Chemical structure and biodegradability of halogenated aromatic compounds

Halogenated muconic acids as intermediates

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Substituted muconic acids were prepared from the corresponding catechols by pyrocatechase II from *Pseudomonas* sp. B13. The stabilities of substituted muconic acids were compared under different pH conditions. 3-Substituted *cis,cis*-muconic acids cycloisomerized readily in slightly acidic solutions, whereas 2-chloro- and 2-fluoro-*cis,cis*-muconic acids were stable under these conditions and could be isolated as crystalline compounds. They were isomerized to the *cis,trans*-form in highly acidic solution (pH 1), particularly when heated to 80°C. Cycloisomerization of 2-chloro-*cis,cis*-muconic acid in 75% (v/v) H₂SO₄ yields 4-carboxymethyl-2-chloro-but-2-en-4-olide (4-chloro-2,5-dihydro-5-oxo-³H-furan-2-ylacetic acid). The *cis,cis*-configuration of 2-chloromuconic acid was certified by ¹H n.m.r. spectroscopy and by enzymic cycloisomerization. Although the *cis,cis*-configuration of 2-fluoromuconic acid was confirmed by corresponding spectroscopic data, it was not cycloisomerized by crude extracts or cycloisomerase II preparations from *Pseudomonas* sp. B13.

Few bacteria are described that can utilize halosubstituted aromatic compounds as sole sources of carbon and energy. Most of these eliminate halide after the aromatic ring has been opened by an *ortho*-cleavage enzyme (Janke & Fritsche, 1978). Halomuconic acids have been incriminated as ring-fission products of halocatechols during catabolism of 2,4-dichlorophenoxyacetate (Fernley & Evans, 1959; Bollag *et al.*, 1968; Tiedje *et al.*, 1969; Evans *et al.*, 1971b), of 4-chlorophenoxyacetate (Evans & Moss, 1957; Evans *et al.*, 1971a), of 4-fluorobenzoate (Harper & Blakley, 1971), of 2-fluorobenzoate (Goldman *et al.*, 1967), of 3-chlorobenzoate (Dorn & Knackmuss, 1978), of 3-chloro-, 4-chloro- and 3,5-dichloro-benzoate (Hartmann *et al.*, 1979) and of 4-chlorophenol (Knackmuss & Hellwig, 1978). The mechanism of halide elimination is not completely understood, partly because *cis,cis*-halomuconic acids were not readily available for enzymic studies. Nevertheless a causal relation appears to exist between cycloisomerization of halomuconic acids and liberation of

halide ions (Knackmuss *et al.*, 1976; Hartmann *et al.*, 1979). The present paper describes the preparation and properties of authentic halomuconic acids and the following paper (Schmidt & Knackmuss, 1980) their cycloisomerization with simultaneous elimination of halide.

Experimental

Chemicals

Methylcatechols were purchased from EGA Chemie, Steinheim, Germany. 3-Chlorocatechol and the monofluorocatechols were prepared as described by Schreiber *et al.* (1980). 4-Chlorocatechol was synthesized by the method of Willstätter & Müller (1911). The catechols were purified by sublimation before use.

cis,cis-Muconic acid was prepared by the method of Wacek & Fiedler (1949). *cis,trans*-Muconic acid was obtained by isomerization of the *cis,cis* isomer as described by Elvidge *et al.* (1950b). *trans,trans*-Muconic acid was bought from Fluka, Buchs, Switzerland.

For high-pressure liquid chromatography, 4-carboxymethyl-2-methyl- and 4-carboxymethyl-4-

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methyl-but-2-en-4-olide (2,5-dihydro-4-methyl- and 2,5-dihydro-2-methyl-5-oxo-3*H*-furan-2-ylacetic acid; Knackmuss *et al.*, 1976) and the *cis*- and the *trans*-isomer of 4-carboxymethylenebut-2-en-4-olide (5-oxo-3*H*-furan-2-ylideneacetic acid; Schreiber *et al.*, 1980) were used as reference substances.

All other chemicals for buffer solutions and solvents were of analytical-grade quality and were purchased from E. Merck, Darmstadt, Germany.

Preparative silica-gel layer chromatography

Methyl ester derivatives, which had been prepared with a slight excess of diazomethane in diethyl ether (De Boer & Backer, 1954), were separated on preparative silica-gel layers (0.2 cm, silica gel 60 PF 254; Merck). Hexane/dichloromethane (1:1, v/v) was used as solvent system. Bands were located under u.v. light and eluted by di-isopropyl ether.

Analytical methods

Substances were characterized spectrometrically by use of a model DMR 10 u.v.-visible recording spectrophotometer from Zeiss, Oberkochen, Germany, a model MAT 731 mass spectrometer (low and high resolution) from Varian, a model SP. 1000 i.r. spectrometer from Pye-Unicam and a model HA-100 n.m.r. spectrometer from Varian. For ¹H-n.m.r. spectra [²H]acetone was used as solvent. *trans,trans*-Muconic acid, which was insoluble in acetone, was measured in [²H]dimethyl sulphoxide.

Concentrations of muconic acids and their reaction products were determined by reverse-phase high-pressure liquid chromatography by using a Hibar-Pre-Packed column (Merck). Vacuum-degassed 10mm-phosphoric acid, containing 0.5% (v/v) propanol and 15% (v/v) methanol, was used as eluent. Peaks were identified with reference substances by retention time and scanning *in situ* of the u.v. spectra with a variable-wavelength model 635 spectrometric detector (Varian-Techtron, Melbourne, Victoria, Australia). Under the described conditions *trans,trans*-muconic acid, which was a commercial compound, exhibited a retention time of 8.7 min.

Enzyme assay

Cycloisomerization of substituted muconic acids was assayed by the method of Ornston (1966) with muconate cycloisomerases I and II, which are described in the following paper (Schmidt & Knackmuss, 1980).

Results

Formation of substituted muconic acids from catechols

3-Chloro-, 4-chloro-, 3-fluoro-, 4-fluoro and 3- and 4-methyl-catechol were ring-cleaved to the corresponding muconic acid by pyrocatechase II from

Pseudomonas sp. B13 (Dorn & Knackmuss, 1978). Substituted catechols were added in portions to a stirred and aerated solution (500 ml) of pyrocatechase II (enough enzyme was added to convert 50 µg of catechol per min) in 50 mM-Tris/HCl buffer, pH 8, supplemented with 2-mercaptoethanol (final concentration 20 mM). Turnover of catechols was monitored by high-pressure liquid chromatography. When 5 mmol of catechol/litre was oxidized the catechol-free solution was re-adjusted to pH 8 by addition of NaOH and stored at -18°C for isolation and isomerization studies.

Stability of substituted muconic acids in aqueous solution

For enzymic isomerization experiments (Schmidt & Knackmuss, 1980) and for the isolation of pure compounds from acidified solutions the stabilities of substituted muconic acids were studied under different pH conditions. The velocities of isomerization and product formation were measured by high-pressure liquid chromatography. With the exception of 3-methylmuconic acid all substituted muconic acids studied were stable at pH 7 for several hours. The stabilities of muconic acids and their isomerization products produced at lower pH values are given in Table 1. It is noteworthy that muconic acids with substituents in the 3-position were already cycloisomerized in slightly acidic solution. 3-Methylmuconic acid was the most unstable compound and cycloisomerized almost completely at pH 6.5 within 40 min. Muconic acids substituted in the 2-position were considerably more stable against cycloisomerization. At pH values greater than 6 no transformation products could be detected chromatographically within 2 h. At pH 1 2-chloro-*cis,cis*-muconic acid isomerized completely to 2-chloro-*cis,trans*-muconic acid (see below) within 20 h. 2-Fluoro-*cis,cis*-muconic acid was even more stable and isomerized at pH 1 with considerable velocity only when heated to 80°C. Under these conditions isomerization was complete after 5 h.

As has been described for unsubstituted *cis,cis*-muconic acid (Elvidge *et al.*, 1950a) cycloisomerization of 2-chloro-*cis,cis*-muconic acid could be effected chemically only when exposed to conc. H₂SO₄ (75%, v/v). However only 5% of the initial muconic acid could be recovered by diethyl ether extraction as a crystalline product. This was identified as 4-carboxymethyl-2-chlorobut-2-en-4-olide (see below). No major by-products could be detected by high-pressure liquid chromatography.

4-Carboxymethyl-2-chlorobut-2-en-4-olide (4-chloro-2,5-dihydro-5-oxo-3*H*-furan-2-ylacetic acid)

The compound derived from 2-chloro-*cis,cis*-muconic acid by H₂SO₄ treatment exhibited the same molecular weight as the initial compound (*m/e*

Table 1. *Stability of substituted muconic acids under different pH conditions*

Substituted muconic acids were prepared in Tris/HCl buffer as described in the text. The initial concentrations of the muconic acids were 5 mM. Concentration of muconic acids and product formation was determined by means of high-pressure liquid chromatography (see the text).

Muconic acid	pH	Time of exposure	Concn. after exposure (mM)	Mode of reaction	Concn. of product (mM)
<i>cis,cis</i> -Muconic acid	1	2 h	5	None	0
2-Chloro- <i>cis,cis</i> -muconic acid	6.1	2 h	5	None	0
	1	1.6 h	2.5	Isomerization	2.5
3-Chloro- <i>cis,cis</i> -muconic acid	6.1	1 h	4	Cycloisomerization*	1
	5	1.5 h	0	Cycloisomerization*	5
2-Methyl- <i>cis,cis</i> -muconic acid	6.1	2 h	5	None	0
	1	1.5 h	4.2	Cycloisomerization†	0.8
3-Methyl- <i>cis,cis</i> -muconic acid	6.5	40 min	0.8	Cycloisomerization‡	4.2
2-Fluoro- <i>cis,cis</i> -muconic acid	6.1	2 h	5	None	0
	1	2 h	5	None	0
3-Fluoro- <i>cis,cis</i> -muconic acid	6.3	1 h	4.2	Cycloisomerization*	0.8
	4	13 min	0	Cycloisomerization*	5

* Yielding a mixture of *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide.

† Yielding 4-carboxymethyl-2-methylbut-2-en-4-olide.

‡ Yielding 4-carboxymethyl-4-methylbut-2-en-4-olide.

Table 2. *¹H-n.m.r. data of two cycloisomerization products*

The samples were dissolved in [²H]acetone and spectra were recorded at 100 MHz. Tetramethylsilane was used as an internal standard. To avoid confusion the numbering convention of all protons were the same as those of the parent muconic acids (see Table 3).

Compound	Proton	δ (p.p.m.)
4-Carboxymethyl-2-chlorobut-2-en-4-olide (X = Cl)	H-3	7.74 (d)
	H-4	5.45 (m)
	H-5	2.74 (dd, J_{gem} 17.7 Hz)
	H-5	2.96 (dd)
	OH	8.00 [s (broad)]
4-Carboxymethyl-2-methylbut-2-en-4-olide (X = CH ₃)	CH ₃	1.83 (t)
	H-3	7.34 (t)
	H-4	5.26 (m)
	H-5	2.57 (dd, J_{gem} = 16.7 Hz)
	H-5	2.84 (dd)
	OH	10.00 [s (broad)]

175.988), but no diene chromophore (λ_{max} 216 nm in methanol). Striking similarities exist between the ¹H-n.m.r. spectra of the isolated product (see Table 2) and 4-carboxymethyl-2-methylbut-2-en-4-olide. In the analogous carboxymethyl-chlorobutenolide structure, H-3 couples only with H-4 (for the numbering convention, see Table 2), whereas in the analogous methyl compound an additional coupling with the methyl group was observed (Knackmuss *et al.*, 1976).

Methyl-substituted muconic acids

Enzymically prepared 2-methyl- and 3-methyl-muconic acid were characterized by quantitative cycloisomerization to 4-carboxymethyl-2-methyl- and 4-carboxymethyl-4-methylbut-2-en-4-olide with cycloisomerase II. Cycloisomerization products were identified with reference to authentic compounds (Knackmuss *et al.*, 1976) by retention time during high-pressure liquid chromatography, and by u.v. and i.r. spectroscopy.

3-Chloro-*cis,cis*-muconic acid dimethyl ester (3-chloro-*cis,cis*-hexa-2,4-dienedioic acid dimethyl ester)

The enzymically prepared solution of 3-chloro-muconic acid in Tris/HCl buffer (500ml) was evaporated at 20°C bath temperature to one-tenth the original volume. After being cooled to 0°C the solution was acidified to pH 2 by addition of concentrated phosphoric acid and extracted repeatedly with 500ml of ice-cold diethyl ether. To stop cycloisomerization the ethereal solution was methylated immediately (De Boer & Backer, 1954). The dimethyl ester was purified by preparative t.l.c., with dichloromethane/hexane (1:1, v/v), and recovered as an oil.

The u.v. spectrum in diethyl ether exhibited a maximum at 266nm indicating a diene chromophore. A mass-spectral determination gave a calculated mass for $C_6H_5O_4Cl$ of 204.0190; the observed mass was 204.0188. Since most of the 3-chloro-*cis,cis*-muconic acid cycloisomerized spontaneously during extraction, the amount of the isolated dimethyl ester was too small for 1H -n.m.r. spectroscopy.

2-Chloro-*cis,cis*-muconic acid (2-chloro-*cis,cis*-hexa-2,4-dienedioic acid) and its *cis,trans* isomer

As described for 3-chloro-*cis,cis*-muconic acid the enzymically prepared solution of 2-chloro-*cis,cis*-muconic acid was concentrated to one-tenth the original volume. After being cooled to 0°C the solution was acidified to pH 2 by addition of concentrated phosphoric acid and extracted several times with cooled diethyl ether until u.v. absorption indicated complete extraction. The *cis,trans* isomer was isolated correspondingly after isomerization by acid treatment (pH 1, 20h, 20°C). The crystalline residues from diethyl ether extraction were rinsed with chloroform. The re-crystallization of the *cis,cis* isomer from tetrahydrofuran was initiated by dropwise addition of benzene, the re-crystallization of the *cis,trans* isomer from diethyl ether by the addition of light petroleum (b.p. 40–60°C). Identical retention time of 9.8min was observed during high-pressure liquid chromatography for the isolated 2-chloro-

cis,cis-muconic acid and the native preparation, whereas retention time of the *cis,trans* isomer was 15.6min. The molecular formula of both isomers was established as $C_6H_5O_4Cl$ (175.9876) by high-resolution mass spectrometry. The u.v.-absorption maxima in Tris/HCl buffer, pH 8, at 267nm ($\epsilon = 12\,500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *cis,cis*-isomer and 271nm ($\epsilon = 23\,900 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *cis,trans* isomer, indicated intact diene chromophores. Melting points of substituted muconic acids could not be determined, because of decomposition above 150°C. In contrast with 2-chloro-*cis,cis*-muconic acid, the *cis,trans* isomer could not be cycloisomerized either by muconate cycloisomerase I or by muconate cycloisomerase II from benzoate- or 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13 (Schmidt & Knackmuss, 1980).

The assignment of the *cis,cis*-configuration to the isolated 2-chloromuconic acid and the *cis,trans*-configuration to the acid-treated product is supported by 1H -n.m.r. spectral data (Table 3). The coupling constant $J_{4,5}$ in the native product was found to be 12 Hz, indicating the *cis*-configuration of the $C_{(4)}-C_{(5)}$ double bond. On the other hand the isomerization product from acid treatment exhibited a coupling constant $J_{4,5} = 15.4 \text{ Hz}$, which clearly favours the *trans*-configuration of the unsubstituted double bond. The assumption that the 2-chloro-*cis,cis*-muconic acid was isomerized by acid treatment to the *cis,trans* isomer is also supported by the observed dislocations of the H-3 and H-4 signals. During isomerization of the $C_{(4)}-C_{(5)}$ double bond the signal of H-3 is significantly shifted to lower frequencies because the anisotropic influence of the $C_{(6)}$ carboxy group is decreased. In contrast this isomerization exposes H-4 to the anisotropic effect of both carboxylic groups. Consequently the signal is shifted to higher frequencies. The dislocations of the corresponding protons are qualitatively the same when unsubstituted *cis,cis*-muconic acid is isomerized (see Table 4). Isomerization from the *cis,cis*- to the *trans,trans*-form would not explain the above dislocation. In this case the H-4 signal would be shifted to lower frequencies, as observed for the unsubstituted *trans,trans*-muconic acid.

Scheme 1. Configurations of isomeric 2-substituted muconic acid ($X = H, Cl, F$)

Table 3. ^1H -n.m.r. data of muconic acids

All compounds were dissolved in $[\text{D}_6]\text{acetone}$ with the exception of *trans,trans*-muconic acid, for which $[\text{D}_6]\text{dimethyl sulphoxide}$ was used. The spectra were recorded at 100 MHz with tetramethylsilane as an internal standard. To avoid confusion we have adopted the numbering convention for all protons as though they were the protons of unsubstituted muconic acid (see Scheme 1). For ^1H -n.m.r. data of unsubstituted muconic acids see also Elvidge & Ralph (1966).

Compound	Proton	δ (p.p.m.)	description		
<i>cis,cis</i> -Muconic acid	H-2	6.03	1H	*	$J_{2,3} = 12 \text{ Hz}$
	H-3	7.93	1H		
	H-4	7.93	1H		$J_{3,4} = 11.4 \text{ Hz}$
	H-5	6.03	1H		$J_{4,5} = J_{2,3}$
	OH	7.15	2H	s (broad)	
<i>cis,trans</i> -Muconic acid	H-2	6.01	1H	d	$J_{2,3} = 11.4 \text{ Hz}$
	H-3	6.85	1H	t	
	H-4	8.48	1H	dd	$J_{3,4} = 11.5 \text{ Hz}$
	H-5	6.19	1H	d	$J_{4,5} = 15.6 \text{ Hz}$
	OH	9.70	2H	s (broad)	
<i>trans,trans</i> -Muconic acid	H-2	6.43	1H	*	$J_{2,3} = 15.4 \text{ Hz}$
	H-3	7.32	1H		
	H-4	7.32	1H		$J_{3,4} = 11.8 \text{ Hz}$
	H-5	6.43	1H		$J_{4,5} = J_{2,3}$
	OH	5.65	2H	s (broad)	
2-Chloro- <i>cis,cis</i> -muconic acid	H-3	8.30	1H	dd	$J_{3,4} = 11.6 \text{ Hz}$
	H-4	7.62	1H	t	
	H-5	6.03	1H	d	$J_{4,5} = 12 \text{ Hz}$
	OH	9.06	2H	s (broad)	
2-Chloro- <i>cis,trans</i> -muconic acid	H-3	7.16	1H	d	$J_{3,4} = 11.8 \text{ Hz}$
	H-4	8.17	1H	dd	
	H-5	6.23	1H	d	$J_{4,5} = 15.4 \text{ Hz}$
	OH	10.10	2H	s (broad)	
2-Fluoro- <i>cis,cis</i> -muconic acid	H-3	7.83	1H	m	$J_{4,5} = 11 \text{ Hz}$
	H-4	7.64	1H		
	H-5	6.01	1H	dd	
	OH	9.31	2H	s (broad)	
2-Fluoro- <i>cis,trans</i> -muconic acid	H-3	6.74	1H		$J_{3,4} = 12 \text{ Hz}$
	H-4	8.23	1H	dd	
	H-5	6.24	1H	d	$J_{4,5} = 15.4 \text{ Hz}$
	OH	11.05	2H	s (broad)	

* Not fully resolved multiplets.

Table 4. Dislocation of proton signals during isomerization of *cis,cis*-muconic acids to *cis,trans*-configuration. Chemical-shift data of *cis,cis*-muconic acids and their *cis,trans* isomers from Table 3 were compared. The differences are expressed as the change in δ (p.p.m.).

Proton	Muconic acid	2-Chloromuconic acid	2-Fluoromuconic acid
H-2	-0.02	—	—
H-3	-1.08	-1.14	-1.09
H-4	+0.55	+0.55	+0.59
H-5	+0.16	+0.20	+0.23

2-Fluoro-*cis,cis*-muconic acid (2-fluoro-*cis,cis*-hexa-2,4-dienedioic acid) and its *cis,trans*-isomer

2-Fluoro-*cis,cis*-muconic acid and its *cis,trans*-isomer were extracted as described for 2-chloromuconic acid. The *cis,cis* isomer was re-crystallized

from methanol and the *cis,trans* isomer from acetone/chloroform. The molecular formula of both compounds was established as $\text{C}_6\text{H}_5\text{O}_4\text{F}$ (160.0171) by high-resolution mass spectrometry. The u.v.-absorption spectrum in Tris/HCl buffer, pH 8, gave

a maximum at 261 nm ($\epsilon = 15\,500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *cis,cis* isomer and at 265 nm ($\epsilon = 30\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *cis,trans* isomer.

The *cis,cis*-configuration of 2-fluoromuconic acid isolated from the native preparation was confirmed by ^1H -n.m.r. spectroscopy. The coupling constant $J_{4,5} = 11 \text{ Hz}$ clearly favours a *cis*-configuration of the $\text{C}_{(4)}-\text{C}_{(5)}$ double bond. To establish that this 2-fluoromuconic acid has the *cis,cis*- rather than the *trans,cis*-configuration, the chemical-shift data were compared with those of 2-chloro-*cis,cis*-muconic acid. In muconic acids with the *cis,cis*-configuration, but not in *trans,cis* isomers, H-4 is exposed to the strong anisotropic effect of the $\text{C}_{(1)}$ carboxylic group. Consequently the deshielding of H-4 in 2-fluoro-*trans,cis*-muconic acid must be considerably smaller than in 2-halo-*cis,cis*-muconic acids. But in fact H-4 of the isolated 2-fluoromuconic acid exhibited a δ value of 7.64 p.p.m., which is also found in 2-chloro-*cis,cis*-muconic acid ($\delta = 7.62 \text{ p.p.m.}$). When 2-fluoro-*cis,cis*-muconic acid was isomerized at pH 1 and 80°C , the δ values and coupling constants were altered in the same way as during isomerization of *cis,cis*-muconic acid and 2-chloro-*cis,cis*-muconic acid to the corresponding *cis,trans*-isomers (see Tables 3 and 4).

2-Fluoro-*cis,trans*-muconic acid could not be cycloisomerized by enzyme preparations from benzoate- or 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13. In this respect it corresponded to its chloro-analogue and to unsubstituted *cis,trans*-muconic acid, all of which were inactive as substrates or inhibitors for the cycloisomerization reaction. It is noteworthy that 2-fluoro-*cis,cis*-muconic acid could also not be cycloisomerized by cycloisomerase I or cycloisomerase II from *Pseudomonas* sp. B13. Nevertheless this compound competitively inhibited ($K_i = 33 \mu\text{M}$) the cycloisomerization of 2-chloro-*cis,cis*-muconic acid by cycloisomerase II. To confirm the inability of *Pseudomonas* sp. B13 to convert 2-fluoro-*cis,cis*-muconic acid, 3-fluorocatechol (1 mM) was co-oxidized by 3-chlorobenzoate-grown cells ($A_{546} = 7$ in 50 mM-phosphate buffer, pH 7) of this organism. The analysis of the buffer solution by high-pressure liquid chromatography revealed rapid accumulation of stoichiometric amounts of 2-fluoro-*cis,cis*-muconic acid. In a control experiment 3-chlorocatechol (1 mM) was totally degraded with release of chloride.

Discussion

Previous investigations on the configuration and biodegradability of halogenated muconic acids were based on the relative biological activities of the isomers of unsubstituted muconic acids. Enzyme preparations from arene-degrading bacteria exhibit high cycloisomerase activities only with the *cis,cis*

isomer of muconic acid. In contrast *cis,trans*-muconic acid was not cycloisomerized enzymically (Tiedje *et al.*, 1969; Gaunt & Evans, 1971), or only at very low rates (0.02%; Sistrom & Stanier, 1954). *trans,trans*-Muconic acid was completely inactive.

3-Chloromuconic acid had been reported as a metabolite in 4-chlorophenoxyacetate-utilizing bacterial cultures (Tiedje *et al.*, 1969; Evans *et al.*, 1971a). The product, which had been extracted from acidic solution (pH < 3), was biologically inert and consequently considered as the *cis,trans*-isomer of 3-chloromuconic acid. The present results are inconsistent with this observation, since under acidic conditions (pH < 5) the cycloisomerization product 4-carboxymethylenebut-2-en-4-olide was rapidly formed in quantitative yield. No other reaction product or intermediates could be detected by high-pressure liquid chromatography.

Evans *et al.* (1971b) reported the formation of 2-chloromuconic acid as a metabolite from 2,4-dichlorophenoxyacetate or 3-chlorocatechol by *Pseudomonas* sp. I. Evans *et al.* (1971a,b) considered the *cis,cis*-configuration of the isolated product, because it stimulated the respiration rates of the 2,4-dichlorophenoxyacetate-grown cells. Surprisingly the biological product could be identified with a chemical preparation, which had been exposed to highly acidic conditions (pH ≤ 1) for several weeks. In contrast the present analysis by high-pressure liquid chromatography clearly demonstrates that at pH 1 2-chloro-*cis,cis*-muconic acid completely isomerized to the *cis,trans*-form within 20 h.

3-Methyl-, 3-chloro- and 3-fluoro-muconic acid were cycloisomerized spontaneously at pH < 7. In contrast the 2-substituted analogues could readily be isolated as native *cis,cis* isomers. The present data have shown that 2-fluoromuconic acid is a dead-end metabolite at least for *Pseudomonas* sp. B13. Its *cis,cis*-configuration was stable under physiological and even acidic conditions. The reason for its biological inertness must be correlated with the dichotomy of the C-F bond and not, as Goldmann *et al.* (1967) postulated, to its rapid isomerization to the *cis,trans*-form. The exceptional character of 2-fluoro-*cis,cis*-muconic acid is in part responsible for the inability of *Pseudomonas* sp. B13 to accept 3-fluorobenzoate as a growth substrate. The same phenomenon explains incomplete degradation of 2-fluorobenzoate by a *Pseudomonas* strain described by Goldmann *et al.* (1967) and by adapted strains of *Pseudomonas* sp. B13. Depending on the selectivity of the first enzyme different amounts of the substrate are channelled into the dead-end pathway of 2-fluoromuconic acid (Engesser *et al.*, 1980).

Since the isolated 2-fluoromuconic acid competitively inhibits cycloisomerization of 2-chloromuconic acid by cycloisomerase II, we can assume

that fluorine as a substituent does not interfere with substrate binding. In contrast 2-fluoro-*cis,trans*-muconic acid, like the *cis,trans*- and *trans,trans*-forms of unsubstituted muconic acid, is inactive as an inhibitor. Apparently formation of the lactone ring rather than substrate binding is the critical reaction step during cycloisomerization of 2-fluoromuconic acid.

Note added in proof (Received 22 August 1980)

By using an authentic sample of 3-chloro-*cis,trans*-muconic acid (generously supplied by Professor W. C. Evans, University College of North Wales, Bangor, U.K.) we resolved the inconsistency described in the Discussion section. High-pressure liquid chromatography clearly showed that at pH 5–2.5 3-chloro-*cis,trans*-muconic acid was quantitatively cycloisomerized yielding 4-carboxymethylenebut-2-en-4-olides. 3-Chloro-*cis,trans*-muconic acid could not be detected under these conditions. However, when rapidly acidified to pH ≤ 1 , the *cis,trans* isomer appeared as an additional product, which was identified by mass spectrometry. The yield was approx. 60% as measured by high-pressure liquid chromatography.

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