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Effect of Methyl Substitution on Microbial Degradation of Linear Styrene Dimers by Two Soil Bacteria

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The microbial degradation of 10 linear unsaturated dimers (I to IV) prepared from styrene and *o*-, *m*-, or *p*-methylstyrene was investigated with two soil bacteria, *Alcaligenes* sp. strain 559 and *Pseudomonas* sp. strain 419. The two strains decomposed styrene dimer I and all styrene-methylstyrene codimers II and III, but methylstyrene homodimers IV remained intact. The degradation rates of codimers II and III of *o*- and *m*-methylstyrenes were found to depend on both their structure and the strain used; i.e., *Alcaligenes* sp. strain 559 decomposed III faster than II, whereas the reverse order (II > III) was obtained with *Pseudomonas* sp. strain 419. In biodegradation by the former strain, the codimers were degraded faster in the presence of styrene dimer I than in its absence, but no such effect of dimer I was observed with the latter.

Synthetic oligomers of aromatic olefins, typically oligostyrene and petroleum resins, have recently been used widely in industry as lubricants, tackifiers, plasticizers, additives, and the like. Along with their wide use, an increasing quantity of these oligomers is now spreading in the biosphere. Because they are potential environmental pollutants, their degradation by microorganisms is of great interest from both biochemical and ecological viewpoints.

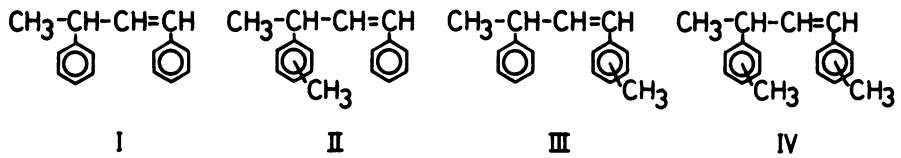
Tsuchii et al. (8) have isolated a strain (*Alcaligenes* sp. strain 559) that can assimilate the linear unsaturated dimer of styrene (St) (1,3-diphenylbut-1-ene; I) as sole carbon source. Later studies by Sielicki et al. (7) showed that the saturated linear dimer of St (1,3-diphenylbutane) is also metabolized by mixed microorganisms in soils, whereas polystyrene, having the same structural units, degrades much more slowly under similar conditions (1, 3, 7). All data reported thus far, however, are confined to the biodegradation of the St dimers and polystyrene; it is unknown whether or not oligomers of other aromatic olefins are biodegradable. This has prompted us to investigate the microbial degradation of a wide variety of synthetic oligomers.

We previously developed a cationic dimerization process to prepare selectively linear unsaturated dimers of St and its derivatives (2, 4, 6). For instance, 1,3-diphenylbut-1-ene (I) can be obtained from St with acetyl perchlorate as catalyst in a very high yield (2, 6). When a mixture of St and *o*-, *m*-, or *p*-methylstyrene (MeSt) is treated with the same catalyst, the product consists of the four unsaturated dimers shown in Fig. 1. I and IV are the homodimers of St and the MeSt, respectively. II and III are codimers of St and *o*-, *m*-, or *p*-MeSt; codimers III have a methyl group on the benzene nucleus attached to the main-chain double bond, whereas in codimers II a methyl group is on the benzene ring linked to a saturated carbon. Compounds I through IV can easily be separated by preparative high-performance liquid chromatography (HPLC).

This investigation deals with the microbial degradation of these structurally different dimers (I to IV) by two soil bacteria, *Alcaligenes* sp. strain 559 and *Pseudomonas* sp. strain 419. Primary attention was focused on the relation between the biodegradability of the dimers and their structure and on the dependence of the degradability upon strains. We report here that all of the St-MeSt codimers (II and III) can be decomposed by the two strains but that the MeSt homodimers (IV) are nondegradable.

MATERIALS AND METHODS

Strains and cultural methods. *Alcaligenes* sp. strain 559 and *Pseudomonas* sp. strain 419 were isolated from soil (8). They were grown on St dimer I for 2 days and then inoculated to 5 ml of the growth medium in a test tube (1.8-cm inside diameter by 18 cm). The procedures for this 2-day precultivation were the same as described below, except that the strain was inoculated directly from a stock slant culture. The growth medium consisted of 1.0 g of a dimer substrate (usually a mixture of I to IV) as sole carbon source, 0.10 g of



II_o, III_o, IV_o: o-CH_3

II_m, III_m, IV_m: m-CH_3

II_p, III_p, IV_p: p-CH_3

FIG. 1. Structure of (co)dimers of St and MeSt used as substrates.

yeast extract (Difco Laboratories), and 80 mg of a surfactant (Plysurf A 210G; Daiichi Kogyo Seiyaku) in 1 liter of a basal mineral solution [K_2HPO_4, 1.6 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 0.20 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; NaCl, 0.10 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $FeSO_4$, 10 mg; Na_2MoO_4, 0.50 mg; Na_2WO_4, 0.50 mg; $MnSO_4$, 0.50 mg; and deionized water; pH 7.5]. Before inoculation, this solution was homogenized by sonication in an ice bath and was sterilized as follows. The culture medium was heated at 100°C in steam for ca. 30 min and then shaken at 30°C overnight on a reciprocal shaker (130 rpm). These procedures were repeated three times and then the strain was inoculated. The culture medium for control runs was the same as above except for the absence of a dimer substrate and the surfactant. Incubations were performed at 30°C on the reciprocal shaker over periods of up to 8 days. Experiments were repeated usually twice and showed good reproducibility.

Assay procedures. After incubation, the culture fluid (5 ml) was acidified to pH 2 with 2 N hydrochloric acid and extracted three times with ethyl acetate (15 ml each time). Cell growth was measured by turbidimetric analysis of the aqueous layer at 660 nm on a Shimadzu UV-190 spectrophotometer. To recover the residual substrate, the ethyl acetate layers were combined and evaporated to dryness under reduced pressure at ca. 40°C. The composition of the recovered substrate was determined by gas chromatography (GC) on a Shimadzu GC-6A chromatograph, with phenylcyclohexane as the internal standard added to the substrate residue just before measurement (ca. 5 mg of the standard in 0.3 ml of CH_2Cl_2 for ca. 5 mg of the residue). The operational conditions for GC were as follows: column, Silicon DC 550, 1 m by 8-mm inside diameter; carrier gas, hydrogen, 1.5 atm; injector temperature, 280°C; oven temperature, 250°C; thermal conductivity detector maintained at 280°C; sample size, 10 to 20 μl. Figure 2 shows typical gas chromatograms. The two peaks of codimers II and III were assigned on the basis of a modified Lemieux-von Rudloff oxidation of a mixture of these dimers (see below). Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded in carbon tetrachloride or deuterated chloroform on JEOL MH-60 and JNM-FX90Q spectrometers.

Substrate synthesis. The dimer substrates were synthesized by the cationic dimerization or codimerization of the corresponding monomer(s) (St and MeSt) with acetyl perchlorate at 50°C in benzene (2, 6). After the reaction mixture was quenched with ammoniacal methanol, it was washed with deionized water to remove the catalyst residue. The products (a mixture of I to IV) were then isolated by evaporating the benzene solution under reduced pressure and vacuum dried. Infrared and ^{1}H-NMR spectra confirmed their expected linear unsaturated structure. When necessary, dimerization products were further fractionated by preparative HPLC (see Table 1).

Lemieux-von Rudloff oxidation of codimers II and III (5). The Lemieux-von Rudloff oxidation method is known to oxidize selectively a carbon-carbon double bond to give two carboxylic acids. The oxidation of codimer II_o thereby, for example, yields benzoic acid and 2-(*o*-methylphenyl)propionic acid, whereas codimer III_o leads to *o*-methylbenzoic and 2-phenylpropionic acids. We can therefore distinguish II from III by analyzing the oxidation products from these codimers by GC and ^{1}H-NMR.

A typical experiment was carried out as follows. A mixture of codimers II_o and III_o (52 mg), purified by HPLC and dissolved in 15 ml of acetone, was mixed

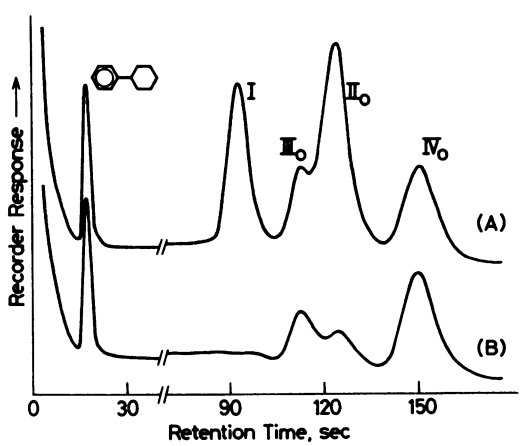


FIG. 2. Typical gas chromatograms of substrates obtained from St and *o*-MeSt. Peak assignments are as indicated. (A) Before incubation; (B) after a 5-day incubation with *Pseudomonas* sp. strain 419.

TABLE 1. Biodegradation of St-MeSt (co)dimers by *Alcaligenes* sp. strain 559 and *Pseudomonas* sp. strain 419[a]

Strain	Substrate	Composition (%, by wt)[b]			% Decomposition[b]			Cell growth[c]
		I	II + III	IV	I	II + III	IV	
Alcaligenes sp. strain 559	St dimer[d]	100	0	0	100			0.40
	o-MeSt–St	23	53	24	100	90	0	0.12
	m-MeSt–St	17	49	34	100	80	0	
	p-MeSt–St	16	57	27	100	76	0	0.095
	o-MeSt–St	2.0	65	33	100	49	0	0.027
	m-MeSt–St	4.0	66	30	100	52	0	0.054
	p-MeSt–St	1.5	87	11	100	41	0	0.058
Pseudomonas sp. strain 419	St dimer[d]	100	0	0	100			0.080
	o-MeSt–St	21	56	23	100	75	0	0.030
	m-MeSt–St	19	52	29	100	68	0	0.030
	p-MeSt–St	17	57	26	100	33	0	0.026
	o-MeSt–St	0	69	31		75	0	0.050
	m-MeSt–St	4.0	66	30	100	37	0	0.016
	p-MeSt–St	3.0	66	31	100	36	0	0.020

[a] Incubation time, 5 days.
[b] Determined by GC.
[c] Maximal absorbance difference at 660 nm between inoculated cultures with and without a (co)dimer substrate and the surfactant.
[d] Incubation time, 1 day.

with 20 ml of deionized water, and the pH of the aqueous phase was adjusted to 9 with sodium bicarbonate. To this mixture an aqueous solution (50 ml) of potassium permanganate (20 mg) and potassium periodate (1.15 g) was added dropwise at 0°C over a 30-min period with stirring. Stirring was continued overnight, and sodium *meta*hydrogensulfite was added to decompose the residual periodate; the reaction media were separated from the manganese dioxide precipitate by filtration, and the acetone was removed under reduced pressure. The remaining aqueous phase was then acidified with hydrochloric acid and extracted with ether. The ether fraction was evaporated to dryness and vacuum dried to give a mixture (66.4 mg) of the expected four acids derived from II_o and III_o nearly in quantitative yield. These acids were identified by ^{1}H-NMR; no by-products were detected. The relative amounts of the benzoic acid (from II_o) and the *o*-methylbenzoic acid (from III_o) were determined by GC under the following conditions: column, Silicon DC 550, 1.5-m by 8-mm inside diameter; carrier gas, hydrogen, 1.5 atm; injector temperature, 190°C; oven temperature, 160°C; thermal conductivity detector at 190°C; sample size, 10 to 20 μl. Comparison of these amounts with those of II_o and III_o in the starting substrate indicated that the retention time of II_o is longer than that of III_o (Fig. 2).

Codimers II_m and III_m were analyzed in the same manner.

Codimers II_p and III_p, on the other hand, could not be separated by GC. Therefore, they were fractionated from the residual substrate by HPLC (as a mixture), and their relative amounts were determined by the Lemieux-von Rudloff method as described above. This analysis was done on samples before and after incubation.

RESULTS AND DISCUSSION

Biodegradation of St-MeSt codimers and MeSt dimers. The biodegradability of St-MeSt codimers II and III and MeSt homodimers IV was examined with soil bacteria, *Alcaligenes* sp.

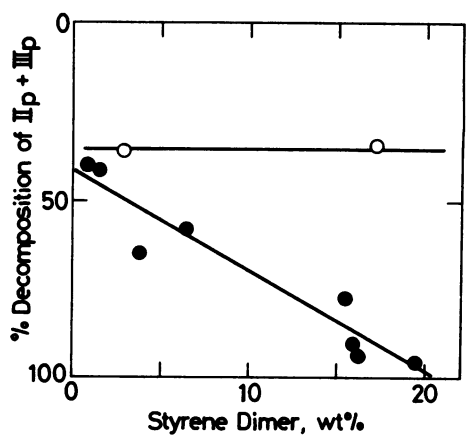


FIG. 3. Effect of St dimer I in the substrate on biodegradation of St–*p*-MeSt codimers (II_p and III_p) after a 5-day incubation. Symbols: (●) *Alcaligenes* sp. strain 559; (○) *Pseudomonas* sp. strain 419.

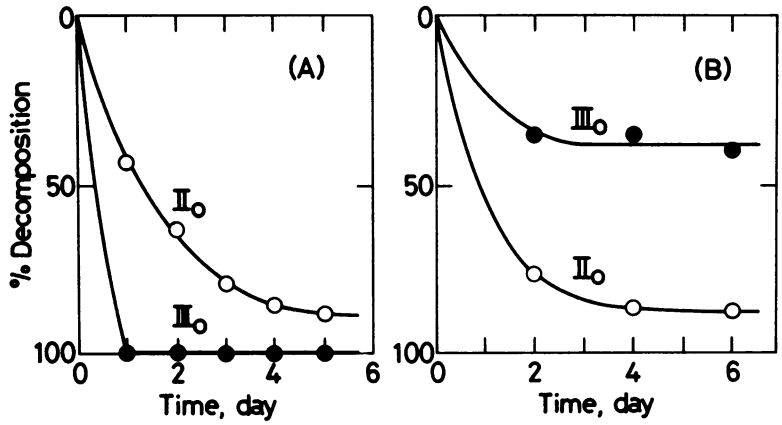


FIG. 4. Time courses of biodegradation of St–*o*-MeSt codimers II_o (○) and III_o (●) by *Alcaligenes* sp. strain 559 (A) and *Pseudomonas* sp. strain 419 (B). Composition of the starting substrate: I, 20% (by weight); II_o, 64% (by weight); III_o, 16% (by weight).

strain 559 and *Pseudomonas* sp. strain 419, both of which are known to degrade effectively St dimer I (8). Table 1 summarizes the results of typical experiments along with the composition of the starting substrates. The cell growth is indicated by the maximal absorbance of the culture at 660 nm. For each strain, two groups of dimer substrates were used: one with 15 to 20% of I and the other practically free of I (0 to 4%). A pure sample of I was also used as substrate for comparison.

Both strains decomposed pure St dimer I completely after 1 day, the maximal absorbance at 660 nm being 0.4 with the *Alcaligenes* strain and 0.08 with the *Pseudomonas* strain. In the degradation of the codimer mixtures, *Alcaligenes* sp. strain 559 decomposed I in the substrate completely within 1 day, at which time the absorbance at 660 nm reached maximum, whereas the codimers (II plus III) were degraded gradually and continuously up to 40 to 90% over 5 days. In sharp contrast, MeSt homodimers IV remained unchanged throughout the experiments. Similar results were obtained with *Pseudomonas* sp. strain 419.

Effect of St dimer I on biodegradation of codimers II and III. Table 1 indicates that with the *Alcaligenes* strain, the percent decomposition of II and III, together with the maximum absorbance at 660 nm, depends on the relative amount of styrene dimer I in the substrate: e.g., decomposition of II_o + III_o, 90% with 23% (by weight) of I; 49% with 2.0% (by weight) of I. We thus examined in detail the effect of the concentration of I on the degradation of St–*p*-MeSt codimers (II_p + III_p). Figure 3 illustrates the relation between the content of I in the substrate and the percent decomposition of the codimers after a 5-day incubation. The decomposition was facilitated with increasing amounts of I in the culture. Such an effect of I was also observed for *o*-MeSt–St and *m*-MeSt–St codimers. With the *Pseudomonas* strain, in contrast, the degradation of the codimers (except for *m*-MeSt–St) was not affected by the presence of dimer I (Fig. 3 and Table 1).

The promotion of the codimer decomposition by dimer I may be because (i) cell growth is facilitated in the presence of this good substrate; (ii) dimer I induces an enzyme that can degrade the codimers effectively; or (iii) the reducing power of the strain required in the initial oxygenation of codimers is effectively regenerated by the presence of I, or all three. Possibilities ii and iii imply the co-oxidation of II or III or both, with I as cosubstrate.

Biodegradability of codimers II and III. So far we have discussed the biodegradability of II and III as their mixture in the substrate. Since we can separately follow the decomposition of these two codimers (see above), we then compared

TABLE 2. Biodegradation of St-MeSt codimers II and III by *Alcaligenes* sp. strain 559 and *Pseudomonas* sp. strain 419

Codimer (I; %, by wt)[a]	% Decomposition by *Alcaligenes* sp.			% Decomposition by *Pseudomonas* sp.		
	II	III	Incubation (days)	II	III	Incubation (days)
o-MeSt–St (20)	67	100	2	75	35	2
o-MeSt–St (0)	37	95	5	90	45	6
m-MeSt–St (4)	24	41	1	87	52	7
p-MeSt–St (10)	17	17	2	84	87	8

[a] Amount of St dimer I in the substrate.

V VI

FIG. 5. Biodegradation products of St–*o*-MeSt codimers.

their biodegradabilities to clarify the effect of their structural difference.

Figure 4 shows the time courses of the biodegradation of II_o and III_o by the two strains. In these experiments the substrates contained some amount (20%, by weight) of styrene dimer I, but similar results were obtained with substrates free of I (Table 2). Two facts of interest were found. First, II_o and III_o have different biodegradabilities, and second, this difference also depends on the strains used; i.e., *Alcaligenes* sp. strain 559 degrades III_o faster than II_o, whereas the reverse is true with *Pseudomonas* sp. strain 419. In addition, the degradation of III_o by the latter strain apparently stopped after 2 days, with ca. 60% of this codimer left intact.

The biodegradabilities of *o*-, *m*-, and *p*-isomers of II and III are compared in Table 2. For codimers of *o*- and *m*-MeSt, biodegradability is II < III with the *Alcaligenes* strain, whereas it is II > III with the *Pseudomonas* strain; these orders were not affected by the relative amount of St dimer I in the substrate. No difference was observed, however, for *p*-MeSt codimers II_p and III_p.

Codimers II and III differ in that II has its methyl group on the benzene ring attached to the main-chain double bond, whereas the methyl group of III is located on the benzene ring linked to a saturated carbon. The present investigations have shown that such a structural difference between II and III, i.e., the relative position of the methyl substituent and the main-chain double bond, plays a vital role in the microbial degradation of St-MeSt codimers. Another important finding is that the *Alcaligenes* and *Pseudomonas* strains gave opposite degadability orders for II and III (Fig. 4 and Table 2), indicating that each microorganism has a suitable substrate structure for biodegradation. Thus, the positions of the carbon-carbon double bond and the methyl substituent in a codimer substrate critically affect the biodegradation by the two strains.

Biodegradation products. To obtain insight into the pathway of biodegradation of St-MeSt codimers, we isolated the biodegradation intermediate(s) or product(s).

In a typical experiment, a mixture of I (21 mg), II_o (43 mg), III_o (13 mg), and IV_o (23 mg) was degraded with the *Pseudomonas* strain. The culture was shaken at 30°C for 1 day, at which time St dimer I was completely consumed, and incubation was further continued under milder conditions, i.e., without shaking at 30°C, for an additional 12 days to accumulate degradation products. The culture fluid was then extracted with ether, and the ether phase was evaporated to dryness under reduced pressure. HPLC analysis indicated that the oily residue consists of not only the residual II_o, III_o, and IV_o but also some new compounds in the dimer region. These new fractions were separated by HPLC, and evaporation of the eluent ($CHCl_3$) gave a white solid (15 mg).

The solid gave the following spectroscopic results: ^{1}H-NMR ($CDCl_3$) 1/M δ 9.0 (CO_2H, broad s), 7.1 to 7.4 (aromatic H), 5.7 to 6.0 (CH=CH, m), 2.3 (ring CH_3, s), and 1.4 (CH_3 [two kinds], d); ^{13}C-NMR ($CDCl_3$, internal Me_4Si)—δ 171.8 (C=C–CO_2H), 42.1 and 37.7 (CH), and 20.0 and 19.3 (CH_3); infrared (KBr)—3,000 (CO_2H), 1,680 and 1,630 (C=C–CO_2H), and 980 cm^{-1} (*trans* C=C). No evidence for the presence of CH_2 units was detected by the three methods. From these observations, we concluded that the biodegradation products were a mixture of two unsaturated carboxylic acids with one aromatic ring (Fig. 5). Similarly, degradation of II_p and III_p resulted in VI and the *para*-isomer of V. With the *Alcaligenes* strain, however, all attempts failed to give any measurable amount of biodegradation products.

The formation of unsaturated carboxylic acids V and VI from II and III suggests that, at least in degradation by the *Pseudomonas* strain, the microbial transformation starts from the benzene ring attached to the main-chain double bond. A similar degradation pathway has been proposed for 1,3-diphenylbutane (saturated linear styrene dimer), which is also decomposed from the benzene ring at the C-1 position (7).

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