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Benzylfumaric, Benzylmaleic, and *Z*- and *E*-Phenylitaconic Acids: Synthesis, Characterization, and Correlation with a Metabolite Generated by *Azoarcus tolulyticus* Tol-4 during Anaerobic Toluene Degradation

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***E*-Phenylitaconic acid has been isolated as a metabolite generated by *Azoarcus tolulyticus* Tol-4 along with benzylsuccinic acid during anaerobic degradation of toluene. Strain Tol-4 converted 1 to 2% of toluene carbon to *E*-phenylitaconate and benzylsuccinate (10:1). The identification of *E*-phenylitaconic acid was based on ^{1}H nuclear magnetic resonance (NMR) characterization of degradation products derived from ^{13}C-labeled toluene followed by comparison of spectroscopic and chromatographic data for the isolated, unlabeled metabolite with those for chemically synthesized benzylfumaric acid, benzylmaleic acid, *E*-phenylitaconic acid, and *Z*-phenylitaconic acid. Spectroscopic comparisons included ^{1}H NMR, ^{13}C NMR, and nuclear overhauser effect correlations. High-pressure liquid chromatography (HPLC) retention times and HPLC coinjections with synthetic dioic acids provided another reliable line of evidence for structure assignment. The formation of *E*-phenylitaconic acid differs from previous reports of benzylfumaric acid generation along with benzylsuccinic acid during anaerobic microbial degradation of toluene. This has important implications relevant to elaboration of the metabolic route for anaerobic toluene degradation by strain Tol-4 and related organisms. Similar amounts of *E*-phenylitaconic acid were also produced by seven other strains of *A. tolulyticus*.**

Benzylsuccinic acid and benzylfumaric acid (compound 1a [Fig. 1]) have been reported to accumulate during anaerobic degradation of toluene under denitrifying conditions by strain T1 (8), *Pseudomonas* sp. strain T (18), and *Thauera aromatica* K172 (1, 18) as well as under sulfate-reducing conditions by strain PRTOL (3, 4). A newly characterized microbe, *Azoarcus tolulyticus* Tol-4 (5), has likewise been discovered to accumulate two metabolites during anaerobic degradation of toluene under denitrifying conditions. One of these metabolites was identified as benzylsuccinic acid (5). However, identification of the second metabolite proved to be more elusive. There was little doubt that the unknown metabolite was either a phenylmethylbutenedioic acid (compounds 1a and 2a [Fig. 1]) or a phenylmethylenebutanedioic acid (compounds 3a and 4a [Fig. 1]). However, beyond the carbon backbone of the second metabolite, the location and substitution pattern of the double bond were open to question. Benzylfumaric acid [*E*-(phenylmethyl)butenedioic acid] (compound 1a), benzylmaleic acid [*Z*-(phenylmethyl)butenedioic acid] (compound 2a), *E*-phenylitaconic acid [*E*-(phenylmethylene)butanedioic acid] (compound 3a), and *Z*-phenylitaconic acid [*Z*-(phenylmethylene) butanedioic acid] (compound 4a) all had to be considered as candidate structures for the second metabolite (Fig. 1).

The previous identification (8, 18) of benzylfumaric acid as a product formed during anaerobic microbial degradation of toluene relied primarily on characterization by using electron impact mass spectrometry (EIMS). However, the use of this spectroscopic technique to distinguish between the structures of the individual phenylmethylbutenedioic acids (compounds 1a and 2a) and phenylmethylenebutanedioic acids (compounds 3a and 4a) was potentially problematic. Double-bond migration and interconversion of *E* and *Z* isomers are phenomena that are documented to occur during MS analysis of olefins (7). Double-bond migration and isomerization would not be a problem with nuclear magnetic resonance (NMR) analysis. However, literature ^{1}H and ^{13}C NMR spectral information was available for only one of the dioic acids (12). The derivatization techniques used in previous analyses of metabolites formed during anaerobic toluene degradation were also a cause for concern. Acid-catalyzed migration and isomerization of the double bonds during derivatization of the dioic acids to the corresponding diesters would greatly complicate the assignment of structures to the degradation metabolites.

Our efforts to identify the unknown metabolite relied heavily on synthesized, authentic samples of benzylfumaric acid (compound 1a), benzylmaleic acid (compound 2a), *E*-phenylitaconic acid (compound 3a), *Z*-phenylitaconic acid (compound 4a), and the corresponding dimethyl diesters (compounds 1b to 4b) (Fig. 1). Published synthetic routes to individual dioic acids and diesters span approximately 30 years of chemical literature (2, 6, 11, 13, 15). Some protocols required substantial optimization to afford the desired products. One instance of misassigned ^{1}H NMR data in the literature (6) was discovered subsequent to synthesis and characterization of all of the dioic acids and diesters. Dioic acids 1a, 3a, and 4a were synthesized as the corresponding dimethyl diesters 1b, 3b, and 4b, and this was followed by hydrolysis under basic conditions to afford the free dioic acids. Because of problematic double-bond migration during base hydrolysis of its dimethyl diester 2b, benzylmaleic acid (compound 2a) was synthesized by deprotection of the corresponding trimethylsilyl diester.

Derivatization of the synthesized dioic acids as dimethyl diesters and subsequent MS analyses were carefully examined.

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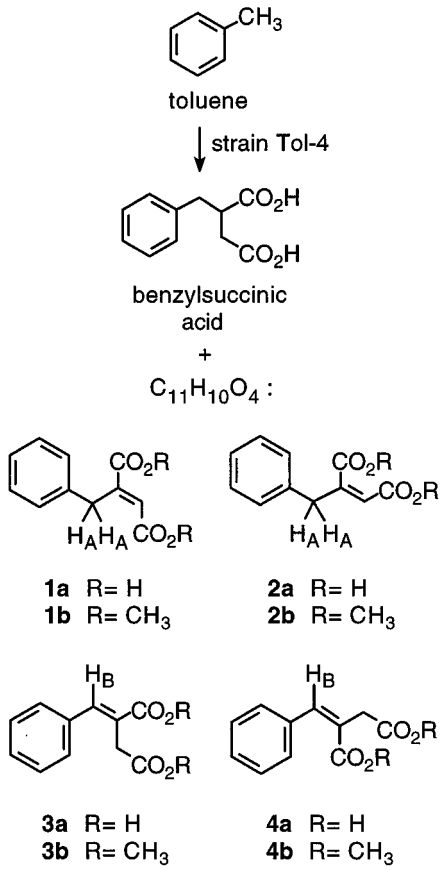

FIG. 1. Possible structures for the second metabolite formed during anaerobic degradation of toluene by *A. tolulyticus* Tol-4.

Ultimately, synthesized phenylmethylenebutanedioic acids and diesters were distinguished from phenylmethylbutenedioic acids and diesters by high-pressure liquid chromatography (HPLC) separation and use of ^{1}H NMR and nuclear overhauser effect (NOE) correlations. These techniques also differentiated between *E* and *Z* isomers within each class of dioic acid and diester. The combined use of ^{1}H NMR, ^{13}C NMR, and HPLC comparisons with synthetic samples ultimately led to the assignment of *E*-phenylitaconic acid (compound 3a) as the structure of the metabolite formed along with benzylsuccinic acid during anaerobic degradation of toluene by strain Tol-4. The identification of *E*-phenylitaconic acid has prompted the proposal of a modified toluene mineralization pathway for strain Tol-4 (5).

MATERIALS AND METHODS

Culture preparation of *A. tolulyticus* Tol-4. Cultures (100 ml) of strain Tol-4 were grown anaerobically in basal salts medium (17) plus 5 mM NO_3^- with 500 μM toluene (98%; Sigma) under a headspace of argon. Cultures were incubated in sealed serum bottles at 30°C. For metabolite analysis, toluene and NO_3^- were added as needed until a total of 500 μmol of toluene was consumed. [*methyl*-^{13}C]toluene (99%; Cambridge Isotope Laboratories) was substituted in order to assess ^{13}C-labeled metabolites by the procedure described above. Separate cultures grown in aerobically prepared basal salts-toluene medium in the absence of NO_3^- were also used for metabolite analysis. Cultures were extracted with ethyl acetate and concentrated for analysis as specified below.

To test the ability of strain Tol-4 to metabolize the dioic acids, cells were grown on 500 μM toluene as described above for 24 h at 30°C. After gas chromatography confirmed toluene depletion, toluene and dioic acids were added to final concentrations of 200 μM toluene and 50 μM *E*-phenylitaconic acid, *Z*-phenylitaconic acid, benzylfumaric acid, or benzylmaleic acid. Samples (1 ml) were removed for HPLC analysis at time zero and then daily for 1 week. After 1 week, cultures were extracted with diethyl ether as specified below. Control cultures consisted of anaerobic toluene-grown cells, one culture to which additional toluene was added, and a second culture to which dioic acids were added. The dioic acids were added from stock solutions prepared in anaerobic basal salts medium and stored at 4°C until use. Authentic undiluted compounds were stored at −20°C.

Metabolite extraction. Cultures were centrifuged at 10,000 × *g* for 20 min at 4°C, and cells were discarded. The supernatant was acidified by addition of 2.5 ml of 10 M H_3PO_4 followed by extraction three times with either ethyl acetate or diethyl ether. The organic fraction was dried with Na_2SO_4 and the solvent was removed under a stream of argon. The residue was dissolved in 1 ml of CH_3OH-H_2O (1:1) and filtered (0.45-μm pore size). Chromatographic analysis and purification of metabolites for spectroscopic characterization followed isocratic elution of a LiChrosorb RP-18 column (10-μm pore size; 4-mm inner diameter [i.d.]; 25-cm length) with H_2O (0.1% in H_3PO_4)-CH_3OH (60:40) with a Hewlett-Packard 1050 HPLC. The metabolite fraction was collected and extracted with hexane as described above. After concentration, the residue was dissolved in ethyl acetate (3 ml) and stored at 4°C until analyzed.

General chemistry. See reference 14 for general experimental information dealing with synthetic manipulations. Photochemical reactions were run in a Rayonet apparatus with an RMR 300-nm light source. Gas chromatography (GC) data were collected on a Hewlett-Packard 5890 apparatus with a DB-1 column (0.25-mm i.d.; 30-m length). HPLC purifications of synthetic dioic acids and diesters were performed on a Rainin instrument with a Microsorb reverse-phase C_{18} semipreparative column (5-μm pore size, 21.4-mm i.d.; 25-cm length). Measurements of retention times and coinjections of synthetic dioic acids and diesters employed a Microsorb reverse-phase C_{18} analytical column (5-μm pore size; 4.6-mm i.d.; 25-cm length). ^{1}H NMR spectra were recorded on either a 300- or 500-MHz spectrometer. Chemical shifts for ^{1}H NMR are reported in parts per million relative to internal tetramethylsilane (δ = 0.0 ppm) when $CDCl_3$ was the solvent and relative to $HD_2C(O)CD_3$ (δ = 2.04 ppm) when acetone-d_6 was the solvent. ^{13}C NMR spectra were recorded at 75 or 125 MHz. Chemical shifts for ^{13}C NMR spectra are reported in parts per million relative to $CDCl_3$ (δ = 77.0 ppm) or $CD_3C(O)CD_3$ (δ = 29.8 ppm) in acetone-d_6. Rotating frame overhauser effect spectra were recorded in the phase-sensitive mode at 500 MHz and a controlled temperature (±0.1°C). A mixing time of 0.08 s was used, while the pulse delay was maintained at 2 s. A spectral window of about 4,000 Hz was used in both the f_1 and f_2 dimensions, and increments of 16 scans were collected. The synthetic procedures and identifying characteristics for each of the synthesized standards follow.

Dimethyl *E*-(phenylmethyl)butenedioate (compound 1b) and dimethyl *Z*-(phenylmethyl)butenedioate (compound 2b). A 2 N solution of benzyl magnesium chloride (12.0 mmol) in dry tetrahydrofuran was added dropwise over 10 min to a suspension of CuI (2.28 g, 12.0 mmol) in tetrahydrofuran (25 ml) kept at −40°C under Ar (15). The heterogeneous solution was stirred for 1 h at −40°C. Dimethyl acetylenedicarboxylate (1.42 g, 10.0 mmol) in tetrahydrofuran (5 ml) was subsequently added to the reaction mixture, which immediately turned dark red. After the mixture was stirred for 2 h at −40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 ml), the mixture was slowly warmed to room temperature, and the organic soluble products were extracted three times with ether (40 ml). The combined organic layers were washed with brine, dried, and concentrated to an oil which was purified first by radial chromatography (hexane) and then by reverse-phase HPLC. Dimethyl benzylfumarate (compound 1b) (0.73 g, 31%) and dimethyl benzylmaleate (compound 2b) (0.94 g, 40%) were obtained as oils. The characteristics of dimethyl benzylfumarate were as follows: ^{1}H NMR ($CDCl_3$) δ 7.30 to 7.05 (m, 5 H), 6.80 (s, 1 H), 4.13 (s, 2 H), 3.73 (s, 3 H), 3.66 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 166.9, 166.0, 145.9, 138.0, 128.8, 128.3, 126.7, 126.3, 52.5, 51.8, 32.9; MS *m/z* (relative intensity) EI 91 (12), 115 (92), 174 (35), 202 (100), 234 (14, M^+); electron impact high-resolution MS [HRMS (EI)] calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; combustion analysis calculated (Anal. Calcd) for $C_{13}H_{14}O_4$, C 66.66 and H 6.02, Found C 66.62 and H 6.03. The characteristics of dimethyl benzylmaleate were as follows: ^{1}H NMR ($CDCl_3$) δ 7.15 to 7.35 (m, 5 H), 5.66 (s, 1 H), 3.77 (s, 3 H), 3.70 (s, 3 H), 3.66 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 168.7, 165.4, 149.0, 135.5, 129.3, 128.7, 127.2, 121.1, 52.3, 51.8, 40.0; MS *m/z* (relative intensity) EI 91 (20), 115 (100), 174 (35), 202 (98), 234 (5, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0893; Anal. Calcd for $C_{13}H_{14}O_4$, C 66.66 and H 6.02, Found C 66.39 and H 6.03.

Dimethyl *E*-(phenylmethylene)butanedioate (compound 3b). Na metal (0.51 g, 22.0 mmol) was slowly added to CH_3OH (50 ml) maintained at 0°C under Ar (11). After complete disappearance of Na, dimethyl succinate (1.61 g, 11.0 mmol), immediately followed by benzaldehyde (1.06 g, 10 mmol), was added. The solution was warmed to room temperature, stirred for 1 h, and then heated at reflux for 2 h. After the reaction mixture was cooled to 0°C, a saturated NH_4Cl solution (25 ml) was added; this was immediately followed by acidification to pH 2 with the dropwise addition of HCl (1 N). The aqueous layer was then extracted three times with ether (50 ml), and the combined organic fractions were washed with brine, dried, and concentrated to an oil which was purified by radial chromatography (hexane). Pure dimethyl *E*-phenylitaconate (compound 3b) was obtained as a yellow oil (1.52 g, 65%): ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1 H), 7.40 to 7.45 (m, 5 H), 3.77 (s, 3 H), 3.66 (s, 3 H), 3.52 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ

171.4, 167.6, 141.9, 134.8, 128.9, 128.8, 128.5, 125.8, 52.1, 52.0, 33.3; MS *m/z* (relative intensity) EI 91 (25), 115 (95), 174 (67), 202 (82), 234 (100, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$, C 66.66 and H 6.02, found C 66.94 and H 6.09.

Dimethyl *Z*-(phenylmethylene)butanedioate (compound 4b). Dimethyl *E*-phenylitaconate (0.34 g, 1.45 mmol) was added to a solution of $CHCl_3$ (10 ml) and acetone (5 ml). After dissolved O_2 was removed by bubbling Ar through the solution for 20 min, the solution was irradiated (2) at 300 nm for 12 h at room temperature. Dimethyl *Z*-phenylitaconate was isolated as the single product of the reaction along with some unreacted material. Purification by radial chromatography (hexane) led to the isolation of dimethyl *Z*-phenylitaconate (compound 4b) (0.20 g, 60%); ^{1}H NMR ($CDCl_3$) δ 7.45 to 7.35 (m, 5 H), 6.88 (s, 1 H), 3.71 (s, 3 H), 3.63 (s, 3 H), 3.47 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 171.1, 168.1, 139.5, 135.4, 128.5, 128.2, 128.1, 128.0, 126.3, 52.0, 51.6, 40.6; MS *m/z* (relative intensity) EI 91 (23), 115 (100), 174 (72), 202 (83), 234 (97, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$, C 66.66 and H 6.02, Found C 66.49 and H 6.08.

Bis(trimethylsilyl)-2-butynedioate. Acetylene dicarboxylic acid (5.70 g, 50.0 mmol) was dissolved (10, 16) under Ar in 33 ml of a tetrahydrofuran-hexane solution (1:2, vol/vol) and slowly added to a solution of hexamethyldisilazane (5.40 g, 33.3 mmol) dissolved in hexane (25 ml). The mixture was stirred for 15 min and then filtered. Pure bistrimethylsilyl acetylenedicarboxylate was obtained as a yellow oil after removal of the solvents (8.97 g, 97%): ^{1}H NMR ($CDCl_3$) δ 0.24 (s); ^{13}C NMR ($CDCl_3$) δ 150.6, 74.8, −0.8.

***E*-(Phenylmethyl)butenedioic acid (compound 1a).** Dimethyl benzylfumarate (compound 1b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). The deprotection was quenched after 12 h of stirring at room temperature by acidification with 5 ml of aqueous HCl (1 N). After extraction of the organic soluble dioic acid three times with ether (10 ml), the combined ether fractions were washed with brine and dried. Pure benzylfumaric acid (compound 1a) was obtained as an oil (0.20 g, 97%) after removal of the solvents: ^{1}H NMR (CD_3COCD_3) δ 7.31 (d, J = 10 Hz, 2 H), 7.24 (dd, J = 10, 10 Hz, 2 H), 7.14 (dd, J = 10, 10 Hz, 1 H), 6.85 (s, 1 H), 4.17 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 167.9, 167.1, 146.7, 139.4, 129.6, 129.0, 127.8, 127.0, 33.2; MS *m/z* (relative intensity) fast atom bombardment (FAB) 205 (100, $M - H^+$), 161 (40), 117 (4), 91 (0.5); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 ($M - H^+$), found 205.04959.

***Z*-(Phenylmethyl)butenedioic acid (compound 2a).** A solution of 2 N benzyl magnesium chloride (11.5 mmol) in dry tetrahydrofuran was added dropwise over 10 min to a suspension of CuI (2.20 g, 11.5 mmol) in tetrahydrofuran (40 ml) maintained at −40°C under Ar (15). The heterogeneous solution was stirred for 1 h at −40°C. Bistrimethylsilyl acetylenedicarboxylate (2.48 g, 9.61 mmol) in tetrahydrofuran (10 ml) was subsequently added to the reaction mixture, which immediately turned dark red. After the mixture was stirred for 2 h at −40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 ml) immediately followed by acidification to pH 2 with dropwise addition of HCl (1 N). The heterogeneous solution was then slowly warmed to room temperature, and the organic soluble products were extracted three times with ether (40 ml). Benzylmaleic acid (compound 2a) was the only dioic acid observable in the crude extract after the combined organic layers were washed with brine, dried, and concentrated. Pure benzylmaleic acid was obtained by reverse-phase HPLC purification as an oil (0.79 g, 40%): ^{1}H NMR (CD_3COCD_3) δ 7.22 to 7.35 (m, 5 H), 5.84 (s, 1 H), 3.71 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 169.5, 166.9, 149.6, 137.5, 130.1, 129.4, 127.6, 122.7, 40.8; MS *m/z* (relative intensity) FAB 205 (100, $M - H^+$), 161 (25), 117 (2.5), 91 (2); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 ($M - H^+$), found 205.05080.

***E*-(Phenylmethylene)butanedioic acid (compound 3a).** Dimethyl *E*-phenylitaconate (compound 3b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). After 15 min of stirring at room temperature, the deprotection was quenched by acidification to pH 2 with 1 N aqueous HCl. The organic soluble dioic acid was immediately extracted three times with ether, and the organic layer was then washed with brine and dried. After removal of the solvents, *E*-phenylitaconic acid (compound 3a) was crystallized from $CHCl_3$ as a white solid (0.19 g, 93%): ^{1}H NMR (CD_3COCD_3) δ 7.92 (s, 1 H), 7.35 to 7.55 (m, 5 H), 3.56 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 172.4, 168.8, 141.8, 136.1, 129.9, 129.7, 129.5, 127.8, 33.8; MS *m/z* (relative intensity) FAB 205 (100, $M - H^+$), 161 (43), 117 (6), 91 (5); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 ($M - H^+$), found 205.05037.

***Z*-(Phenylmethylene)butanedioic acid (compound 4a).** Dimethyl *Z*-phenylitaconate (compound 4b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). The deprotection was quenched after 12 h of stirring at room temperature by acidification with 5 ml of aqueous HCl (1 N). After extraction of the organic soluble dioic acid three times with ether (10 ml), the combined ether fractions were washed with brine and dried. Pure *Z*-phenylitaconic acid (compound 4a) was obtained as an oil (0.18 g, 88%) after removal of the solvents: ^{1}H NMR (CD_3COCD_3) δ 7.4 (d, J = 10 Hz, 2 H), 7.20 to 7.34 (m, 3 H), 6.89 (s, 1 H), 3.44 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 173.0, 169.6, 138.5, 136.7, 129.6, 128.6, 41.5; MS *m/z* (relative intensity) FAB 205 (35, $M - H^+$), 161 (17), 117 (9), 91 (1); HRMS (FAB) calculated for $C_{11}H_9O_4$, 205.05008 ($M - H^+$), found 205.05091. Another method (13) to synthesize *Z*-phenylitaconic acid (compound 4a) from *E*-phenylitaconic acid (compound 3a) was used to confirm the structural assignment of compound 4a.

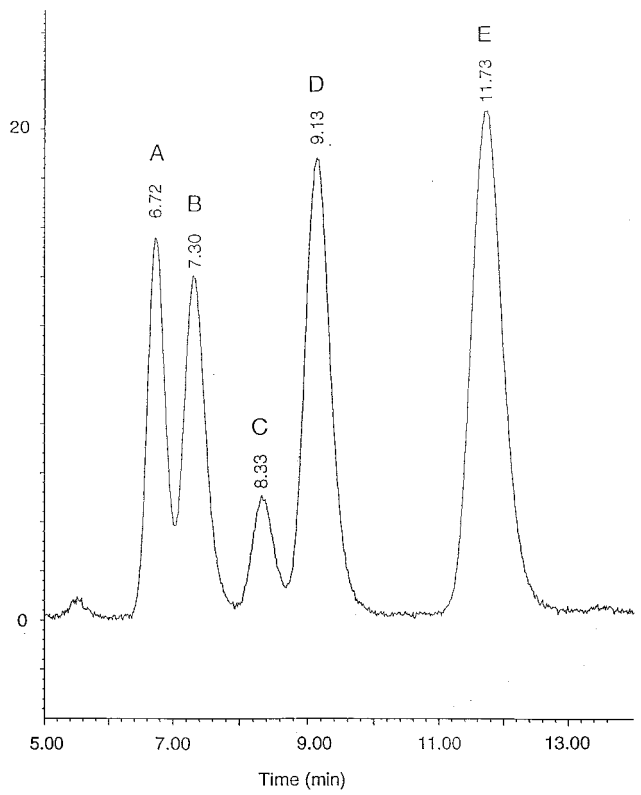

FIG. 2. HPLC analysis and retention times (minutes) of a mixture containing chemically synthesized *Z*-phenylitaconic acid (A), benzylmaleic acid (B), benzylfumaric acid (C), benzylsuccinic acid (D), and *E*-phenylitaconic acid (E). Samples were analyzed on a reverse-phase C_{18} analytical column with UV detection at 218 nm and an isocratic eluting solvent composed of 60% phosphate buffer (0.1% H_3PO_4 in water) and 40% methanol.

E-Phenylitaconic acid (0.21 g, 1 mmol) was dissolved in 10 ml of an acetone-H_2O solution (1:1, vol/vol) containing 1.1 equivalents of Na_2HCO_3. Dissolved O_2 was removed by bubbling Ar through the solution for 20 min, after which the solution was irradiated for 12 h. The reaction mixture was extracted three times with ether (15 ml), and the combined ether fractions were washed with brine, dried, and concentrated. *Z*-Phenylitaconic acid (0.12 g, 60%) was separated from *E*-phenylitaconic acid (0.08 g, 40%) by reverse-phase HPLC.

RESULTS AND DISCUSSION

^{13}C labeling experiments. Anaerobic degradation of [*methyl*-^{13}C]toluene (Fig. 2) by strain Tol-4 provided the first clues relevant to the identity of the second metabolite. The ^{13}C-labeled unknown metabolite was isolated by HPLC with a C_{18} reverse-phase column and then analyzed by ^{1}H NMR. If the metabolite was benzylfumaric acid (compound 1a), a methylene carbon would be ^{13}C labeled. The ^{1}H NMR resonance of the protons (H_A; Fig. 1) attached to the ^{13}C-labeled methylene carbon would then be split into a large doublet relative to the same resonance in a metabolite derived from unlabeled toluene. Rather surprisingly, the ^{1}H NMR resonance displaying the expected splitting caused by ^{13}C labeling occurred at a frequency well downfield of what could be assigned to a methylene proton. This was not consistent with the degradation product being either benzylfumaric acid (compound 1a) or benzylmaleic acid (compound 2a). Such a chemical shift was, however, consistent with ^{13}C labeling of a vinyl carbon (H_B; Fig. 1) of *E*-phenylitaconic acid (compound 3a) or *Z*-phenylitaconic acid (compound 4a). Additional evidence was needed to confirm that the unknown metabolite was a phenylmethyl-

enebutanedioic acid (compound 3a or 4a) and to differentiate between an *E* or *Z* olefin substitution pattern. This necessitated the synthesis and detailed spectroscopic characterization of each phenylmethylbutenedioic acid (compounds 1a and 2a) and phenylmethylenebutanedioic acid (compounds 3a and 4a).

Derivatization and EIMS Analysis. Our original strategy for identifying the second metabolite formed during anaerobic degradation of toluene by *A. tolulyticus* Tol-4 was based on the previously reported derivatization (8) of the metabolites to form dimethyl diesters. These esterified components were then to be analyzed by GC according to retention times and coinjection with synthesized samples. GC interfaced with EIMS was to provide further avenues for analysis with interpretation and correlation of fragmentation patterns. Synthetic dimethyl benzylfumarate (compound 1b), dimethyl benzylmaleate (compound 2b), dimethyl *E*-phenylitaconate (compound 3b), and dimethyl *Z*-phenylitaconate (compound 4b) were separated by GC with baseline resolution.

However, there remained the potential problem of deceptive GC analyses. Double-bond isomerizations were so problematic during attempted hydrolysis of dimethyl benzylmaleate (compound 2b) that an independent route to benzylmaleic acid (compound 2a) had to be developed. Similar isomerizations might occur during derivatization of the dioic acids under acidic conditions. This possibility prompted examination of the previously employed derivatization methods (8), which included treatment with methanol (MeOH)-H_2SO_4-H_2O (2:1:1, vol/vol), MeOH-H_2SO_4 (1:1, vol/vol), and MeOH in the presence of BCl_3.

Treatment of benzylfumaric acid (compound 1a), benzylmaleic acid (compound 2a), and *E*-phenylitaconic acid (compound 3a) at 50°C for 20 min with MeOH-H_2SO_4-H_2O (2:1:1, vol/vol) led to the exclusive formation of dimethyl diesters 1b, 2b, and 3b with no apparent double-bond migration or isomerization. However, treatment of *Z*-phenylitaconic acid (compound 4a) under these same reaction conditions resulted in only partial derivatization, with no dimethyl *Z*-phenylitaconate (compound 4b) observable by GC. Similar results were observed when dioic acids 1a, 2a, 3a, and 4a were treated at 50°C for 20 min with MeOH-H_2SO_4 (1:1, vol/vol). Overall, reaction of the dioic acids 1a, 2a, 3a, and 4a with BCl_3 at 50°C for 20 min proved to be the most useful derivatization protocol. Each of the four dioic acids was dimethylated without any detectable double-bond migration or isomerization.

The next concern was the EIMS analysis of derivatized metabolites. A key argument in previous work (8) was the presence of a tropylium ion ($C_7H_7^+$) at *m/z* 91, which was interpreted as being indicative of the benzyl substituent in dimethyl benzylfumarate (compound 1b) and dimethyl benzylmaleate (compound 2b). The tropylium ion's presence seemed inconsistent with the absence of a benzyl substituent in dimethyl *E*- and *Z*-phenylitaconate (compounds 3b and 4b). The synthesis of dimethyl diesters 1b, 2b, 3b, and 4b provided an opportunity to study MS fragmentation patterns in detail. A fragment at *m/z* 91 consistent with a tropylium ion was observed in the MS for dimethyl benzylfumarate (compound 1b) and dimethyl benzylmaleate (compound 2b). However, a similarly intense fragment at *m/z* 91 was also observed for dimethyl *E*-phenylitaconate (compound 3b) and *Z*-phenylitaconate (compound 4b). In fact, the EIMS fragmentation patterns of compounds 1b, 2b, 3b, and 4b were essentially identical. Ionization evidently was introducing enough energy into these systems for double-bond migration and isomerization to occur.

Identification of the second metabolite. Fortunately, dioic acids 1a, 2a, 3a, and 4a were separable with nearly baseline resolution by HPLC with a C_{18} reverse-phase column (Fig. 2).

TABLE 1. ^{1}H NMR chemical shift values

Proton	Chemical shift (ppm) in compound:			
	1a	2a	3a	4a
H_b (vinyl)	6.85	5.84	7.92	6.89
H_a (methylene)	4.17	3.71	3.56	3.44

The HPLC retention times and coinjection with synthesized dioic acids along with high-field NMR analysis provided the independent lines of evidence needed for the identification of toluene degradation products. By circumventing the previously employed metabolite derivatizations, HPLC and NMR provided the appealing option of direct analysis of the solution matrix attendant with anaerobic microbial degradation of toluene.

The ^{1}H NMR chemical shifts (Table 1) of the vinyl and methylene proton resonances were generally the most useful for identification of dioic acid and diester structures. Critical supporting evidence for the ^{1}H NMR and ^{13}C NMR assignments followed from use of rotating frame overhauser effect spectroscopy. The observed NOEs are summarized in Table 2. Measured NOEs attendant with irradiation of vinyl protons (H_B) were the most diagnostic.

With synthesized samples of the dioic acids on hand, multiple lines of spectroscopic evidence for the assigned dioic acid structures, and baseline HPLC resolution of the dioic acids, attention turned to the unknown metabolite formed by *A. tolulyticus* Tol-4. The isolated quantities of the metabolite were adequate for ^{13}C NMR, ^{1}H NMR, and assignment of NOE correlations. Measured retention times in addition to coinjection with synthetic samples of compounds 1a, 2a, 3a, and 4a by using an HPLC fitted with a C_{18} reverse-phase column provided the necessary confirming data. On the basis of this information, the second metabolite formed along with benzylsuccinic acid during anaerobic degradation of toluene by *A. tolulyticus* Tol-4 is *E*-phenylitaconic acid (compound 3a). These two metabolites were not produced during aerobic toluene degradation.

Previous reports of formation of benzylfumaric acid (compound 1a) (8, 18) need to be reconciled with the results of this

TABLE 2. NOE intensities

Compound and proton	NOE intensity[a] of proton:		
	H_A (methylene)	H_B (vinyl)	H_C (aromatic)
1a			
H_A		w	m
H_B	w		a
H_C	a	a	
2a			
H_A		s	m
H_B	s		a
H_C	a	a	
3a			
H_A		a	m
H_B	a		m
H_C	m	m	
4a			
H_A		m	a
H_B	s		m
H_C	a	m	

[a] s, strong; m, medium; w, weak; a, absent.

study. The sample derivatization techniques used in those earlier studies are not likely to be problematic. In our hands, incomplete dimethyl diester formation was observed when some of the previously employed derivatization conditions were used, although double-bond migration and isomerization were not observed. Reliance on MS analysis for structure identification is, however, best avoided. The identical MS spectra obtained for synthesized samples of compounds 1b, 2b, 3b, and 4b clearly indicate that MS characterization is of limited utility in identifying the structures of these dioic acids. Resolution of the microbial products of anaerobic toluene metabolism in other organisms should now be straightforward, since all products can be resolved by HPLC analysis. Examination of isolated dioic acids by ^{1}H NMR and use of NOE experiments can take advantage of the chemical shift values and NOE correlations reported in this account.

Dioic acid metabolism and biosynthesis. No degradation of benzylfumaric acid, benzylmaleic acid, *E*-phenylitaconic acid, or *Z*-phenylitaconic acid could be detected even after a 1-week incubation of these dioic acids with strain Tol-4. All four dioic acids were stable in culture fluids and during acidification and extraction on the basis of comparison with the HPLC retention times of chemically synthesized dioic acids. A unique feature of benzylmaleic acid was its complete inhibition of toluene metabolism when added to the culture fluid of strain Tol-4. Toluene metabolism was not discernibly affected when strain Tol-4 was cultured in the presence of the other three dioic acids.

Strain Tol-4 converted 1 to 2% of the toluene carbon to *E*-phenylitaconic acid and benzylsuccinic acid in a 10:1 ratio. This differs markedly from the case for strain T1, which was reported to convert up to 17% of the toluene carbon to benzylsuccinic acid and benzylfumaric acid (8). A 0.5% conversion of toluene into these same dioic acids has been observed for strains K172 and T (18). Seven other strains of *A. tolulyticus* with the ability to degrade toluene anaerobically (9, 19) also synthesized similar amounts of a product identified by its HPLC retention time as *E*-phenylitaconic acid during toluene metabolism (data not shown).

Although strain Tol-4 was unable to metabolize benzylsuccinic acid added to its culture medium, benzylsuccinic acid could still be an intermediate during *E*-phenylitaconic acid biosynthesis. *E*-Phenylitaconic acid is always generated by strain Tol-4 in a sizable excess relative to benzylsuccinic acid, which is consistent with oxidation of an intermediate pool of benzylsuccinic acid to *E*-phenylitaconic acid. The inability of strain Tol-4 to metabolize extracellular benzylsuccinic acid may be due to cellular uptake limitations. In addition, the coenzyme A (CoA) derivatives of the dioic acids postulated for the toluene pathway would likely be derived from the conjugation of toluene with acetyl-CoA (5) or even succinyl-CoA. Strain Tol-4 may lack the ligase activity necessary for conversion of free dioic acids into the CoA derivatives required for metabolism.

The formation of *E*-phenylitaconic acid (compound 3a) may be an important clue for defining the pathway of anaerobic toluene metabolism. The outline of a modified microbial route based on this finding is provided in the accompanying paper (5). While strain Tol-4 produces *E*-phenylitaconic acid, it is not clear whether the strains studied in other laboratories produce this product or benzylfumaric acid. Resolving this question could help define whether an altered pathway also occurs.

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