*N*-methylurea to form diazomethane, which was trapped in the ethereal phase. The ethereal phase was carefully removed and used directly for derivatization of acidic metabolites.

**Resting cell incubation experiments.** The preparation of resting cells and the procedure used for resting cell incubation experiments have been described previously (3). Because oxygen uptake experiments performed with strain P166 provided no evidence that 4-CBP induced a different set of enzymes than biphenyl, biphenyl-grown resting cells were used to study 4-CBP metabolism.

**Recovery of metabolites for gas chromatography.** Resting cell incubation experiments were performed as described above, except that all volumes were increased fivefold and each substrate was added at a concentration of 500 μg/ml. After incubation, samples were centrifuged, and the supernatants were extracted three times with an equal volume of diethyl ether. The organic phases were pooled, dried over sodium sulfate, and concentrated to a few hundred microliters with a Buchi rotory evaporating unit. The samples were derivatized by adding an ethereal solution of diazomethane until a yellow color persisted. Samples were then evaporated to about 100 μl under a gentle stream of $N_2$ and analyzed by gas chromatography-infrared spectrometry-mass spectrometry.

**Analytical procedures. (i) Chloride determination.** Amounts of chloride were determined as described previously (3).

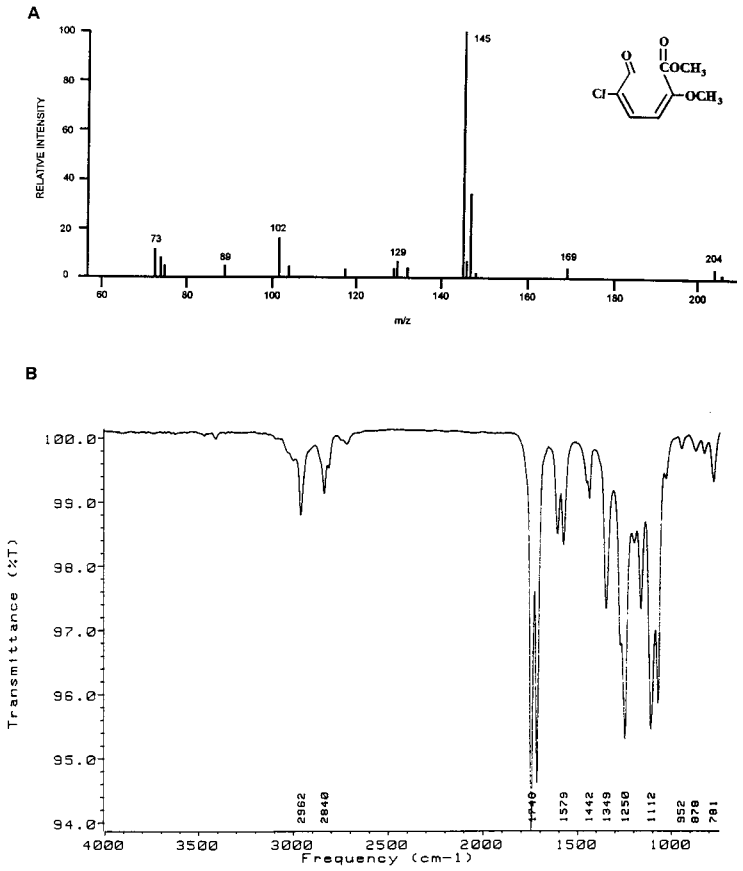


FIG. 1. Mass spectrum (A) and infrared spectrum (B) of the metabolite identified as the dimethyl ester of 5-chloro-2-hydroxymuconic semialdehyde (the *meta* cleavage product of 4-chlorocatechol).

**(ii) Gas chromatography-infrared spectrometry-mass spectrometry.** A Hewlett-Packard model 5989A gas chromatograph-mass spectrometer was used for gas chromatography-infrared spectrometry-mass spectrometry. The samples flowed through a model 5965B infrared detector before they entered the mass analyzer. The mass spectrometer was used in the 70-eV electron impact mode, and a quadrupole mass filter (100°C) was scanned over the range from *m/z* 40 to *m/z* 400 each second. The injector, ion source, and detector temperatures were 250, 200, and 280°C, respectively. Metabolites were separated on a DB-5 capillary column (length, 30 m; inside diameter, 0.24 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, Calif.). The temperature was increased from 30 to 180°C at a rate of 5°C $min^{-1}$ after an initial holding time of 1 min.

**(iii) HPLC.** A high-performance liquid chromatography (HPLC) analysis of 4-chlorobenzoate was performed by using a $C_{18}$ column as described previously (3). Chloroacetate was quantified by detection at 210 nm after separation on an organic acid column (length, 300 mm; inside diameter, 7.8 mm; Interaction Chemicals, Inc.). HCl (50 mM) was used isocratically as the mobile phase at a flow rate of 0.5 ml $min^{-1}$.

## RESULTS

A key chlorinated metabolite of 4-CBP metabolism in strain P166 has been identified previously as 4-chlorobenzoate (3). When 4-chlorobenzoate was incubated with resting cells of strain P166, two chlorinated acidic metabolites were identified by a gas chromatography-mass spectrometric analysis of derivatized supernatant extracts. The mass spectrum of the first compound was consistent with the structure of the dimethyl derivative of the 4-chlorocatechol *meta* cleavage product, 5-chloro-2-hydroxymuconic semialdehyde (Fig. 1). The molecular ion (M) at *m/z* 204 had the characteristic M/M + 2 ratio of 3:1 resulting from the $^{35}Cl/^{37}Cl$ isotope ratio of a single Cl atom. Many of the fragments in the spectrum clearly had the same 3:1 ratio. The major fragment ions were *m/z* 169 (M-Cl), *m/z* 145 (M-$COOCH_3$), *m/z* 130 (M-$COOCH_3$-$CH_3$), *m/z* 102 (M-$COCH_3COOCH_3$), and *m/z* 73 (M-$COCH_3COOCH_3$-CHO). The mass spectrum obtained for this *meta* cleavage product was consistent with the spectra reported previously for the same compound (15, 23). The infrared spectrum of the *meta* cleavage product had two carbonyl stretching bands, one at 1,746 $cm^{-1}$ and one at 1,715 $cm^{-1}$, which were contributed by the alpha-keto ester and the aldehyde, respectively (Fig. 1B). The unusually high frequency of the aldehyde carbonyl stretch band suggests that a vicinal chloride was present. There were also a characteristic aldehyde C-H stretch at 2,820 $cm^{-1}$ and a methyl ether C-H stretch at 2,840 $cm^{-1}$. In addition, the methyl ester of the keto tautomer of the *meta* cleavage product (molecular weight, 190) was also identified.

The second product obtained from 4-chlorobenzoate metabolism was identified as the methyl ester of chloroacetic acid. The mass spectrum, which matched the spectrum of an authentic standard, had a molecular ion at *m/z* 108 and major fragment ions at *m/z* 77 (M-$OCH_3$), *m/z* 59 ($COOCH_3$), *m/z* 49 (M-$COOCH_3$), and *m/z* 42 (M-$OCH_3$-Cl).

4-Chlorobenzoate was quickly depleted when it was incubated with resting cells of strain P166, but the release of chloride was slow, although it was stoichiometric (Fig. 2). Chloroacetate transiently accumulated during this process. Several

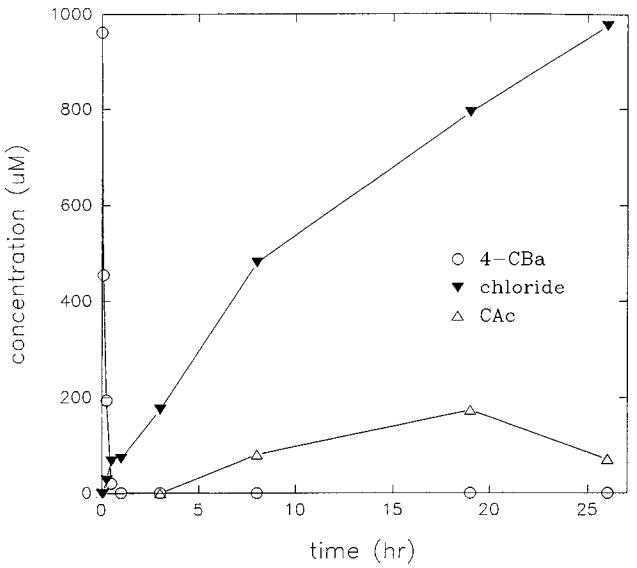


FIG. 2. Resting cells of strain P166 incubated with 0.96 M 4-chlorobenzoate. 4-Chlorobenzoate (4-CBa) was rapidly transformed, leading to the transient accumulation of chloroacetate (CAc) and the stoichiometric release of chloride.

additional polar metabolites were detected by HPLC before chloroacetate accumulated, but these metabolites were not detected by gas chromatography-mass spectrometry. Chloroacetaldehyde was never detected, but chloride release was observed when chloroacetaldehyde was added to resting cells (data not shown).

When resting cells were incubated with 4-chlorocatechol, three additional metabolites were isolated and identified. The first was the dimethyl ester-enol ether of 5-chloro-2-hydroxymuconate. The mass spectrum of this dimethyl ester-enol ether, which was consistent with the mass spectrum reported previously for the same compound (15), had a molecular ion (M) at *m/z* 234 and major fragment ions at *m/z* 199 (M-Cl), *m/z* 175 (M-$COOCH_3$), *m/z* 160 (M-$COOCH_3$-$CH_3$), *m/z* 132 (M-$COCH_3COOCH_3$), *m/z* 73 (M-$COCH_3COOCH_3$-$COOCH_3$), and *m/z* 59 ($COOCH_3$).

The other metabolites identified were 5-chloro-2-oxopent-4-enoate and 5-chloro-4-hydroxy-2-oxopentanoate. The mass spectrum of the methyl ester of 5-chloro-2-oxopent-4-enoate (Fig. 3) had a molecular ion (M) at *m/z* 162 and major fragment ions at *m/z* 127 (M-Cl), *m/z* 113 (M-$CH_2Cl$), *m/z* 103 (M-$COOCH_3$), *m/z* 75 (M-$COCOOCH_3$), *m/z* 68 (M-$COOCH_3$-Cl), *m/z* 59 ($COOCH_3$), and *m/z* 49 ($CH_2Cl$). The mass spectrum of the methyl ester of 5-chloro-4-hydroxy-2-oxopentanoate (Fig. 4) lacked a molecular ion (molecular weight, 180) but had fragment ions at *m/z* 121 (M-$COOCH_3$), *m/z* 103 (M-$COOCH_3$-$H_2O$), *m/z* 85 (M-$COOCH_3$-HCl), *m/z* 79 (M-$CH_2COCOOCH_3$), *m/z* 71 (M-$COOCH_3$-H-$CH_2Cl$), and *m/z* 59 ($COOCH_3$).

The major fragmentation pattern for all of the methyl esters was M-59 rather than the more typical M-31. Fragment M-59 represents loss of $COOCH_3$ and is characteristic of the methyl esters of 2-oxo-carboxylic acids. This cleavage arises from repulsion of the alpha carbonyl, which has a slight carbocation nature. This pattern was also evident in the mass spectra of the methyl derivatives of 5-chloro-2-hydroxymuconic semialdehyde (Fig. 1A) and 5-chloro-2-hydroxymuconate. It is also characteristic of the methyl esters of 2-oxo-carboxylic acids to have very weak molecular ions. Accordingly, these methyl ester species have very weak or no molecular ions, and the Cl isotope peak of the molecular ion is sometimes not detected. An infrared spectrum of 5-chloro-4-hydroxy-2-oxopentanoate (methyl ester) was also obtained, and the presence of the hydroxyl group was confirmed by an O-H stretch at 3,620 $cm^{-1}$.

5-Chloro-4-hydroxy-2-oxopentanoate is a gamma-hydroxy acid and upon acidification should largely be converted to a gamma-lactone. Accordingly, the detection of this gamma-lactone is further evidence that 5-chloro-4-hydroxy-2-oxopentanoate is a metabolite. The mass spectrum of the gamma-lactone of 5-chloro-2,4-dihydroxypent-2-enoate (Fig. 5) had a molecular ion (M) at *m/z* 162 and major fragment ions at *m/z* 130 (M-$CH_3OH$), *m/z* 113 (M-$CH_2Cl$), *m/z* 99 (M-$CH_2Cl$-$CH_2$), and *m/z* 85 (M-$CH_2Cl$-CO). The infrared spectrum of this compound (data not shown) was characterized by a carbonyl stretch band at 1,822 $cm^{-1}$.

## DISCUSSION

The pathway for metabolism of 4-CBP proposed in Fig. 6 is consistent with all of the metabolites identified in this study. 4-Chlorobenzoate, a central intermediate, is transformed to 4-chlorocatechol, which is metabolized by a typical *meta* cleavage pathway. Although *meta* cleavage of 4-chlorocatechol has been described previously (15, 20, 23), an entire *meta* cleavage pathway is usually associated only with the metabolism of nonchlorinated catechols produced as central intermediates in the degradation of such aromatic hydrocarbons as naphthalene (9)

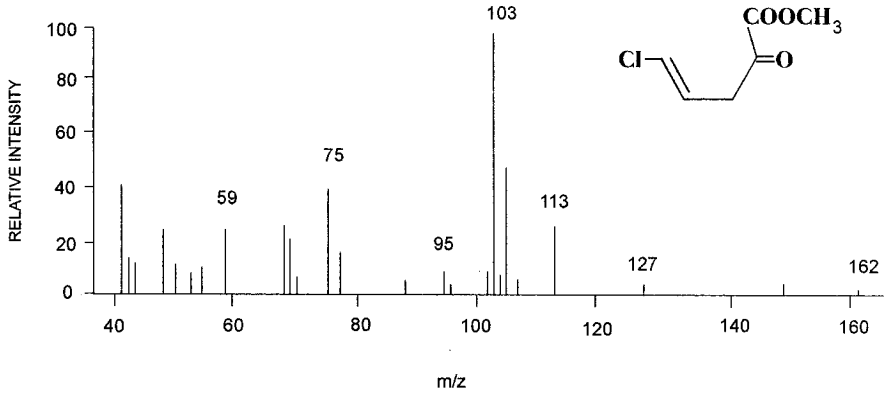


FIG. 3. Mass spectrum of the metabolite identified as the methyl ester of 5-chloro-2-oxopent-4-enoic acid.

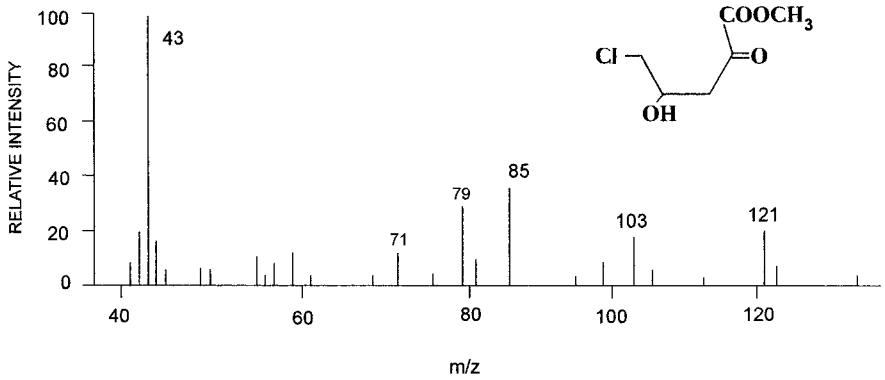


FIG. 4. Mass spectrum of the metabolite identified as the methyl ester of 5-chloro-4-hydroxy-2-oxopentanoic acid.

and toluene (7). The *meta* cleavage pathway which has been studied most extensively is the pathway associated with the TOL plasmid (4, 7), which contains genes for the catabolism of toluene. An upper pathway converts toluene to catechol, which is then metabolized by the lower or *meta* cleavage pathway. The *meta* cleavage pathway of the TOL plasmid has two alternate branches (13, 21). A hydrolytic branch converts the *meta* cleavage product to 2-hydroxypent-2,4-dienoate with a hydrolase. The dehydrogenative branch also produces 2-hydroxypent-2,4-dienoate, but does so in three steps. The *meta* cleavage product is first oxidized by a dehydrogenase to a 2-hydroxymuconate, which is converted to 4-oxalocrotonate by 4-oxalocrotonate isomerase. Finally, 4-oxalocrotonate is converted to 2-hydroxypent-2,4-dienoate by a carboxylase. A hydratase then converts 2-hydroxypent-2,4-dienoate to 4-hydroxy-2-oxopentanoate, which is cleaved by an aldolase to form pyruvate and acetaldehyde.

Identification of the 4-chlorocatechol *meta* cleavage product, 5-chloro-2-hydroxymuconic semialdehyde, confirmed that strain P166 catabolizes 4-chlorobenzoate to 4-chlorocatechol which is *meta* cleaved. The high aldehyde carbonyl stretching frequency in the infrared spectrum of the semialdehyde and the production of chloroacetate (rather than acetate) indicate that proximal rather than distal *meta* cleavage of 4-chlorocatechol occurs. The presence of 5-chloro-2-hydroxymuconate indicates that the *meta* cleavage pathway for 4-chlorocatechol in strain P166 is dehydrogenative. No chlorinated 4-oxalocrotonate was identified. However, we cannot eliminate the possibility that strain P166 also has a hydrolytic branch of the *meta* cleavage pathway.

5-Chloro-2-hydroxypent-2,4-dienoate was not detected, although its keto tautomer, 5-chloro-2-oxopent-4-enoate, was identified. It has been demonstrated for the *meta* cleavage pathway of the TOL plasmid that the enol tautomer, 2-hydroxypent-2,4-dienoate, is the product of the decarboxylase in the dehydrogenative branch (14), and the enol tautomer again serves as the substrate for the hydratase. It has also been shown that 2-hydroxypent-2,4-dienoate is unstable and upon accumulation is converted to its keto tautomer, which cannot serve as a substrate for the hydratase. If 5-chloro-2-hydroxypent-2,4-dienoate is as unstable as its nonchlorinated analog, it is not surprising that this metabolite was detected only in the keto form.

This pathway for metabolism of 4-chlorobenzoate is very slow. High-density resting cells took 1 day to completely mineralize the substrate, as determined by stoichiometric release of chloride. Chloroacetate, the most highly oxidized metabolite identified, transiently accumulated later. The fate of chloroacetate has not been determined although biochemical dehalogenation eventually occurs.

The formation of 4-chlorobenzoate from 4-CBP coincides with the formation of 2-hydroxypent-2,4-dienoate, which is derived from the nonchlorinated ring. The pathway for metabolism of this compound in biphenyl degraders has not been determined. It should be noted that this compound is the nonchlorinated analog of 5-chloro-2-hydroxypent-2,4-dieno-

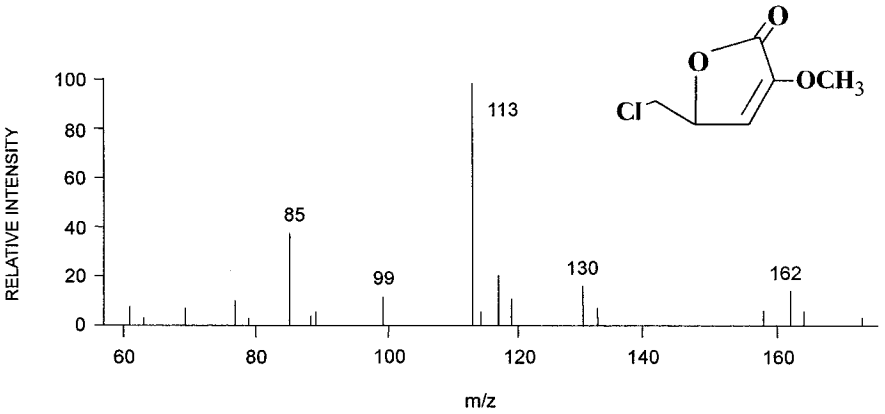


FIG. 5. Mass spectrum of the metabolite identified as the gamma-lactone of 5-chloro-4-hydroxy-2-oxopentanoic acid.

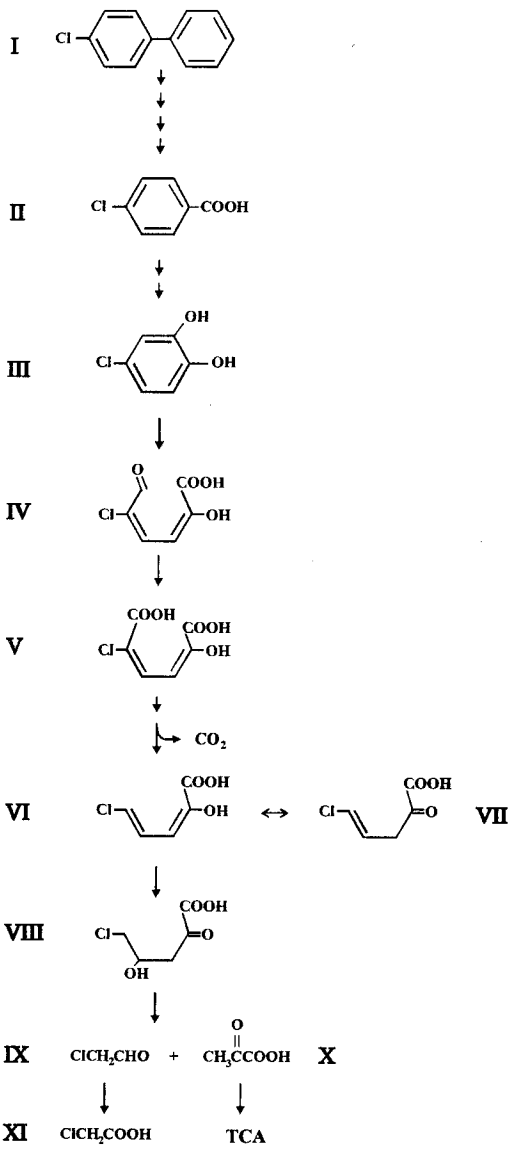


FIG. 6. Proposed pathway for the catabolism of 4-CBP by *P. cepacia* P166. I, 4-CBP; II, 4-chlorobenzoate; III, 4-chlorocatechol; IV, 5-chloro-2-hydroxymuconic semialdehyde; V, 5-chloro-2-hydroxymuconic acid; VI, 5-chloro-2-hydroxypent-2,4-dienoic acid (enol); VII, 5-chloro-2-oxopent-4-enoic acid (keto); VIII, 5-chloro-4-hydroxy-2-oxopentanoic acid; IX, chloro-acetaldehyde; X, pyruvic acid; XI, chloroacetic acid; TCA, trichloroacetic acid cycle.

ate, which is produced from 4-chlorobenzoate, as described above.

## ACKNOWLEDGMENTS

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