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Enzymatic Formation, Stability, and Spontaneous Reactions of 4-Fluoromuconolactone, a Metabolite of the Bacterial Degradation of 4-Fluorobenzoate

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Enzymatic conversion of 4-fluorocatechol in the simultaneous presence of partially purified preparations of catechol 1,2-dioxygenase from *Pseudomonas cepacia* and muconate cycloisomerase from *Alcaligenes eutrophus* 335 yielded a product that was unambiguously identified as (+)-4-fluoromuconolactone [(+)-4-carboxymethyl-4-fluoro-but-2-en-4-olide]. This compound was shown to be the only major product formed from 3-fluoro-*cis*,*cis*-muconate by the action of muconate cycloisomerases from *A. eutrophus* 335, *A. eutrophus* JMP134, and *P. cepacia* as well as by the action of dichloromuconate cycloisomerase from *A. eutrophus* JMP134. This finding implies that dichloromuconate cycloisomerase, like the muconate cycloisomerases, catalyzes primarily a cycloisomerization reaction, which only in the case of chloro- and bromo-substituted substrates is connected to a dehalogenation. 4-Fluoromuconolactone at pH 7 decomposes by spontaneous reactions mainly to maleylacetate, which then decarboxylates to give *cis*-acetylacrylate. Although significant amounts of an unidentified compound are also formed from the fluorolactone, HF elimination to the two isomeric dienelactones (4-carboxymethylenebut-2-en-4-olides) is negligible. However, all spontaneous reactions proceed so slowly that an enzymatic conversion of 4-fluoromuconolactone must be assumed. Participation of dienelactone hydrolases in this reaction is indicated by their induction during growth of various strains with 4-fluorobenzoate. However, experiments with cell extracts of *P. putida* A3.12 suggest that at least one other hydrolytic enzyme is able to contribute to 4-fluoromuconolactone conversion. In light of these observations, earlier proposals for a 4-fluorobenzoate degradative pathway are discussed.

Many chloroaromatics have been shown to be degraded by bacteria via chlorosubstituted catechols (for recent reviews, see references 27, 33, 35, and 36). The catechols are cleaved in a 1,2-dioxygenation reaction, yielding the corresponding muconates (Fig. 1). Dechlorination occurs during or directly after cycloisomerization of the chloro-*cis*,*cis*-muconates (15, 16, 18, 38, 46). Although several studies have been performed on the mechanism of cycloisomerization of unsubstituted *cis*,*cis*-muconate (25, 28, 29; G. Avigad and S. Englard, Fed. Proc. **28**:345, 1969), until now relatively little was known about the dechlorination reaction.

The knowledge about the corresponding reaction sequence in fluoroaromatic metabolism is not only sparse but also contradictory. It resulted mainly from the investigation of the bacterial degradation of 4-fluorobenzoate (4FB). After earlier studies on 4FB-cooxidizing strains had shown that the initial conversion of 4FB is carried out by enzymes of the benzoate catabolism via the 3-oxodipate pathway (2, 6, 10, 43), Harper and Blakley (20) isolated a *Pseudomonas* sp. that was able to utilize 4FB as the sole source of carbon and energy. From conversion experiments with resting cells (no nitrogen source), they deduced a pathway (Fig. 2A) according to which 3-fluoro-*cis*,*cis*-muconate is further metabolized via two parellel branches, one being initiated by a cycloisomerization to 4-fluoromuconolactone (4-carboxymethyl-4-fluorobut-2-en-4-olide) and the other resulting from cycloisomerization and defluorination to a dienelactone (4-carboxymethylenebut-2-en-4-olide), which was supposed to be reduced to (−)-muconolactone. In contrast to Harper and Blakley, Schreiber et al. (40) assumed that the 4-fluoromuconolactone would be unstable, yielding the *cis*-dienelactone in a spontaneous α,β-elimination reaction (Fig. 2B). This reaction would be analogous to the corresponding one in chlorocatechol catabolism (38). The pathway proposed by Schreiber et al. (40) was later used by other authors to interpret results obtained with different experimental systems (7, 22).

Enzymatic studies with several 4FB-utilizing *Alcaligenes* strains and *Pseudomonas cepacia* were consistent with the catabolic route proposed by Schreiber et al. (40) insofar as all strains examined contained catechol 1,2-dioxygenase, muconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase activities after growth with 4FB (37). However, the majority of 4FB-assimilating strains had a type I dienelactone hydrolase, exhibiting only extremely low activity toward the *cis*-dienelactone, which was suggested by Schreiber et al. (40) to be the isomer produced during spontaneous elimination of HF from 4-fluoromuconolactone.

In this report we show that normal muconate cycloisomerases from several strains as well as the dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134 convert 3-fluoro-*cis*,*cis*-muconate to 4-fluoromuconolactone as the only main product. The investigation of its stability and spontaneous reactions proves that nonenzymatic α,β-elimination of HF does not play a significant role in the further metabolism of 4-fluoromuconolactone. Preliminary evidence suggests that it is converted to maleylacetate by

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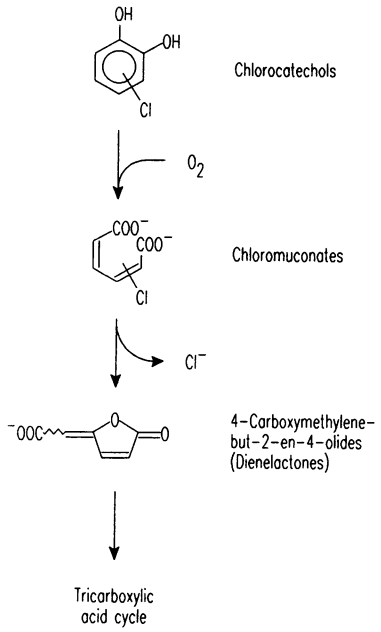


FIG. 1. Initial reactions in the bacterial degradation of chlorocatechols.

dienelactone hydrolases and/or 3-oxoadipate enol-lactone hydrolases.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this investigation are specified in the accompanying paper (37). To obtain enough cells for the enrichment of enzymes from *A. eutrophus* JMP134, we grew this strain aerobically in a 10-liter fermentor at 30°C with the mineral medium described in the accompanying paper (37), supplemented with repeated additions of 5 mM 4FB. Large-scale growth of *A. eutrophus* 335 and *P. cepacia* took place at 30°C in a 250-liter fermentor (no. IF-250; New Brunswick Scientific Co., Inc., Edison, N.J.) with a mineral medium of the following composition: 680 g of KH_2PO_4, 710 g of Na_2HPO_4, 200 g of $(NH_4)_2SO_4$, 60 g of $MgSO_4$, 13.2 g of $CaCl_2 \cdot 2H_2O$, 1.6 g of $FeSO_4 \cdot 7\ H_2O$, 0.34 g of $MnSO_4 \cdot H_2O$, 40 g of NaOH, and 200 liters of tap water. The cultures were stirred at 100 rpm, while aeration was gradually increased from 100 to 250 liters/min. Antifoam A concentrate (Sigma Chemical Co., St. Louis, Mo.) was added when necessary. For *A. eutrophus* 335 a mixture of 4 mM benzoate plus 1 mM 4FB, added four times, was used as the carbon source. The wet weight of cells obtained was 530 g. For *P. cepacia* the carbon source was changed stepwise from 3 mM benzoate plus 1 mM 4FB via substrate ratios of 2 mM benzoate–2 mM 4FB and 1 mM benzoate–3 mM 4FB to 4 mM 4FB (added twice) as the only carbon source. To keep the pH near 7.0 5 mM, NaOH was added with the first portion of 4 mM 4FB. The wet weight of cells obtained was 640 g. The fermentors were inoculated with 20-liter precultures grown in a 28-liter fermentor (no. MF-128 S; New Brunswick) containing medium with the same concentration of salts and carbon sources as the main cultures at the beginning.

Cell harvest and preparation of cell extracts. Cells of *A. eutrophus* JMP134 were harvested and disrupted as described in the accompanying paper (37). The buffer (50 mM Tris hydrochloride [pH 7.0]) contained 0.1 mM dithiothreitol in addition to 4 mM $MnSO_4$. For *A. eutrophus* 335 and *P. cepacia* the procedure was modified as follows. Biomass obtained from 200-liter cultures was collected by using a continuous-flow centrifuge. The cell pastes of *A. eutrophus* 335 and *P. cepacia* were stored at −20°C for 6 months and for 2 years, respectively. Portions of the cells were suspended in 50 mM Tris hydrochloride (pH 7) with 4 mM $MnSO_4$ and 0.1 mM dithiothreitol (*A. eutrophus* 335) or in 20 mM histidine hydrochloride (pH 6) (*P. cepacia*). After disintegration of the cells the cell extracts were separated from whole cells and cell debris by centrifugation at 15,000 × *g* for 90 min (*A. eutrophus* 335) or at 100,000 × *g* for 30 min (*P. cepacia*).

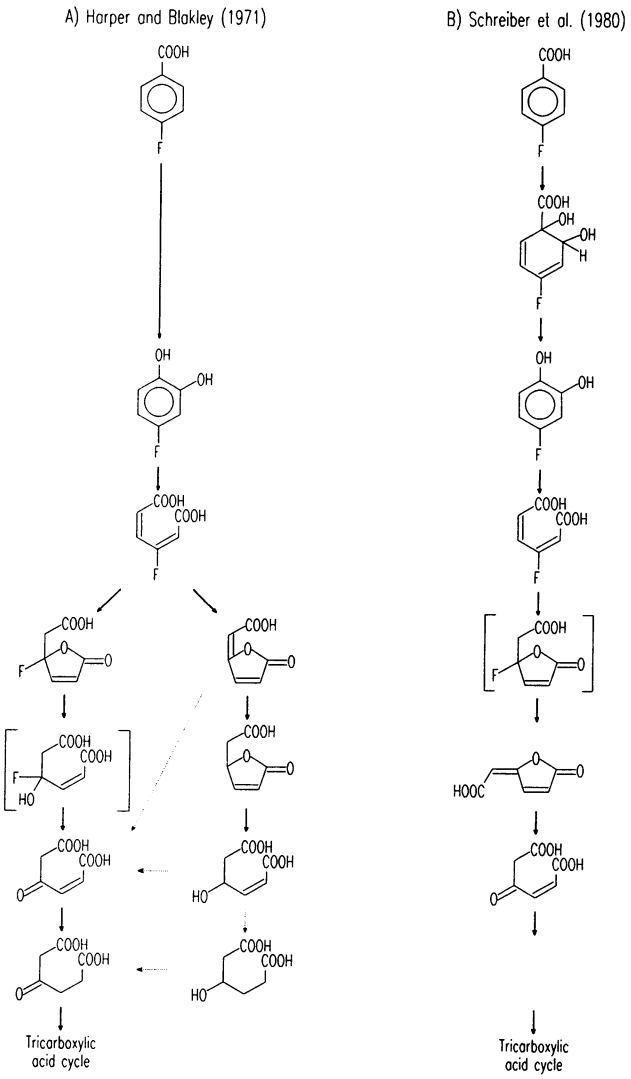


FIG. 2. Comparison of the pathways described for the bacterial degradation of 4FB by Harper and Blakley (20) (A) and by Schreiber et al. (40) (B).

Enzyme assays and estimation of protein concentration. The procedures for enzyme assays and for the estimation of protein concentrations are described in the accompanying paper (37).

Enzyme enrichments. A homogeneous preparation of dichloromuconate cycloisomerase from *A. eutrophus* JMP134 was available from a previous purification (26). All

other enzyme preparations used in this investigation were obtained as by-products during attempts to purify the respective dienelactone hydrolases (to be published separately). Therefore, the conditions given below were not necessarily optimal for the stability of the catechol 1,2-dioxygenase and the muconate cycloisomerases. Except for the purification of the enzymes from *P. cepacia*, which was carried out at room temperature, column chromatography and ultrafiltration were performed at 4°C in a cold room.

Partially purified muconate cycloisomerse from *A. eutrophus* JMP134 was obtained by gel filtration chromatography of a cell extract (22 ml; 1.37 g of protein) from 4FB-grown cells. The extract was applied to a K 50/60 column (Pharmacia, Uppsala, Sweden) filled to a bed length of 46 cm with Sephacryl S-200 Superfine (Pharmacia). Tris hydrochloride (50 mM; pH 7.0) with 4 mM $MnSO_4$ and 0.1 mM dithiothreitol was used to equilibrate the column and to elute the proteins (flow rate, 80 ml/h). This procedure resulted in a complete separation of muconate cycloisomerase and dienelactone hydrolase activities, whereas the activity peaks of muconate cycloisomerase and catechol 1,2-dioxygenase overlapped considerably. Enzyme assays with 2,4-dichloro-*cis*,*cis*-muconate proved the muconate cycloisomerase preparation so obtained to be free from any detectable dichloromuconate cycloisomerase activity. The enzyme was stored at −20°C.

Muconate cycloisomerase from *A. eutrophus* 335 was partially purified by the following steps. A cell extract (895 ml; 5.74 g of protein) from cells grown on a mixture of 4 mM benzoate and 1 mM 4FB was subjected to an ammonium sulfate fractionation. The fraction from 30 to 60% saturation was redissolved in 10 mM Tris hydrochloride (pH 7.5) with 4 mM $MnSO_4$ and dialyzed against two 10-liter batches of the same buffer (final volume, 154 ml; 3.14 g of protein). Further purification was achieved by DEAE-cellulose chromatography. A K 50/100 column (Pharmacia), filled to a bed length of 81 cm with DE 52 (Whatman, Inc., Clifton, N.J.), was equilibrated with 20 mM Tris hydrochloride (pH 7.5) with 4 mM $MnSO_4$. After the column had been washed with 1.2 liters of the equilibration buffer proteins were eluted by a linear gradient from 0 to 0.5 M NaCl in the same buffer (gradient volume, 3.2 liters; flow rate, 200 ml/h). The dienelactone hydrolase pool from the DEAE-cellulose column (363 ml; 670 mg of protein) still contained most of the muconate cycloisomerase activity. After concentration of this pool to 47 ml by ultrafiltration (Amicon PM10 membrane), a sufficient separation of both enzymes was accomplished by gel filtration chromatography. Tris hydrochloride (50 mM; pH 7.5) containing 4 mM $MnSO_4$ and 0.1 M NaCl was used at a flow rate of 123 ml/h in a K 50/60 column filled with Fractogel HW-55 (S) (bed length, 46 cm; E. Merck AG, Darmstadt, Federal Republic of Germany). By using the above procedure, muconate cycloisomerase was enriched 11-fold with a yield of 32%. The preparation (59 ml; 167 mg of protein) still contained 1570 U of activity (as measured with *cis*,*cis*-muconate at pH 8) and was stored at −20°C.

The catechol 1,2-dioxygenase and the muconate cycloisomerase from *P. cepacia* were partially purified from a cell extract of cells, which had been grown by changing the growth substrate from benzoate-4FB to 4FB. The extract (180 ml; 4.37 g of protein) was applied to a K 50/60 column filled with Q Sepharose Fast Flow (Pharmacia) to a bed length of 40 cm. Histidine hydrochloride (20 mM; pH 6) was used for the equilibration and for washing the column (with 11 liters of buffer) after sample application. The relevant enzymes were eluted with two NaCl gradients (0 to 0.3 M over 3.2 liters and 0.3 to 2.0 M over 0.2 liters) in the same buffer (flow rate, 300 ml/h). Both muconate cycloisomerase and catechol 1,2-dioxygenase were efficiently separated from dienelactone hydrolase and 3-oxoadipate enol-lactone hydrolase. The fractions (20 ml each) with catechol 1,2-dioxygenase and muconate cycloisomerase activity were collected in tubes that already contained 0.8 ml of 2 M Tris hydrochloride (pH 8) for the stabilization of the dioxygenase, which was shown to be considerably less stable at pH 6 than at pH 8. By using this chromatographic procedure, a catechol 1,2-dioxygenase preparation (101 ml; 385 mg of protein) was obtained which contained about 1,200 U of a 14-fold-purified enzyme (determined yield, 119%). The muconate cycloisomerase pool (224 ml; 100 mg) also contained about 1,200 U, which corresponded to a 58% yield (25-fold enrichment). However, upon storage at −20°C for 6.5 months most of the muconate cycloisomerase activity was lost (residual activity, 0.3%). Despite the relatively efficient separation mentioned above, the muconate cycloisomerase preparation after storage contained considerable amounts of 3-oxoadipate enol-lactone hydrolase activity. The stability of the latter enzyme under the storage conditions had increased its activity relative to the muconate cycloisomerase.

Preparative conversion of 4-fluorocatechol to 4-fluoromuconolactone. The reaction mixture initially contained the following components (total volume, 1.4 liters): 70 ml of catechol 1,2-dioxygenase preparation from *P. cepacia* (total activity of 168 U as measured with 0.3 mM 4-fluorocatechol at pH 6.5), 14 ml of muconate cycloisomerase preparation from *A. eutrophus* 335 (total activity of 2,040 U as measured with 0.1 mM 3-fluoro-*cis*,*cis*-muconate at pH 6.5), and 1.32 liters of 100 mM bis-Tris hydrochloride buffer (pH 6.5). After the reaction had been started by addition of 144 mg of 4-fluorocatechol, giving a concentration of 0.8 mM, the mixture was incubated at 30°C on a magnetic stirrer. The progress of the reaction was monitored by high-pressure liquid chromatography (HPLC) analyses. After 27 min the reaction was stopped by extraction of residual 4-fluorocatechol with 1.4 liters of diethyl ether. During this procedure, precipitated protein was removed by centrifugation for 10 min at 15,000 × *g* and 5°C. The aqueous phase was then acidified with HCl to pH 2.3 and extracted twice with 1.4 liters of ethyl acetate. The ethyl acetate phases were combined, dried over $MgSO_4$, and vaporized to dryness. HPLC analyses were used to make sure that the isolated product had the same chromatographic characteristics as the immediate product of enzymatic 4-fluorocatechol conversion.

HPLC. Quantitative analyses of low-molecular-weight compounds were performed by reversed-phase HPLC with Hyperchrome SC columns (125 by 4.6 mm) filled with 5-μm particles of LiChrospher 100 RP8 (Bischoff, Leonberg, Federal Republic of Germany). An aqueous solution of 10% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid was used as the mobile phase for most experiments (flow rate, 1 ml/min). Under these conditions typical retention volumes were as follows: maleylacetic acid, 2.6 ml; compound X, 3.0 ml; *cis*-acetylacrylic acid acylale [5-hydroxy-5-methyl-2(5*H*)-furanone], 3.2 ml; *trans*-dienelactone (*trans*-4-carboxymethylenebut-2-en-4-olide), 3.9 ml; *trans*-acetylacrylic acid; 4.4 ml; 3-fluoro-*cis*,*cis*-muconic acid, 5.3 ml; compound Y, 5.6 ml; 4-fluoromuconolactone (4-carboxymethyl-4-fluorobut-2-en-4-olide), 6.5 ml; *cis*-dienelactone (*cis*-4-carboxymethylenebut-2-en-4-olide), 7.5 ml; and 4-fluorocatechol, 10.1 ml (first salt signal, 1.7 ml). In experiments in which exact quantitation of maleylacetic acid could be compromised for the sake of speed, 16% instead of 10%

acetonitrile was used in the mobile phase. Detection was performed simultaneously at 205 nm and 280 nm. Samples from enzyme-catalyzed reactions were acidified and centrifuged before injection.

Spectroscopic methods and melting point determination. UV spectra were recorded either in a double-beam spectrophotometer or under stopped-flow conditions during HPLC. In the latter system wavelengths were changed stepwise (2 nm per step; 2 s per step) from 200 to 400 nm. Absorption of the mobile phase was subtracted from the spectrum initially obtained. Infrared spectra were obtained with a model PE 457 spechrometer (The Perkin-Elmer Corp., Norwalk, Conn.) from a sample embedded in KBr as well as from a sample dissolved in dichloromethane. ^{13}C and high-resolution ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in a Bruker CXP 300 (7.046 T) by using the pulsed Fourier transform technique. The nominal frequence was 75.47 MHz for ^{13}C NMR and 300.13 MHz for ^{1}H NMR spectra. Tetramethylsilane was used as internal standard. The mass spectrum was obtained with a mass spectrometer (MAT 711; Varian, Palo Alto, Calif.) at a vaporization temperature of 30°C with an electron beam of 70 eV for the ionization. Specific rotation was determined with a polarimeter (no. 241; Perkin-Elmer) at 20°C in methanol. Melting points were measured with a heatable microscope (Reichert Thermovar) after calibration with benzil (95°C) and acetanilide (114.5°C).

Reactions under UV irradiation. Aqueous solutions (2 ml) of the compounds to be irradiated were placed in 10-ml beakers under a MinUVis-lamp (type 131200; Desaga, Heidelberg, Federal Republic of Germany). Irradiation was performed from a distance of 6 cm with 254-nm UV light. A control solution of identical composition was incubated under the same conditions, except that it was covered. Reactions were monitored by HPLC analyses.

Chemicals. Sources and syntheses of most chemicals used in this investigation are described in the accompanying paper (37). *trans*-Acetylacrylic acid was obtained from Lancaster Synthesis. 4-Fluorocatechol was synthesized from 5-fluoro-2-hydroxyacetophenone (obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.) by Dakin oxidation as described by Corse and Ingraham (11). The product had the same melting point (not depressed by mixing both samples) and the same HPLC retention volume as a standard derived from 4-aminoveratrole (40). UV spectra of both compounds showed maxima at 279 to 280 nm. Solutions of 3-fluoro-*cis,cis*-muconate in 30 mM Tris hydrochloride (pH 8) were prepared by enzymatic 1,2-dioxygenation of 4-fluorocatechol with partially purified catechol 1,2-dioxygenase from *P. cepacia*. If necessary these solutions were diluted with other buffers to change pH values.

RESULTS

Preliminary experiments on the enzymatic conversion of 4-fluorocatechol and 3-fluoro-*cis,cis*-muconate. Because of its instability under acidic conditions, 3-fluoro-*cis,cis*-muconic acid was not readily available as a crystalline compound until recently (31, 39). Even in solution at pH 6.3, 3-fluoro-*cis,cis*-muconate is relatively unstable (16% conversion in 1 h) (39). To avoid the formation of artifacts during the enzymatic cycloisomerization of 3-fluoro-*cis,cis*-muconate, it was therefore decided to use 4-fluorocatechol as the initial substrate and to convert it in the simultaneous presence of catechol 1,2-dioxygenase and muconate cycloisomerase. A catechol 1,2-dioxygenase preparation from *P. cepacia* and a muconate cycloisomerase preparation from *A. eutrophus* 335 with sufficient purity and activity were available from the purification of the respective dienelactone hydrolases.

Possible lactonic cycloisomerization products were expected to be unstable under alkaline conditions (16, 20). On the other hand, catechol 1,2-dioxygenases had been shown to have optimal activity at relatively high pH values (12, 21). Therefore, the activity of the catechol 1,2-dioxygenase from *P. cepacia* was assayed in several buffers of various pH values. Activity was optimal between pH 7 and 8, decreasing faster toward lower than toward higher pH values. From this experiment, bis-Tris hydrochloride (pH 6.5) was judged to be a suitable buffer for the conversion of 4-fluorocatechol (80% of the activity at pH 8). A pH value of 6.5 apparently is also favorable for cycloisomerizing enzymes (26, 30).

The catechol 1,2-dioxygenase of *P. cepacia* showed a relative activity of 24% for 4-fluorocatechol as compared with catechol. If freshly prepared solutions of 4-fluorocatechol were used, concentrations higher than 2 mM resulted in inhibition of the enzyme. The muconate cycloisomerase of *A. eutrophus* 335 converted 3-fluoro-*cis,cis*-muconate almost twice as fast as *cis,cis*-muconate (relative activity, 191%). In bis-Tris hydrochloride buffer (pH 6.5) the enzyme was slightly more active than in Tris hydrochloride (pH 8) (109% compared with pH 8).

Isolation and identification of 4-fluoromuconolactone. Of 144 mg of 4-fluorocatechol initially added to the solution of catechol 1,2-dioxygenase and muconate cycloisomerase, 38 mg was recovered at the end of the experiment by extraction with diethyl ether at pH 6.5. Following acidification to pH 2.3 and extraction with ethyl acetate, 123 mg of slightly yellowish crystals was obtained. Dissolution of this raw product in chloroform and subsequent partial vaporization of the solvent yielded colorless crystals, with the yellow material left in the remaining solvent. After careful drying, the yield of the crystalline product was 82 mg. According to HPLC analysis it contained the *cis*- and the *trans*-dienelactone as contaminants in relative amounts of 0.7 and 0.4%, respectively.

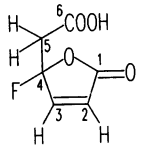
The crystals had a melting point of 104°C, which is exactly the same as that obtained by Harper and Blakley (20). Solutions of the compound in Tris hydrochloride (pH 7) showed no maximum of UV absorption between 200 and 350 nm. Mass-spectrometric analysis at 12,000-fold resolution yielded a mass of 160.0173 for the molecular ion. With a deviation of 0.0001 mass unit, this corresponds to the formula weight of $C_6H_5O_4F$. The fragmentation pattern showed all signals mentioned by Harper and Blakley (20), as well as some others of lower intensity (m/z = 140, 115, and 96, respectively), all in accordance with the proposed structure. In infrared absorption spectra characteristic bands were discernible for the carboxyl group (1,719 cm^{-1}), for the lactone carbonyl group (1,798 cm^{-1}), and for the olefinic double bond (1,609 cm^{-1}).

^{1}H NMR spectra were recorded in $CDCl_3$ and in CD_3COCD_3 (Table 1). They show two olefinic protons at 7.604/7.810 ppm and at 6.326/6.475 ppm, as well as the typical AB spectrum of two diastereotopic methylene protons (with additional coupling to the ^{19}F nucleus). Chemical shifts and ^{3}J coupling constants of the olefinic protons correspond to those published for 2,5-dihydrofuran-2-one (23), muconolactone (29), and 4-methylmuconolactone (24). The assignment, however, has to be reversed with respect to that made by Harper and Blakley (20). The pronounced low-field shift of one of the olefinic signals is due to the negative mesomeric effect of the carbonyl group and there-

TABLE 1. ^1H and ^{13}C NMR data for the cycloisomerization product[a]

Nucleus	Chemical shift (ppm)[b] in:		Coupling assignment	Coupling constant (Hz)[b] in:	
	$CDCl_3$	CD_3COCD_3		$CDCl_3$	CD_3COCD_3
2-H	6.326	6.475 (7.81)	^3J (2-H,3-H)	5.6	5.7 (6)
			^4J (2-H,4-F)	1.6	1.8 (−)
3-H	7.604	7.810 (6.46)	^3J (2-H,3-H)	5.6	5.5 (6)
			^3J (3-H,4-F)		(2)
5-H_A	3.374	3.357 (3.0–3.54)	^3J (5-H_A,4-F)	8.1	9.2
			^2J (5-H_A,5-H_B)	(−)16.7	(−)16.6
5-H_B	3.098	3.249 (3.0–3.54)	^3J (5-H_B,4-F)	23.1	20.9
			^2J (5-H_A,5-H_B)	(−)16.8	(−)16.6
6-OH		(8.75)			
C-1	170.73[c]				
C-2	124.76		^3J (C-2,4-F)	4.7	
C-3	149.80		^2J (C-3,4-F)	19.2	
C-4	113.24		^1J (C-4,4-F)	227.5	
C-5	40.05		^2J (C-5,4-F)	33.6	
C-6	167.56[c]				

[a] The product has the following structure:



[b] Values obtained by Harper and Blakley (20) are shown in parentheses.
[c] Assignments to C-1 and C-6 are tentative.

fore must be assigned to the proton in the β-position. Consequently the 1.6/1.8-Hz H,F coupling represents the allylic 2-H,4-F coupling, while a vicinal 3-H,4-F coupling is not detectable. This can be rationalized in terms of an essentially axial position of the fluorine substituent with respect to the lactone ring. The anisochrony of the diastereotopic methylene protons 5-H_A,H_B was found in a similar magnitude in muconolactone (29) and 3-methylmuconolactone (32). The large difference in coupling constants ^3J (H,F) between 5-H_A and 5-H_B must be due to H_B being in an antiperiplanar orientation and H_A in a synclinal orientation relative to the fluorine substituent.

In the ^{13}C NMR spectrum (Table 1), the C-3 signal occurs downfield of the C-2 signal because of the electron-withdrawing effect of the C=O group. This assignment is supported by the 19.2-Hz ^2J (C,F) coupling. C-4 is characterized by a ^1J (C,F) coupling of 227.5 Hz, whereas the signal at 40.05 ppm, because of the chemical shift and the pronounced ^2J (C,F) coupling, can be attributed to C-5.

Like muconolactone (42) and 2-methyl- and 4-methylmuconolactone (24), the product showed a rotation of polarized light to the right. Specific rotation, measured at a concentration of 127.5 mg/ml, was found to be +277.6° at 365 nm, +140.4° at 436 nm, +72.2° at 546 nm, +61.9° at 578 nm, and +58.8° at 589 nm. From the above evidence, the isolated compound is therefore assumed to be (+)-4-carboxymethyl-4-fluorobut-2-en-4-olide [(+)-4-fluoromuconolactone].

Cycloisomerization of 3-fluoro-*cis*,*cis*-muconate to 4-fluoromuconolactone by different cycloisomerases. For the preparation of 4-fluoromuconolactone from 4-fluorocatechol, the catechol 1,2-dioxygenase from *P. cepacia* and the muconate cycloisomerase from *A. eutrophus* 335 were used. The data summarized in Table 2 show that all investigated muconate cycloisomerases, from *A. eutrophus* 335 as well as from *A. eutrophus* JMP134 and *P. cepacia*, formed 4-fluoromuconolactone as the main cycloisomerization product of 3-fluoro-*cis*,*cis*-muconate. This was also true for the dichloromuconate cycloisomerase from *A. eutrophus* JMP134. The two dienelactones were detected in comparatively small amounts. The lack of a preference in the formation of the two isomers indicates that they resulted from spontaneous reactions of the substrate 3-fluoro-*cis*,*cis*-muconate (39). However, since 3-fluoro-*cis*,*cis*-muconate cannot be quantitated by reversed-phase HPLC because of its instability under acidic conditions, it was not possible to determine the rate of its nonenzymatic decomposition to the dienelactones under the given experimental conditions.

TABLE 2. Formation of 4-fluoromuconolactone by different cycloisomerases[a]

Strain	Enzyme[b]	Product concn (μM):			
		Maleylacetate	*trans*-Dienelactone	4-Fluoromuconolactone	*cis*-Dienelactone
A. eutrophus 335	MCI	3	4	88	5
A. eutrophus JMP134	MCI		2	89	5
	DMCI		4	86	7
P. cepacia	MCI	39[c]	1	52[c]	5

[a] To a 0.1 mM solution (1 ml) of 3-fluoro-*cis*,*cis*-muconate in 30 mM bis-Tris hydrochloride (pH 6.5) the cycloisomerase preparations were added to a final activity (as measured with 3-fluoro-*cis*,*cis*-muconate at pH 6.5) of 0.1 U/ml (0.02 U/ml for *P. cepacia*). The reaction was monitored at 260 nm in a spectrophotometer. Product concentrations were determined by HPLC.
[b] Abbreviations: MCI, muconate cycloisomerase; DMCI, dichloromuconate cycloisomerase.
[c] The muconate cycloisomerase preparation contained some 3-oxoadipate enol-lactone hydrolase activity, which presumably resulted in enzymatic conversion of 4-fluoromuconolactone to maleylacetate.

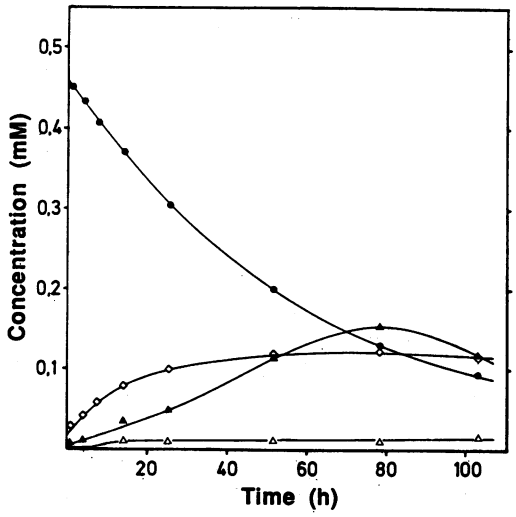


FIG. 3. Decomposition of 4-fluoromuconolactone by spontaneous reactions at pH 7.2. A 0.5 mM solution of 4-fluoromuconolactone (●) in 10 mM sodium phosphate (pH 7.2) was incubated at 23°C in the dark. The concentrations of maleylacetate (◇), *cis*-acetylacrylate, (▲) and *trans*-acetylacrylate (△) were determined by HPLC. The *trans*- and *cis*-dienelactones were formed in approximately the same amount (≤3 μM) (not shown).

Stability and spontaneous reactions of 4-fluoromuconolactone. The isolation of 4-fluoromuconolactone, described above, contradicted the assumption of Schreiber et al. (40) that this compound would be unstable at neutral pH, yielding the *cis*-dienelactone in a spontaneous reaction. Because of the relevance of this question for further metabolism, the stability and the spontaneous reactions of 4-fluoromuconolactone were investigated in some detail. During incubation at pH 7.2 the concentration of 4-fluoromuconolactone decreased to half its initial value in 43 h (Fig. 3; Table 3). According to HPLC analyses, seven different products were formed by spontaneous reactions. *trans*-Acetylacrylate and *cis*- and *trans*-dienelactone could easily be identified, since standard compounds were available commercially or from previous syntheses (34). Whereas the former product finally made up 3% of the initial-4-fluoromuconolactone concentration, the dienelactones did not exceed 1% each during the experiment. Since both dienelactones at pH 7.2 were more stable than 4-fluoromuconolactone (half-life of 93 h for the *cis*-isomer and 165 h for the *trans*-isomer versus 43 h for 4-fluoromuconolactone), spontaneous decomposition of the dienelactones cannot be responsible for the low concentrations observed. A fourth substance, maleylacetate, could be identified by using reference solutions, which were prepared from the *trans*-dienelactone by alkaline hydrolysis (16) or by enzymatic hydrolysis with partially purified dienelactone hydrolase (38). Maleylacetate initially was the main product, but the concentration ceased to increase after about 50 h (Fig. 3), indicating decomposition to a further product. As described below, this compound could be identified as being *cis*-acetylacrylate. Its concentration reached a maximum after about 80 h, when it was higher than that of maleylacetate. Two additional products, preliminarily named compound X and compound Y, had not yet been identified. Their UV spectra, measured under stopped-flow conditions during HPLC, showed absorption maxima at 228 and 241 nm, respectively.

TABLE 3. Stability of 4-fluoromuconolactone at different pH values[a]

Solution for incubation	pH	Half-life (h)[b]
10 mM sodium phosphate	11.0	0.05[c]
10 mM Tris hydrochloride	8.5	2.0
10 mM sodium phosphate	7.2	43
10 mM sodium citrate	5.3	52
10 mM sodium citrate	3.2	34
0.1 N HCl	1.1	32

[a] 4-Fluoromuconolactone at an initial concentration of 0.5 mM was incubated in the presence of different buffers or 0.1 N HCl at 23°C in the dark. Concentration changes were monitored by HPLC (compare Fig. 3).

[b] Logarithmic plots of the 4-fluoromuconolactone concentration against time gave a linear relationship. Half-lives were obtained from the slopes of these plots.

[c] In addition to HPLC, the time course was monitored by photometric methods.

Incubation of 4-fluoromuconolactone at different pH values revealed that it is comparatively stable under acidic conditions, whereas it is readily decomposed at high pH values (Table 3). Under the latter conditions maleylacetate and the two isomeric acetylacrylates were the main products. At low pH values compounds X and Y played a more important role than at pH 7.2, reaching their highest concentrations at pH 1.1 and pH 3.2, respectively. In all cases compound Y was formed at detectable levels considerably later than compound X was.

***cis*-Acetylacrylate as the product of spontaneous maleylacetate decarboxylation.** As mentioned above, the concentration of maleylacetate formed by spontaneous reactions of 4-fluoromuconolactone at pH 7.2 ceased to increase after about 50 h (Fig. 3). This suggested that further maleylacetate decomposition could have given rise to those products, which initially could not be identified. To test this hypothesis, maleylacetate was incubated in the same buffer solutions as 4-fluoromuconolactone (except for pH 11). The rate of maleylacetate decomposition was strongly dependent on the pH value (Fig. 4). The maleylacetate (or maleylacetic acid) concentration decreased to half the initial value in 27 h at pH 8.5, in 23 h at pH 7.2, in 0.85 h at pH 5.3, in 0.35 h at pH 3.2, and in 25 h at pH 1.1. Different ratios of two products were found by HPLC measurements. Although compounds X and Y could not be detected, *trans*-acetylacrylate (or *trans*-acetylacrylic acid) and the third originally unidentified product of 4-fluoromuconolactone decomposition were formed in considerable amounts. Although quantitation of the latter compound was not possible until it was identified, the HPLC signals suggested that its formation at all pH values preceded the formation of *trans*-acetylacrylate. This finding indicated that the unidentified compound could be an intermediate of the overall reaction from maleylacetate to *trans*-acetylacrylate. Depending on whether *cis*,*trans*-isomerization or decarboxylation was the first reaction of maleylacetate decomposition, fumarylacetate or *cis*-acetylacrylate would be the intermediate.

Sugiyama et al. (44, 45) had shown that *trans*-acetylacrylic acid can be isomerized to the *cis*-isomer on exposure to UV light. We therefore examined whether UV light would convert *trans*-acetylacrylic acid to the product, which was to be identified. HPLC measurements showed that the compound arising from decomposition of 4-fluoromuconolactone and maleylacetate indeed had the same retention volume as the product which was obtained by UV irradiation of 2 mM *trans*-acetylacrylic acid for 4 h. In addition, UV spectra of both products, measured under stopped-flow conditions during HPLC, were identical in showing no maximum be-

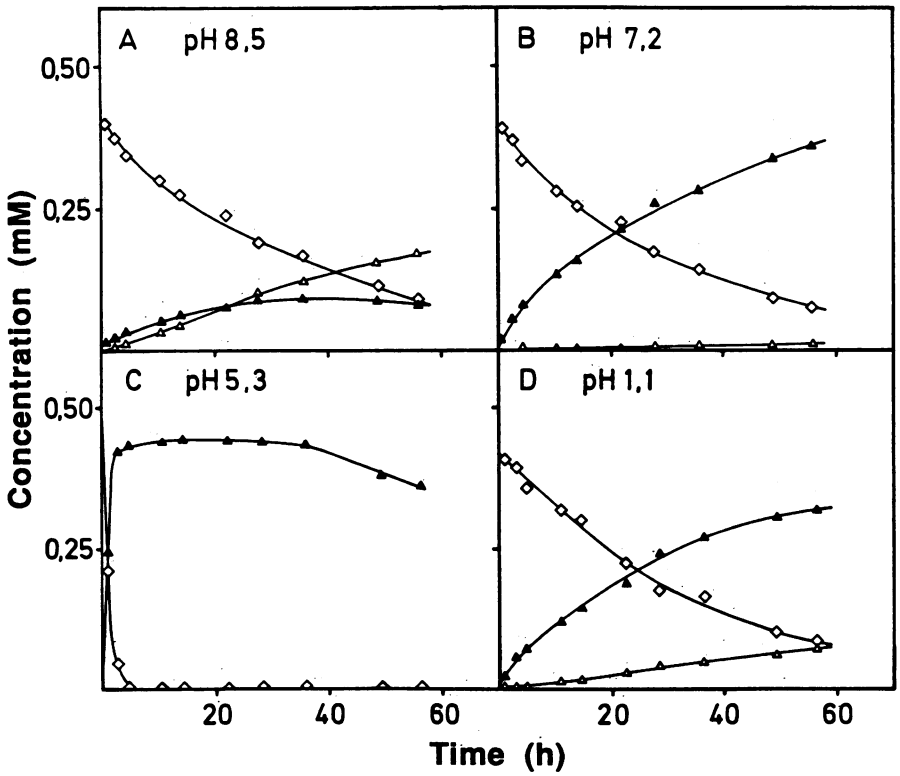


FIG. 4. Decomposition of maleylacetate by spontaneous reactions at various pH values. Maleylacetate was incubated in (A) 10 mM Tris hydrochloride (pH 8.5), (B) 10 mM sodium phosphate (pH 7.2), (C) 10 mM sodium citrate (pH 5.3), (D) 0.1 N HCl (pH 1.1), and 10 mM sodium citrate (pH 3.2) (not shown) at 23°C in the dark. The concentrations of maleylacetate (◇), *cis*-acetylacrylate (▲), and *trans*-acetylacrylate (△) were determined by HPLC analysis.

tween 200 and 400 nm. This absorption spectrum occurs because on acidification, as during HPLC, the *cis*-acetylacrylate anion (λ_{max}, 198 and 240 nm) tautomerizes to give the cyclic acylal, 5-hydroxy-5-methyl-2(5*H*)-furanone, which was reported to have a λ_{max} of 195 nm (41). From extinction coefficients obtained from Seltzer and Stevens (41) for *trans*-acetylacrylic acid (ε_{205}, 6,920 M^{-1} cm^{-1}) and for cyclic *cis*-acetylacrylic acid acylale (ε_{205}, 6,600 M^{-1} cm^{-1}), the latter compound was quantitated in HPLC chromatograms by comparison of the signals with those of *trans*-acetylacrylic acid.

Preliminary evidence for the nature of compound X. To examine the feasibility of a preparative isomerization of the *trans*-dienelactone to the *cis*-isomer (for analogous experiments, see reference 13), we exposed an aqueous solution of the former to UV irradiation (Fig. 5). In addition to the expected product (*cis*-dienelactone), low concentrations of maleylacetic acid, considerable amounts of *cis*-acetylacrylic acid acylale, and a fourth product were detected. The fourth product had the same HPLC retention volume and the same UV absorption characteristics under stopped-flow conditions as the above-mentioned compound X. This indicated that they might be identical. In a control, which was incubated in the dark, only *cis*-acetylacrylic acid acylale was detected as a product. Incubation of an aqueous solution of 0.1 mM maleylacetic acid (pH 3.1) for 1 h under UV irradiation yielded only the decarboxylation product *cis*-acetylacrylic acid acylale. This proves that in the preceding experiment, compound X was not formed from either of these products, but directly from the *cis*- or the *trans*-dienelactone.

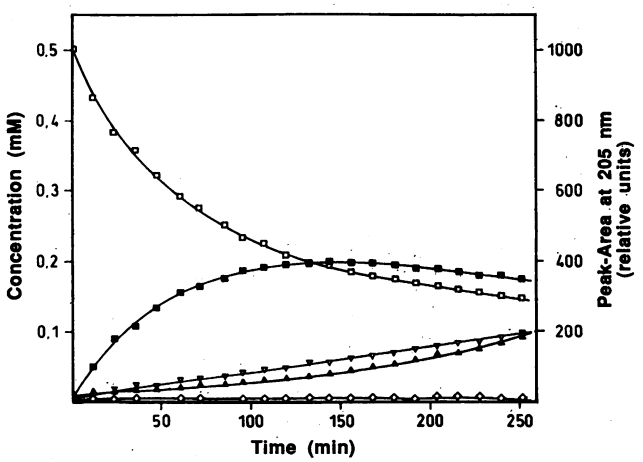


FIG 5. Products formed from *trans*-dienelactone under UV irradiation. An aqueous solution of 0.5 mM *trans*-dienelactone (pH 3.6) (□) was incubated under a UV lamp. *cis*-Dienelactone (■), maleylacetic acid (◇), and *cis*-acetylacrylic acid acylale (▲) were identified and quantitated by HPLC. For the fourth product, compound X (▽), peak areas in relative units are given. A peak area of 200 relative units was assumed to be equivalent to 0.1 mM, since for each time point this would result in an overall concentration of all compounds detected of approximately 0.5 mM. In a control solution, which was incubated in the dark, the concentration of the *trans*-dienelactone decreased from 0.50 to 0.48 mM. *cis*-Acetylacrylic acid acylale was the only detectable product.

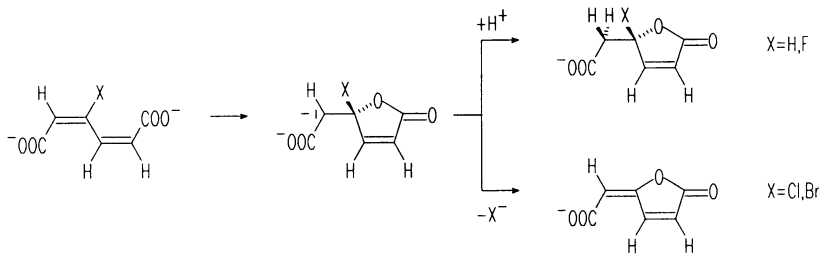


FIG. 6. Model for the cycloisomerization of *cis,cis*-muconate and 3-halo-*cis,cis*-muconate by various bacterial cycloisomerizing enzymes based on published results (25, 28, 29, 38; Avigad and Englard, Fed. Proc., 1969) and on findings of the present investigation.

Conversion of 4-fluorocatechol by crude cell extracts of *P. putida* A3.12. Since the preparation of 4-fluoromuconolactone by the use of two partially purified enzymes was very laborious, an easier method of enzymatic synthesis of this compound was highly desirable. Therefore, the conversion of 4-fluorocatechol by a crude cell extract of benzoate-grown cells of *P. putida* A3.12 was investigated by enzyme assays and HPLC analyses. The extract did not have any dienelactone hydrolase activity (with the *cis*- or the *trans*-dienelactone), whereas catechol 1,2-dioxygenase (2.1 U/mg of protein) and muconate cycloisomerase (2.4 U/mg of protein) were present at considerable levels. Conversion of 4-fluorocatechol by this extract (pH 7; 3.5 mM in 2 h) gave rise to maleylacetate, *cis*-acetylacrylate, and two unidentified products, while 4-fluoromuconolactone could not be detected.

DISCUSSION

Many mono- and dichlorinated aromatic compounds are degraded by bacteria via chlorosubstituted catechols and muconates as intermediates (Fig. 1). The (first) dehalogenation in this pathway occurs during or directly after cycloisomerization of the chloromuconate (15, 16, 18, 38, 46). Schmidt and Knackmuss (38) observed that the muconate cycloisomerase and the chloromuconate cycloisomerase of *Pseudomonas* strain B13 both convert 2- or 3-chloro-*cis,cis*-muconate to the corresponding dienelactone and 2- or 3-methylmuconate to the corresponding methylmuconolactone. They concluded that both enzymes catalyze essentially a cycloisomerization reaction and that 5- and 4-chloromuconolactone, respectively, should be formed from the 2- and 3-chloromuconates as intermediates. Dehalogenation was postulated to be a spontaneous secondary reaction yielding the *trans*- or the *cis*-dienelactone by α,β-elimination of HCl.

4-Fluoromuconolactone is the fluorosubstituted analog of the proposed reaction intermediate 4-chloromuconolactone. It was first isolated from the culture fluid of 4FB converting resting cells (no nitrogen source) of a *Pseudomonas* sp. (20). Doubts on whether 4-fluoromuconolactone was a major product of 3-fluoro-*cis,cis*-muconate cycloisomerization were raised by the isolation of (−)-muconolactone, which led Harper and Blakley (20) to postulate a second branch of 4FB degradation, in which cycloisomerization of 3-fluoro-*cis,cis*-muconate resulted in the formation of a dienelactone (Fig. 2A). In addition, Schmidt et al. (39) observed a nonenzymatic conversion of 3-fluoro-*cis,cis*-muconic acid to a mixture of the *cis*- and the *trans*-dienelactone at pH 6.3 and at pH 4. It was concluded (38) that the 4-fluoromuconolactone, which was assumed to be an intermediate of this reaction, could not be stable at least under acidic conditions.

The present investigation shows that the muconate cycloisomerases of *A. eutrophus* 335, *A. eutrophus* JMP134, and *P. cepacia*, as well as the dichloromuconate cycloisomerase of *A. eutrophus* JMP134, cycloisomerize 3-fluoro-*cis,cis*-muconate to 4-fluoromuconolactone as the only major product. The compound isolated after a preparative conversion of 3-fluoro-*cis,cis*-muconate by *A. eutrophus* 335 muconate cycloisomerase was characterized by UV, infrared, ^{1}H NMR, ^{13}C NMR, and mass-spectroscopic methods. All data obtained are consistent with the proposed structure; an identification of the isolated product as 3-fluoromuconolactone or as one of the isomeric 3-fluoromuconates can be excluded. The results of Harper and Blakley (20) were similar or even identical to ours, apart from a different interpretation of the ^{1}H NMR spectrum (Table 1). It is of special interest that not only the muconate cycloisomerase of different strains, but also the dichloromuconate cycloisomerase of *A. eutrophus* JMP134, converts to 3-fluoro-*cis,cis*-muconate to 4-fluoromuconolactone, showing that the latter enzyme does not necessarily catalyze a dehalogenation during the reaction with a halo-substituted substrate. This supports the proposal of Schmidt and Knackmuss (38) that the cycloisomerases for chlorosubstituted substrates, as the normal muconate cycloisomerases, catalyze primarily a cycloisomerization reaction, while dehalogenation must be regarded as a gratuitous secondary reaction.

Since several studies have shown that the muconate cycloisomerases of different bacterial sources, the chloromuconate cycloisomerase of *P. putida* (pAC27), and the dichloromuconate cycloisomerase of *A. eutrophus* JMP134 are evolutionary closely related (1, 19, 47), it can be assumed that they use the same mechanism for the conversion of their respective substrates. The muconate cycloisomerase of *P. putida* has been shown to catalyze a *syn* addition to a double bond via a carbanionic intermediate (25, 28; Avigad and Englard, Fed. Proc., 1969). The manganese ion present in each subunit seems to be located close to the C-6 carboxylate group of the substrate; however, a direct coordination of the metal ion to the carboxylate group could not be shown (29). Application of these findings to the cycloisomerization of 3-halo-*cis,cis*-muconates suggests a model as depicted in Fig. 6. Chloride or bromide should be eliminated at the stage of the carbanionic intermediate to give the *cis*-dienelactone. In the case of the unsubstituted and the fluorosubstituted substrate, the reaction is completed by addition of a proton to give muconolactone and 4-fluoromuconolactone, respectively. It is remarkable that fluoride is not eliminated, as are chloride and bromide.

Although Schreiber et al. (40) agreed with Harper and Blakley (20) that 4-fluoromuconolactone could be the initial product of 3-fluoromuconate cycloisomerization, they assumed this compound to be unstable under neutral conditions, giving rise to the *cis*-dienelactone in a spontaneous

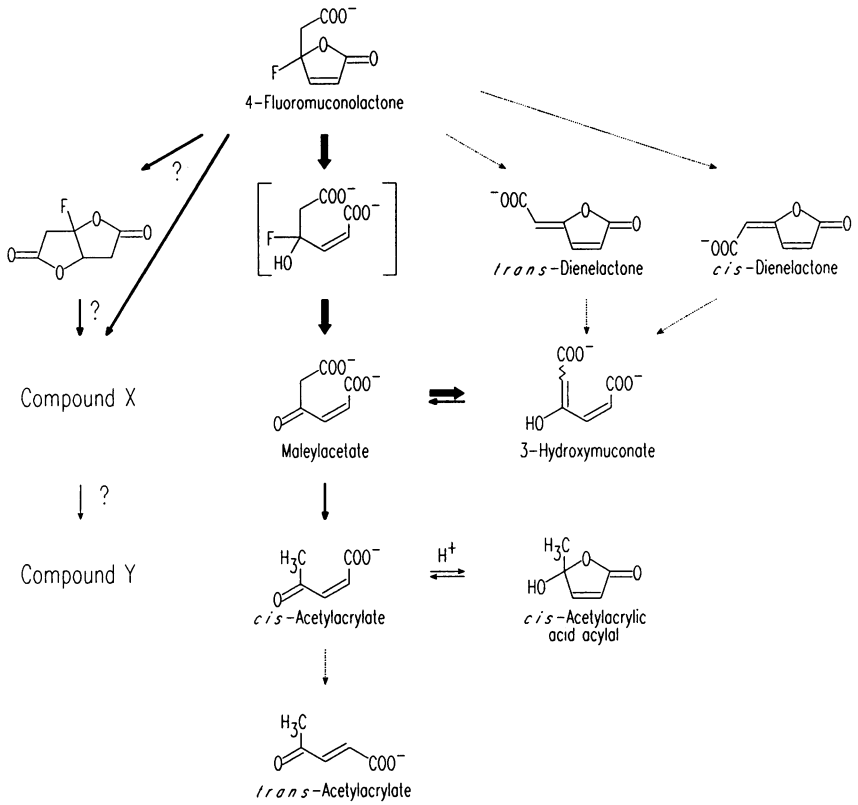


FIG. 7. Products formed from 4-fluoromuconolactone during decomposition by spontaneous reactions at pH 7.2. The thickness of the arrows illustrates the contribution of the reaction to overall product formation. Further explanations are given in the text.

elimination reaction (Fig. 2B). However, the results of the present investigation clearly show that 4-fluoromuconolactone is fairly stable under neutral and acidic conditions, although it is easily hydrolyzed at high pH values. At pH 7.2 the *cis*- and *trans*-dienelactones were formed in only insignificant amounts, whereas initially maleylacetate and then its decarboxylation product *cis*-acetylacrylate were the predominant products (Fig. 3 and 7). Maleylacetate due to keto-enol tautomerization is in equilibrium with 3-hydroxymuconate, the latter being favored at neutral pH (16). Despite the importance of maleylacetate and 3-hydroxymuconate as an intermediate in the microbial degradation of aromatic and haloaromatic compounds, respectively (for examples, see references 3 and 33), the significance of *cis*-acetylacrylate for the quantitation of maleylacetate has generally not been recognized, except perhaps by Bollag et al. (4), who described the acylale of chloro-*cis*-acetylacrylic acid. Several groups found *trans*-acetylacrylic acid during attempts to isolate maleylacetic acid under acidic conditions (5, 9, 17, 38). However, depending on the pH value, the *trans*-isomer might represent only a very small portion of the acetylacrylates present (Fig. 3 and 4). There was no indication that fumarylacetate, which was mentioned by Tiedje et al. (46) as being formed from maleylacetate, would be an intermediate of *trans*-acetylacrylate formation from maleylacetate.

In addition to the above-mentioned products, the unidentified compounds X and Y were formed by spontaneous reactions of 4-fluoromuconolactone at pH 7.2 and even more so at lower pH values. None of these substances were detected when maleylacetate was indubated at various pH values. Since compound X was also a product of UV irradiation of the dienelactones, but not of UV irradiation of maleylacetic acid, it is concluded that in the formation of compound X from 4-fluoromuconolactone the fluorine substituent is lost, while the lactonic structure is at least initially retained. The preferred appearance at low pH values and analogy to reactions of 4-methylmuconolactone (8) suggest that formation of a dilactonic structure might be a first step in 4-fluoromuconolactone decomposition to compound X (Fig. 7). Compound Y was detected only when compound X was also present, and always appeared after a considerable delay, suggesting that it might be formed from compound X by further reactions.

The relatively high stability of 4-fluoromuconolactone at neutral pH implies that its further metabolism should be enzyme catalyzed. Almost all 4FB-utilizing strains investigated induced dienelactone hydrolase activity during growth with the fluorosubstituted substrate, and even in the only exception the enzyme was present at low uninduced levels (37). This observation suggests that dienelactone hydrolases might be responsible for the conversion of 4-fluoromuconolactone. However, incubation of 4-fluorocatechol in the presence of crude cell extract from benzoate-grown cells of *P. putida* A3.12 did not allow accumulation of the fluorosubstituted lactone. Maleylacetate and *cis*-acetylacrylate were formed despite the total absence of dienelactone hydrolase activity from the extract. This finding indicated that at least one other hydrolytic enzyme, for example the 3-oxoadipate enol-lactone hydrolase, is also able to catalyze the conversion of 4-fluoromuconolactone to maleylacetate. The contribution of the dienelactone hydrolases and the 3-oxoadipate enol-lactone hydrolases to 4-fluoromuconolactone hydrolysis could be established with some certainty

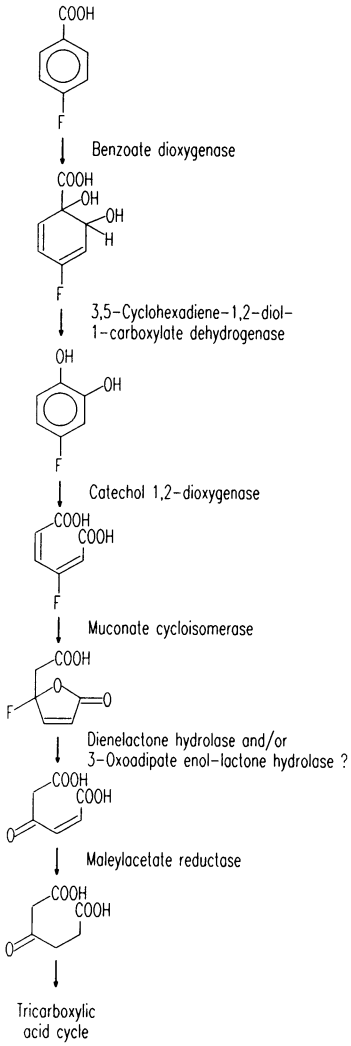


FIG. 8. Suggested pathway for 4FB degradation in *A. eutrophus* 335, *A. eutrophus* JMP134, and *P. cepacia*. The proposed catabolic route is based on published observations with other bacterial strains (2, 6, 10, 20, 22, 40, 43), on enzyme assays (37), and on the identification of 4-fluoromuconolactone as a cycloisomerization product (see the text).

only after at least a partial purification of these enzymes. The results of these investigations show considerable differences among the strains and will be published separately.

From evidence presented here and from the pattern of enzyme induction described in the accompanying paper (37), it can be concluded that 4FB is degraded by *A. eutrophus* 335, *A. eutrophus* JMP134, and *P. cepacia* (Fig. 8). As one branch of the catabolic route described by Harper and Blakley (20) (Fig. 2A), this pathway assumes dehalogenation to be a reaction connected to lactone hydrolysis rather than to lactone formation, which is the dehalogenating step in the degradation of many chloroaromatic compounds (Fig. 1). There was no indication that the second branch of 4FB conversion, which was postulated by Harper and Blakley (20), would be present in the bacteria investigated here. Since not only 4FB but also 3-fluorobenzoate (14), 2,5-difluorobenzoate (7), and 4-fluoroaniline (48) are catabolized via 4-fluorocatechol, the pathway proposed above might also be relevant for the degradation of these fluoroaromatic compounds.

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