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Synthesis of 3-Arylmuconolactones Using Biphenyl Metabolism in *Aspergillus*

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Abstract: Strains of the fungus *Aspergillus* form 3-arylmuconolactones as secondary products of the metabolism of biphenyl and substituted biphenyls. The 3-arylmuconolactones are not found to support growth and can accumulate to significant concentrations. The metabolic pathway leading from hydroxylated biphenyls to lactones appears to proceed through 3,4-catechol intermediates.

INTRODUCTION

The fungal metabolism of biphenyl has been studied as a model of mammalian metabolism,[1] and as a synthetic tool for producing hydroxylated chemical intermediates.[2,3,4] Biphenyl is initially mono-hydroxylated by fungi. The hydroxylation favors the 4-position, but the 2- and 3-positions are also hydroxylated by some fungal species.[2,5,6] Further conversion results in dihydroxylated products. In *Aspergillus parasiticus*, hydroxylation of biphenyl is exclusively *para* and the hydroxylation readily proceeds to 4,4'-dihydroxybiphenyl as the predominant product.[6] The regiospecific hydroxylation by *A. parasiticus* extends to substituted biphenyls, providing an attractive synthetic route to a variety of phenolic chemicals.[7,8]

Biphenyl metabolism by *A. parasiticus* was reported to yield the sulfate ester conjugates (*O*-sulfonic acids) of 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl, apparently as secondary metabolites from the hydroxylated products.[9] By contrast, the metabolism of biphenyl by *Cunninghamella elegans* leads to the formation of both sulfate and glucuronate conjugates.[5] Fungal metabolism of 4-hydroxybenzoic acid and 4-methylcatechol are known to yield 3-carboxymuconolactone and 3-methylmuconolactone, respectively, as intermediate products.[10,11]

In this paper we describe the formation of 3-arylmuconolactones as secondary products of the metabolism of biphenyl and substituted biphenyls by *A. parasiticus* and other *Aspergillus* species. Unlike the 3-carboxy- and 3-methylmuconolactones, the 3-arylmuconolactones do not appear to support growth and can accumulate to significant concentrations in liquid cultures. The only other reported synthesis of 3-arylmuconolactones yields the product along with the 2-aryl isomer.[12] The 3-arylmuconolactones could be of interest as members of the larger class of unsaturated γ-lactones, a number of which possess biological activity.[13]

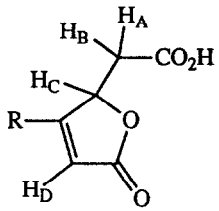
RESULTS AND DISCUSSION

3-(4'-hydroxyphenyl)muconolactone from biphenyl

We have been conducting studies to maximize the yield of hydroxylated products from the fungal conversion of biphenyls. As part of this work, it was important to understand the further metabolism, if any, of the hydroxylated products. By incubating cultures of *A. parasiticus* ATCC 15517 with 4,4'-dihydroxybiphenyl, we were able to confirm the formation of the mono- and di-sulfate esters as post-hydroxylation products. However, our standard analysis (HPLC with UV detection at 254 nm) was unable to account for all of the starting 4,4'-dihydroxybiphenyl. In related experiments, we had screened a number of *Aspergillus nidulans* strains for biphenyl hydroxylation activity. While none of these strains had significant

hydroxylation activity, four *A. nidulans* strains converted 4,4'-dihydroxybiphenyl to products not detected by our standard analysis. These observations led us to look for other secondary metabolites of biphenyl.

HPLC analysis with UV detection over a range of wavelengths revealed a product of 4,4'-dihydroxybiphenyl metabolism (Compound I) with an absorbance maximum of 293 nm in acid and 355 nm in base. Extended incubations of *A. parasiticus* ATCC 15517 cultures with biphenyl and 4,4'-dihydroxybiphenyl yielded sufficient amounts of Compound I for spectral characterization. The proton-proton spin coupling of Compound I is very similar to the ^{1}H-NMR spectra reported for 3-*tert*-butylmuconolactone[14] and 3-carboxymuconolactone,[15] leading to the identification of Compound I as structure **3** shown below. High resolution mass spectral analysis of Compound I yielded a molecular weight of 234 and a cracking pattern showing loss of CO_2 and CH_2COOH, which is consistent with structure **3**. Treatment of Compound I with methanol/HCl yielded the expected methyl ester.



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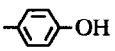
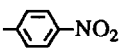
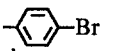
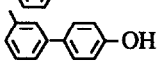
2, R = -$C(CH_3)_3$

3, R = 

4, R = 

5, R = 

6, R = 

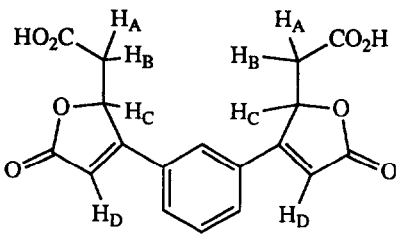


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Table 1. Comparison of ^{1}H NMR Spectra of 3-Substituted Muconolactones

	1[a]	2[b]	3	4	5	6	7
δ_A	2.79	2.54	2.48	2.62	2.32	2.59	2.62
J_{AB}	16.8	16.0	17.0	16.8	15.9	17	17
J_{AC}	8.1	9.2	9.8	8.9	9.3	9	9
δ_B	3.24	3.08	3.05	3.07	2.84	3.10	3.09
J_{AB}	16.8	16.0	17.0	16.8	15.9	17	17
J_{BC}	3.3	3.2	2.5	2.9	3.0	3	3
δ_C	5.59	5.46	5.93	6.12	6.01	6.11	6.10
J_{AC}	8.0	9.2	9.8	8.9	9.3	9	9
J_{BC}	3.3	3.2	2.5	2.9	2.9	3	3.3
J_{CD}	2.2	1.7	1.5	1.7	1.6	1.5	1.8
δ_D	NR	5.91	6.33	6.76	6.48	6.63	6.69
J_{CD}	NR	1.7	1.5	1.7	1.5	1.8	1.8

[a] Data from Kirby, et al. (Ref. 15)
[b] Data from Rogic, et al. (Ref. 14)
δ in ppm; J in Hz; NR = not reported

Lactones from substituted biphenyls

A. parasiticus hydroxylates a variety of substituted biphenyls.[7,8] After the initial identification of the lactone from biphenyl, we have found the corresponding 3-arylmuconolactones as metabolites of

A. parasiticus incubated with 4-bromobiphenyl, 4-nitrobiphenyl, and *m*-terphenyl. Table 1 shows the similarity among ^{1}H NMR spectra of the 3-arylmuconolactone products reported here and the 3-substituted muconolactones reported previously. *m*-Terphenyl is hydroxylated to form 4,4"-dihydroxy-*m*-terphenyl, which in turn yields both a monolactone (**6**) and a bislactone (**7**) from lactonization of one or both of the pendent phenyl rings, respectively.

To our knowledge, this is the first report of 3-arylmuconolactones as metabolic products. The analogous 3-carboxymuconolactone is an intermediate in the fungal metabolism of 4-hydroxybenzoic acid,[10] and 3-methylmuconolactone is formed by the fungal metabolism of 4-methylcatechol.[11] Both 3-carboxymuconolactone and 3-methylmuconolactone are further degraded by fungi to feed into the growth-supporting 3-ketoadipate metabolic pathway. In contrast, we have found no evidence that the 3-arylmuconolactones are intermediates that support growth. In minimal medium cultures of *A. parasiticus*, 4,4'-dihydroxybiphenyl did not support growth as the sole carbon source. This was true even when the lactone-forming and 3-ketoadipate metabolic systems were induced by prior growth of the culture on 4-hydroxybenzoic acid as the sole carbon source.

m-Terphenyl has been a convenient substrate for following the formation and fate of 3-arylmuconolactones, since both the monolactone and bislactone, as well as the hydroxylated and sulfate ester products, are easily quantified by our standard HPLC analysis. The ratio of lactones to total products is strongly influenced by culture conditions. In particular, when *m*-terphenyl is converted in stirred cultures of *A. parasiticus* grown on a corn steep liquor medium and provided with a steady supply of carbon source to extend the period of conversion, the lactones can become the predominant product. For example, after 84 hours of conversion, an experiment of this type yielded a total product concentration of 11.3 mM with the following mole percent composition: 5% 4-hydroxy-*m*-terphenyl, 14% 4,4"-dihydroxy-*m*-terphenyl, 10% 4,4"-dihydroxy-*m*-terphenyl sulfate, 44% monolactone (**6**), and 27% bislactone (**7**). Together with the unconverted substrate, essentially all (98%) of the added *m*-terphenyl was accounted for. This was a typical mass balance for these experiments. The mass balance and the absence of further metabolites, even under conditions producing high levels of lactones, is evidence that the lactones are dead-end products in the metabolism of substituted biphenyls.

Stereochemistry

The fungal metabolism leading to 3-carboxymuconolactone[15] and 3-methylmuconolactone[16] have been shown to be stereospecific. Thus, we would expect the 3-arylmuconolactones from *Aspergillus* to be stereochemically pure. Prior to recrystallization from methanol, the bislactone from *m*-terphenyl was found to be a 50:50 mixture of compounds with very similar HPLC retention times and identical UV spectra. The ^{1}H NMR of this mixture differed from that of the pure material from methanol in that most of the resonances were replaced by a double set of peaks. When purified material was briefly exposed to the acidic conditions used in isolation of this compound, a mixture containing both of the closely eluting compounds resulted. We believe the conditions of isolation scramble the stereochemistry, so that the isolated mixture contained both the *meso* and racemic diastereomers of the bislactone from *m*-terphenyl. We cannot therefore comment on the stereochemical purity of the metabolic products.

Catechol intermediates and metabolic pathway

The production of 3-carboxymuconolactone from 4-hydroxybenzoic acid proceeds through protochatechuic acid (3,4-dihydroxybenzoic acid) as the initial product.[10] If the pathway for formation of the 3-arylmuconolactones is similar, the 3,4-catechols of the corresponding substituted biphenyls are expected intermediates. 3,4-catechols were never found in high concentrations under our culture conditions, but we have isolated 3,4-dihydroxy-4'-nitrobiphenyl as an intermediate in the conversion of 4-nitrobiphenyl by *A. parasiticus*.

Subsequently, we produced two catechols of *m*-terphenyl (3,4,4"-trihydroxy-*m*-terphenyl and 3,4,3",4"-tetrahydroxy-*m*-terphenyl) by enzymatic hydroxylation of 4,4"-dihydroxy-*m*-terphenyl using tyrosinase from *Neurospora crassa*. Careful analysis of *A. parasiticus* cultures actively hydroxylating *m*-terphenyl, using HPLC with a diode array detector, revealed a small peak at the correct retention time and with a UV spectrum identical to that of the enzymatically synthesized 3,4,4"-trihydroxy-*m*-terphenyl. We found no evidence, however, of the 3,4,3",4"-tetrahydroxy-*m*-terphenyl in the cultures. In the cultures, the 3,4,4"-trihydroxy-*m*-terphenyl appears subsequent to the appearance of 4,4"-dihydroxy-*m*-terphenyl and disappears by the time that lactones from *m*-terphenyl become prominent products. This is the pattern expected for an intermediate in the pathway between the diol and the lactones.

Biphenyl and *m*-terphenyl each present two phenyl rings for metabolic attack by Aspergillus. With such substrates, we have never detected the 3-arylmuconolactone that would be formed after hydroxylation of just one of the phenyl rings. This suggests that each available phenyl must be *para*-hydroxylated before proceeding to lactone formation.

Comparison of Aspergillus strains

Most of these studies were conducted with *A. parasiticus* ATCC 15517 and an aflatoxin-minus mutant derived from it, *A. parasiticus* ATCC 74022. There was no significant difference in 3-arylmuconolactone formation between these strains. Two other *A. parasiticus* strains that are mutants blocked in the aflatoxin synthesis pathway, ATCC 24690 and ATCC 56774, formed the lactones (**6** and **7**) from the conversion of *m*-terphenyl. We have also observed the formation of **6** and **7** from 4,4"-dihydroxy-*m*-terphenyl by *Aspergillus* sp. NRRL A-13647 and *A. tamarii* NRRL 3280, indicating that the ability to form 3-arylmuconolactones may be widely distributed among fungal species.

EXPERIMENTAL

Fungal strains

A. parasiticus ATCC strains 15517, 24690, and 56774 were obtained from the American Type Culture Collection, Rockville, MD. Aflatoxin-deficient strain *A. parasiticus* ATCC 74022, formerly designated JS 1-89, was generated from ATCC 15517 as previously described.[8] *Aspergillus* sp. NRRL A-13647 and *A. tamarii* NRRL 3280 were obtained from the Midwest Area National Center for Agricultural Utilization Research, Peoria, IL.

Culture media and conditions

Aspergillus strains were grown to sporulation on Sabouraud Dextrose Agar (Difco) plates. Spores were harvested into an aqueous solution of 1% NaCl, 0.1% Triton X-100, and 20% glycerol and stored at -80 °C at a density of about 10^8 spores/mL.

Flask cultures containing filamentous, pipettable mycelia were grown by inoculating 1 L of sterile Sabouraud Dextrose Broth (Difco; 20 g/L dextrose, 10 g/L Neopeptone) with 1 mL of spore suspension and incubating at room temperature with vigorous stirring on a stir plate. When the glucose in the medium was 50% depleted (determined by Sigma diagnostic kit 115-A), the flask was shake incubated at 200 rpm and 30 °C until the glucose was depleted (about 3 days from inoculation). For conversion of the biphenyl substrates, two methods were used. In the first, 50 mL of the grown culture was transferred to a sterile 250 mL flask, the substrate was added and the flask was shake incubated at 30 °C. In the second, 250 mL of the flask-grown culture was transferred to a sterile 2 L roller bottle, the substrate was added, and the bottle was incubated on a roller with air sparging into the air-space. In both methods, the substrate was added as a concentrated (typically 100 mg/mL) solution in DMF while swirling vigorously to produce a finely dispersed precipitate.

A variety of conditions and media have been used in stirred fermentor conversions of biphenyl substrates by *A. parasiticus*. These different methods and media consistently result in the same products, though at different rates and yields. The following is a representative procedure. Starter culture was prepared by inoculating 100 mL of Sabouraud Dextrose Broth in a 500 mL trypsinizing flask with 10^7 - 10^8 spores and shake incubating at 37 °C, 200 rpm for 24 hours. Stirred fermentor culture was prepared by inoculating 15 L of sterile corn syrup/corn steep liquor medium (22 g/L Karo light corn syrup and 43 g/L Argo Steepwater E801 (both from CPC International)) in a Braun Biostat E fermentor with the starter culture and growing at 37 °C with air sparging at 2 to 5 standard liters per minute. After 24 hours of growth, the substrate (biphenyl or substituted biphenyl), Triton X-100 (nonionic surfactant; Union Carbide) and 50 mg/L 4,4'-dihydroxybiphenyl were added. During the substrate conversion phase, corn syrup was added at a rate up to 0.4 g/L/hr, but staying below the rate that would lead to accumulation of glucose (as measured with Diastix glucose strips; Miles, Inc.). More substrate was added as necessary to prevent substrate depletion.

To test for growth on 4,4'-dihydroxybiphenyl, *A. nidulans* minimal medium[17] (50 mL per 250 mL Trypsinizing flask) containing 1 g/L 4,4'-dihydroxybiphenyl instead of glucose as the sole carbon source was inoculated with *A. parasiticus* ATCC 74022 and shake incubated at 150 rpm and 35 °C. Inocula were: 1) 10^6 spores/mL medium, 2) 200 µL homogenized culture grown on the same minimal medium, but with 10 g/L glucose as the carbon source, and 3) 200 µL homogenized culture grown on the same minimal medium, but with 1 g/L 4-hydroxybenzoic acid (added as a concentrated sodium salt solution, pH 6.5) as the sole carbon source. In no case was there evidence of growth on the 4,4'-dihydroxybiphenyl.

Analytical HPLC

For routine analysis, culture samples were diluted with methanol, centrifuged to remove cell debris, and analyzed by high pressure liquid chromatography (HPLC) using a C_{18} column and a solvent gradient from water to acetonitrile, each containing 0.06% acetic acid, and UV detection at 254 nm.

Synthesis, isolation and characterization

4,4"-dihydroxy-m-terphenyl. A stirred fermentor culture of *A. parasiticus* ATCC 15517 was given a total of 1.0 g/L *m*-terphenyl, added as the gelatin dispersion described earlier,[7] and 3 g/L surfactant. After 90 hours of conversion a portion of the culture was diluted with four volumes of methanol and filtered. Most of the methanol was removed by evaporation and the residue basified to pH 12.5 with 50% NaOH. This was continuously extracted with ether overnight to remove most of the surfactant. The aqueous phase was acidified to pH 5 with acetic acid and continuously extracted overnight with ether again. This ether extract was dried with $MgSO_4$, evaporated to dryness and blown with N_2 to remove volatile biological material. The residue was dissolved in chloroform and separated by silica gel chromatography using a gradient from chloroform to 10% ethyl acetate in chloroform. The residue from evaporation of the 10% ethyl acetate fraction was taken up in ether, decolorized with charcoal, and recrystallized from toluene and then from ethanol/water. 200 MHz ^1H NMR (CD_3OD) δ: 6.87 (m, 4H; 3, 3", 5, 5"), 7.40 (m, 3H; 4', 5', 6'), 7.47 (m, 4H; 2, 2", 6, 6"), 7.67 (broad s, 1H, 2'); ^{13}C NMR (CD_3OD): 116.6 (3, 3", 5, 5"), 125.6 (2', 4', 6'), 129.2 (2, 2", 6, 6"), 130.1 (5'), 134.0 (1, 1"), 142.9 (1', 3'), 158.2 (4, 4"); MS (FD): M^+= 262. The NMR spectra, HPLC retention times and UV spectra of this material were indistinguishable from that of 4,4"-dihydroxy-*m*-terphenyl synthesized chemically by the method of Muellar and Honaker.[18]

3,4,4"-trihydroxy-m-terphenyl and 3,4,3",4"-tetrahydroxy-m-terphenyl. These compounds were synthesized from 4,4"-dihydroxy-*m*-terphenyl using a tyrosinase isolated from *Neurospora crassa* that is known to catalyze the *o*-hydroxylation of phenols and aromatic amines.[19,20] The tyrosinase was isolated by the procedure of Lerch[21] except that tyrosinase was precipitated with ammonium sulfate directly from the suspension of ground mycelia in phosphate buffer and the 30-60% ammonium sulfate precipitate was resuspended in phosphate buffer and dialyzed against phosphate buffer containing 30 % glycerol before storing at -20 °C. 12.5 µmole 4,4"-dihydroxy-*m*-terphenyl (from 10 mM solution in methanol) was reacted at 30 °C in

5 mL of 50 mM sodium phosphate buffer (pH 7.2) containing 75 µmole ascorbic acid and 115 units tyrosinase (1 unit oxidizes 1 µmole L-DOPA per minute at 30 °C). After 1 hour, >60% of the substrate had been converted. Reaction products were extracted with ether, dried, derivatized with bis(trimethylsilyl)–trifluoroacetamide (BSTFA) and analyzed by GC/MS. The mass spectra confirmed that the products were trihydroxy-*m*-terphenyl (m/z: 494 (M^+, tri(trimethyl)silylated), 479 (-CH_3), 406 (-$Si(CH_3)_4$), 391 (-$Si(CH_3)_4$ -CH_3)) and tetrahydroxy-*m*-terphenyl (m/z: 582 (M^+, tetra(trimethyl)silylated), 494 (-$Si(CH_3)_4$), 406 (-2$Si(CH_3)_4$)) with adjacent hydroxyl groups, since the loss of $Si(CH_3)_4$) is characteristic of 1,2-bis-trimethylsiloxy derivatives. HPLC analysis (solvent gradient: water containing 0.1% trifluoroacetic acid (TFA) to acetonitrile with 0.1% TFA) with UV detection by diode array yielded UV spectra consistent with 3,4,4"-trihydroxy-*m*-terphenyl and 3,4,3",4"-tetrahydroxy-*m*-terphenyl when compared to that of 4,4"-dihydroxy-*m*-terphenyl (λ_{max} = 264 nm for all three compounds, with broadening of the band for the triol and a second maximum at 296 nm for the tetraol).

3-(4'-hydroxyphenyl)muconolactone (3). This product was initially detected by HPLC (with UV detection over a range of wavelengths) in standard shake flask cultures of *A. parasiticus* ATCC 15517 incubated with 500-1000 mg/L biphenyl and 50 mg/L 4,4'-dihydroxybiphenyl (as inducer[9]). HPLC fractions containing Compound I were analyzed by high resolution mass spectrometry (m/z: 234 (M^+, $C_{12}H_{10}O_5$), 190 ($C_{11}H_{10}O_3$), 175 ($C_{10}H_7O_3$), 147 ($C_9H_7O_2$), 119(C_8H_7O), 118 (C_8H_6O)). To isolate more material, two liters of similar culture were incubated for five days after biphenyl depletion to maximize secondary product formation. The mycelial mass was removed by filtration, the filtrate was centrifuged to remove a fine precipitate, and the resulting supernatant was concentrated by rotary evaporation to 300 mL. An equal volume of acetonitrile was added, followed by acidification (to pH 1-2) with HCl, and the mixture was refluxed for one hour to hydrolyze any sulfate esters. The acetonitrile was removed by rotary evaporation and the pH adjusted to 5.0. A solid residue was removed by centrifugation and the resulting liquid was extracted with chloroform to remove lipid materials which otherwise produced a gel upon ether extraction. The aqueous phase was reacidified with HCl and extracted with ether. The ether was evaporated and the resulting solid was washed with ice water. The isolated solid was further purified by preparative HPLC by dissolving in methanol/water and running on a Waters 19 mm X 150 mm µBondapak C_{18} column. The solvents were water and acetonitrile containing 0.1% trifluoroacetic acid, the flow rate was 8.0 mL/min, and the gradient was 15 to 25% acetonitrile phase over 12 minutes, hold for 2 minutes, 25 to 50% acetonitrile over 4 minutes and hold for 2 minutes. Collected fractions were dried and characterized. IR (film): ~3200 (broad, COOH), 1730 (C=O), 1610 (C=C-C=O), 1175 cm^{-1} (C-O); 200 MHz ^1H NMR (CD_3OD) δ: 2.48 (dd, J_{AB} = 17.0, J_{AC} = 9.8 Hz, 1H, methylene), 3.05 (dd, J_{AB} = 17.0, J_{BC} = 2.5 Hz, 1H, methylene), 5.93 (ddd, J_{AC} = 9.8, J_{BC} = 2.5, J_{CD} = 1.5 Hz, 1H, allylic), 6.33 (d, J_{CD} = 1.5 Hz, 1H, alkene), 6.99 (m, 2H, *m*-AR-H), 7.59 (m, 2H, *o*-Ar-H); ^{13}C NMR (CD_3OD): 40.4 (5), 80.6 (4), 111.7 (2), 117.2 (3', 5'), 122.1 (1'), 130.6 (2', 6'), 162.3 (4'), 169.6 (3), 173.2 (1), 175.5 (6), where primes indicate the hydroxyphenyl moiety, unprimed positions are in the lactone moiety. The methyl ester was obtained by heating in methanol/HCl: ^1H NMR (CD_3OD) δ: 2.53 (dd, J_{AB} = 16.4, J_{AC} = 8.8 Hz, 1H, methylene), 3.07 (dd, J_{AB} = 16.5, J_{BC} = 2.5 Hz, 1H, methylene), 5.96 (m, 1H, allylic), 6.30 (1H, alkene), 6.91 (m, 2H, *m*-AR-H), 7.51 (m, 2H, *o*-Ar-H); MS (FD): M^+ = 248.

3-(4'-nitrophenyl)muconolactone (4). 250 mL of *A. parasiticus* ATCC 15517 culture were incubated with 1 g/L 4-nitrobiphenyl and 50 mg/L 4,4'-dihydroxybiphenyl using the roller bottle method. After acidification with HCl (pH 1-2) the whole culture was extracted with ether, yielding 900 mL extract which was dried with $MgSO_4$ and evaporated. The residue was dissolved in methanol and separated by preparative HPLC; column: Waters 7.8 mm X 300 mm µBondapak C_{18}, solvents: water and acetonitrile; program: 0 to 60% acetonitrile over 20 min., to 80% acetonitrile in 5 min., flow rate: 4 mL/min. The fraction containing 3-(4'-nitrophenyl)muconolactone was recrystallized from methanol. 200 MHz ^1H NMR (CD_3OD) δ: 2.62 (dd, J_{AB} = 16.8, J_{AC} = 8.9 Hz, 1H, methylene), 3.07 (dd, J_{AB} = 16.8, J_{BC} = 2.9 Hz, 1H, methylene), 6.12 (ddd, J_{AC} = 8.9, J_{BC} = 2.9, J_{CD} = 1.7 Hz, 1H, allylic), 6.76 (d, J_{CD} = 1.7 Hz, 1H, alkene), 8.02 (m, 2H, *m*-AR-H), 8.38 (m, 2H, *o*-Ar-H); MS (FD): M^+ = 263; MS (EI): 219 (M^+- 44, CO_2), 176, 149, 115, 102, 76, 44.

3,4-dihydroxy-4'-nitrobiphenyl. This compound was found in another fraction from the preparative HPLC purification of 3-(4'-nitrophenyl)muconolactone. 200 MHz ^{1}H NMR (CD_3OD) δ: 6.98 (d, J = 8.6 Hz, 1H, 6), 7.17 (dd, J = 8.3 and 2.5 Hz, 1H, 5), 7.27 (d, J = 2 Hz, 2), 7.85 (m, 2H, 3' and 5'), 8.27 (m, 2H, 2' and 6'), 8.85 and 9.67(broad, -OH); MS (FD): M^+ = 231. Formation of the BSTFA derivative with analysis by GC/MS (30 m DB1 FSWCOT column, 100-290 °C at 12 °C/min; m/z: 375 (M^+), 287 (- $Si(CH_3)_4$)) and formation of the phenylboronic acid derivative[22] with analysis by GC/MS (15 m DB1 column, 150-290 °C at 15 °C/min; m/z: 317(M^+)) were both consistent with dihydroxynitrobiphenyl with adjacent hydroxyl groups.

3-(4'-bromophenyl)muconolactone (5). A stirred fermentor culture of *A. parasiticus* ATCC 74022 was given 4 g/l Triton X-100 and a total of 6 g/L 4-bromobiphenyl. After 90 hours of conversion, 1 L of culture was filtered. The filtrate was acidified to pH 2 with HCl and extracted twice with 100 mL ethyl acetate. The extract was evaporated to about 15 mL and extracted twice with 1 M $NaHCO_3$. The aqueous extract was brought to pH 5.5 with acetic acid and back-extracted with ethyl acetate. After evaporation, the ethyl acetate extract yielded 170 mg of a glassy solid. 300 MHz ^{1}H NMR (CD_3OD) δ: 2.32 (dd, J_{AB} = 15.9, J_{AC} = 9.3 Hz, 1H, methylene), 2.84 (dd, J_{AB} = 15.9, J_{BC} = 3.0 Hz, 1H, methylene), 6.01 (ddd, J_{AC} = 9.3, J_{BC} = 2.9, J_{CD} = 1.6 Hz, 1H, allylic), 6.48 (d, J_{CD} = 1.5 Hz, 1H, alkene), 7.58 (m, 2H, *m*-AR-H), 7.67 (m, 2H, *o*-Ar-H).

3-arylmuconolactones from m-terphenyl. A stirred fermentor culture of *A. parasiticus* ATCC 15517 was given a total of 2.1 g/L *m*-terphenyl, added as the gelatin dispersion described earlier,[7] and 5 g/L surfactant. After 270 hours of conversion, two liters of the culture were filtered and the solids were washed with one liter of 1 M $NaHCO_3$ and refiltered. The filtrates were combined to give about 2.6 L at pH 8-9, which was washed three times with 1.2 L ether, acidified to pH 5.6-5.8 with acetic acid, and extracted three times with ether (2 L total) to recover one of the metabolites. The aqueous layer was washed with 1 L ethyl acetate, acidified to pH 1 with HCl and extracted with ethyl acetate twice (2L total) to recover the second metabolite. Salt was added to both extracts and they were allowed to settle overnight under refrigeration. The organic layers were each decanted, dried with magnesium sulfate, and evaporated to dryness under vacuum.

Monolactone from m-terphenyl (6). The ether extract residue was triturated with ether to give a pale yellow powder (700 mg) and crystallized from toluene/ethyl acetate. 200 MHz ^{1}H NMR (acetone-d_6) δ: 2.59 (dd, J_{AB} = 17, J_{AC} = 9 Hz, 1H, methylene), 3.10 (dd, J_{AB} = 17, J_{BC} = 3 Hz, 1H, methylene), 6.11 (ddd, J_{AC} = 9, J_{BC} = 3, J_{CD} = 1.5 Hz, 1H, allylic), 6.63 (d, J_{CD} = 1.8 Hz, 1H, alkene), 6.96 (m, 2H; 3", 5"), 7.6 (m, 4H; 5', 6', 2", 6"), 7.77 (m, 1H, 4'), 7.92 (m, 1H, 2'); ^{13}C NMR (CD_3OD): 39.63 (5), 80.69 (4), 115.48 (2), 116.83 (3", 5"), 126.47 (2',4'), 129.29 (2", 6"), 130.61 & 130.81 (5', 6'), 131.56 (1"), 132.46 (3'), 143.7 (1'), 158.86 (4"), 169.67 (3), 172.95 (1), 174.89 (6); where double primes refer to the hydroxyphenyl moiety, primes refer to the central phenyl, and unprimed positions are in the muconolactone moiety; MS (FD): 310 (M^+).

Bislactone from m-terphenyl (7). The ethyl acetate extract residue was fractionated on a 160 g C18 column using a step gradient from 0.5% acetic acid/15% acetonitrile/water to 0.5% acetic acid/22.5% acetonitrile/water. The 20-22.5% acetonitrile fractions were extracted with ethyl acetate. The extract was dried with $MgSO_4$ and evaporated to yield a residue (750 mg) of yellow glassy solid. Crystallization from methanol gave a white powder. 200 MHz ^{1}H NMR (acetone-d_6) δ: 2.62 (dd, J_{AB} = 17, J_{AC} = 9 Hz, 2H, methylene), 3.09 (dd, J_{AB} = 17, J_{BC} = 3 Hz, 2H, methylene), 6.095 (ddd, J_{AC} = 9, J_{BC} = 3.3, J_{CD} = 1.8 Hz, 2H, allylic), 6.69 (d, J_{CD} = 1.8 Hz, 2H, alkene), 7.72 (m, 1H, 5'), 7.88 (m, 2H; 4', 6'), 8.11 (t, 1H, 2'); ^{13}C NMR (DMSO-d_6) 38.1 (5), 78.34 (4), 115.89 (2), 126.64 (2', 5'), 130.05 (4', 6'), 130.49 (1', 3'), 166.07 (3), 170.50 (1), 171.95 (6), where primes refer to central phenyl and unprimed positions are in the muconolactone moiety; MS (FD): 359 ($M+H^+$), with small peaks at 381 ($M+Na^+$) and 397 ($M+K^+$). The ^{1}H NMR spectum of the yellow glassy solid before crystallization from methanol showed "doubling" of several of the peaks relative to the spectrum of the cystallized material. Thus at 2.62 and 3.10 there are two quartets each, at 6.10 there is a multiplet with at least fourteen peaks, the doublet at 6.69 is accompanied by another at 6.65, the multiplet at 7.9 has more peaks, and an apparent quartet is found at 8.1. The additional peaks are all equal in intensity to the peaks found in the

spectrum of the crystallized material, indicating the presence of approximately equal amounts of *meso* and racemic diastereomers.

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