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Medium-Scale Preparation of Useful Metabolites of Aromatic Compounds via Whole-Cell Fermentation with Recombinant Organisms

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Abstract:

The whole-cell fermentation of aromatic coumpounds with *Escherichia coli* JM109 (pDTG601) on a medium scale (10−15 L) produces enantiopure cyclohexadienediols. A detailed procedure for the fermentation is described, and yields for several metabolites are provided. A similar procedure using *E. coli* JM109 (pDTG602) affords catechols. The dienediols are useful for asymmetric synthesis, and several important targets originating from these metabolites are tabulated.

Introduction

More than 35 years ago Gibson et al. described the isolation and structure determination of an optically active diol derived from the biooxidation of an aromatic compound with a blocked mutant of *Pseudomonas putida* Pp F1.[1] Despite this and other reports from his laboratory, the organic synthesis community waited 20 years before the first application of these compounds to synthesis appeared in the literature in the form of the polyphenylene process by ICI[2] and the synthesis of racemic pinitol by Ley.[3] Our group pioneered this field in the U.S. by the disclosure in 1988 of the conversion of toluene to a prostaglandin synthon in just three steps,[4] Figure 1.

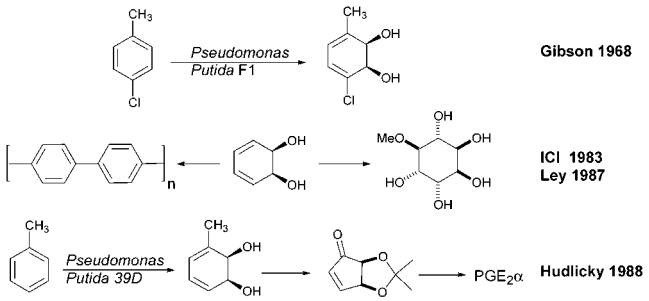

Figure 1. **Historically important isolation and applications of arene *cis*-diols in synthesis.**

The past decade witnessed an almost explosive growth of applications of these metabolites to asymmetric synthesis—

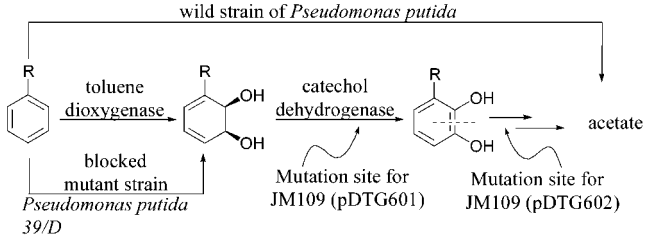

Figure 2. **Natural degradation pathway of aromatics by eukaryotic organisms and mutation points for specific enzyme expression.**

the accomplishments from our group as well as those from several others have been amply reviewed as recently as 1999.[5] Many of the metabolites as well as some of the synthetically useful intermediates derived from them have become catalog items (see Table 1).

Because of the increased interest in these compounds, we published an *Organic Syntheses*[6] procedure for the small-scale (shake-flask) preparation of diols by fermentation with the blocked mutant *P. putida* 39D. The use of this organism requires induction with a known substrate, usually toluene or chlorobenzene. This is an obvious disadvantage when different substrates are used as the resulting diols require separation from the diol derived from the inducer. As Gibson has subsequently developed organisms in which the enzyme expression is initiated by a nonaromatic promoter on the plasmid,[7] it is more convenient to prepare the metabolites by whole-cell fermentation with recombinant *Escherichia coli* organisms in which the protein synthesis is induced by isopropylthiogalactose (IPTG). These recombinant organisms contain the plasmid for expression of either the aromatic dioxygenases [toluene (TDO), naphthalene (NDO), or bi-

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Table 1. **List of diol metabolites and intermediates derived from them, available from Aldrich catalog (prices are from 2001 edition)**

Aldrich catalog number	diol metabolites	price
48,949-2	(1*S*-*cis*)-3-bromo-3,5-cyclohexadiene-1,2-diol, 96%	1 g $35.00; 5 g $139.00
48,950-6	(1*S*-*cis*)-3-chloro-3,5-cyclohexadiene-1,2-diol, 98%	1 g $31.00; 5 g $130.00
48,963−-	(1*S*-*cis*)-3-phenyl-3,5-cyclohexadiene-1,2-diol, 98%	1 g $76.00
49,032-6	(1*R*-*cis*)-1,2-dihydro-1,2-naphthalenediol, 98%	1 g $76.00
	Synthetically useful intermediates prepared from diols	
49,035-0	[3a*S*-(3aα,4α,5α,7aα]-7-bromo-3a,4,5,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-4,5-diol, 99%	500 mg $135.00
49,038-5	[3a*S*-(3aα,4α,5α,7aα]-]-3a,4,5,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-4,5-diol, 98%	500 mg $180.00
49,085-7	[3a*S*-(3aα,5aα,6aα,6bα]-4-bromo-3a,5a,6a,6b-tetrahydro-2,2-dimethyloxireno[*e*]−1,3-benzodioxole-4,5-diol, 98%	500 mg $160.00
49,088-1	[3a*R*-(3aα,5a*β*,6a*β*,6bα]-3a,5a,6a,6b-tetrahydro-2,2-dimethyloxireno[*e*]−1,3-benzodioxole, 98%	500 mg $185.00
49,340-6	[3a*S*-(3aα,4α,5*β*,7aα]-5-azido-7-bromo-3a,4,5,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxol-4-ol, 99%	500 mg $220.00
49,388-0	(3a*S*,7*R*,7a*S*)-7,7a-dihydro-7-hydroxy-2,2-dimethyl-1,3-dibenzodioxol-4(3a*H*)-one, 98%	500 mg $130.00
49,389-9	(3a*R*,7*R*,7a*S*)-7-(carbobenzyloxyamino)-7,7a-dihydro-2,2-dimethyl-1,3-dibenzodioxol-4(3a*H*)-one, 98%	500 mg $135.00
49,390-2	(3a*R*,4*S*,7*R*,7a*S*)-7-(carbobenzyloxyamino)-3a,4,7,7a-tetrahydo-2,2-dimethyl-1,3-benzodioxol-4-ol, 98%	500 mg $130.00
49,391-0	(3a*R*,4*S*,7*R*,7a*S*)-3a,4,7,7a-tetrahydro-7-(methoxycarbonylamino)-2,2-dimethyl-1,3-benzodioxol-4-ol-4-acetate, 98%	500 mg $130.00

phenyl (BDO)] or the dioxygenases and the corresponding catechol dehydrogenases that convert either the diols or aromatics to the corresponding catechols. The mutations of the natural degradation pathway are shown in Figure 2.

The availability of the organisms for the fermentation now offers the opportunity to prepare both homochiral diols and the corresponding catechols, some of which are very difficult to prepare in traditional ways.[8] This contribution reports the details of medium-scale synthesis of some of the more common metabolites in the aromatic series.

Results and Discussions

The *Organic Syntheses* procedure for the use of *P. putida* 39D is straightforward and can be adapted for practice in a laboratory not equipped for microbiology. It is useful for small (<3 g) amounts of self-inducing substrates (toluene, benzene. and chloro- and bromobenzene). By contrast, the use of recombinant organisms requires more sophistication and a relatively costly fermentor. However, the cost and sophistication of these requirements is offset by the yields of metabolites and the ease of handling the *E. coli* strains. Table 2 lists many of the diols and Table 3 lists many of the catechols that have been prepared by this procedure with yields and references to the isolation and characterization of each. The direct preparation of catechols in one step represents an important "green" alternative to the sometime arduous and lengthy chemical synthesis.[8,17]

A detailed description of the 10- and 15-L fermentation follows, including the handling of metabolites. For a complete listing of all known metabolites consult ref 5a.

Conclusions

The medium-scale fermentation of aromatics with recombinant organisms expressing toluene dioxygenase has been described in detail. These fermentations yield, in some cases, 100-g quantities of metabolites, enantiopure *cis*-cyclohexadiene diols. A listing of important applications of these compounds is provided in Table 4 divided into asymmetric and nonasymmetric synthetic applications. We hope that this disclosure, together with a detailed procedure, will further stimulate the growth of this fascinating area.

Experimental Section

General Overview of Fermentation. The whole-cell dihydroxylation of aromatic compounds is a three-day process. On day one the cell cultures and fermentor are

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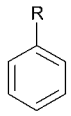
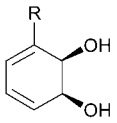
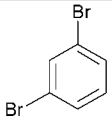
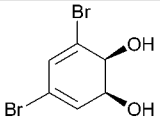
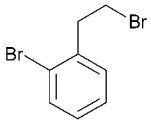
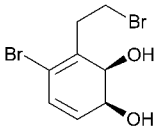
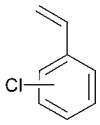
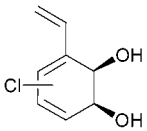
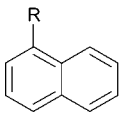
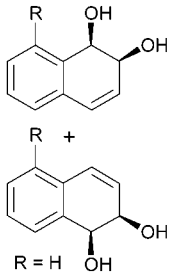
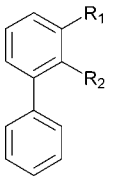
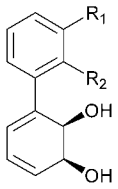
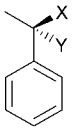
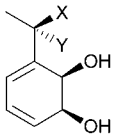
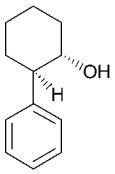
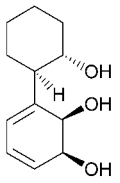
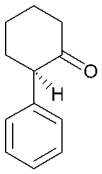
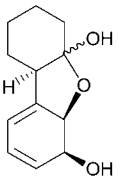
Table 2. **Survey of useful diol metabolites prepared by fermentation**

Substrate	Product from *E. coli* JM109 (pDTG601)	10-15L Fermentation yield (g)	Ref.
			
R=Br	R=Br	90–120	1, 9
Cl	Cl	90–110	1,10
I	I	70–90	1, 9
Me	Me	120–150	4,10,11
Ph	Ph	20-50	12
OPh	OPh	30–50	13
$CH=CH_2$	$CH=CH_2$	60-80	4a
CH=CHBr	CH=CHBr	10–25	14
CH_2CH_2Br	CH_2CH_2Br	60-90	15
CH_2CH_2CN	CH_2CH_2CN	60–90	16
$CH_2CH_2N_3$	$CH_2CH_2N_3$	30–50	16
CH_2CH_2OAc	CH_2CH_2OAc	30-50	16
		17-22	17
		27-35	17
		7-12	17
		30-35	4
		35-40	18
		1.2-2	19
		5-8	20

Substrate	Product from *E. coli* JM109 (pDTG601)	10-15L Fermentation yield (g)	Ref.
			
R = H	R = H	90-100	21
Br	Br	15-20	22
			
$R_1=R_2=H$	$R_1=R_2=H$	25– 30	23
$R_1=H$, $R_2=OMe$,	$R_1=H$, $R_2=OMe$,	20–25	23
$R_1=OMe$, $R_2=OMe$	$R_1=OMe$, $R_2=OMe$	5-9	23
			
X = H, Y = OH	X = H, Y = OH	9-12	17
X = OH, Y = H	X = OH, Y = H	9-12	17
		9-15	17
		9-16	17

prepared. Preparation of the cell cultures involves making the necessary solutions and sterilizing all solutions, glassware, and spatulas in an autoclave. Flasks containing solutions are capped with gauze. All other glassware and spatulas are capped or wrapped with aluminum foil in preparation for autoclaving. After sterilization the flasks are allowed to cool before being inoculated with the cells and being placed on an orbital shaker. The fermentor is made ready by adding water and the necessary materials for the culture medium then sterilizing at 120 °C for 20 min.

On day two the pH of the medium in the fermentor is adjusted to 6.8, and preculture solutions are transferred to

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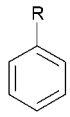
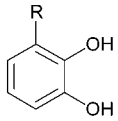
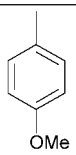
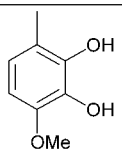
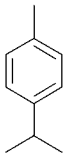
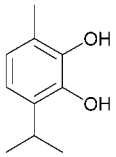
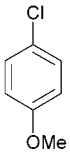
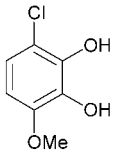
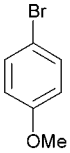
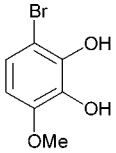
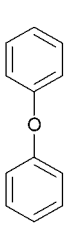
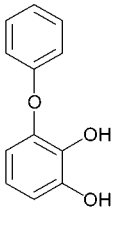
Table 3. **Survey of useful catechol metabolites prepared by fermentation**

Substrate	Product from *E. coli* JM109 (pDTG602)	10-15L Fermentation yield (g)	Ref.
			
R = Br	R= Br	9-11	8
Cl	Cl	10-13	8
I	I	6-8	8
Me	Me	20-25	8
OMe	OMe	9-10	8
		1-1.2	8
		8-9	24
		10-11	24
		5-7	24
		4-5	24
		5-6	24
		4-5	24
		1-1.4	24

the fermentor. The cells are allowed to grow while being fed glucose. The amount of dissolved oxygen is monitored to ensure proper conditions for growth of the cells. When the amount of cells is sufficient, IPTG (isopropyl β-D-thiogalactopyranoside) is added to induce the production of toluene dioxygenase.

On day three the substrate is added, and the transformation is monitored. When the reaction is complete, the fermentation

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(45) Hudlicky, T.; Boros, C. H. *Tetrahedron Lett.* **1993**, *34*, 2557.
(46) Frey, D. A.; Duan, C.; Hudlicky, T. *Org. Lett.* **1999**, *1*, 2085.
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(49) (a) Davies, I. W.; Senanayake, C. H.; Castonguay, L.; Larsen, R. D.; Verhoeven, T. R.; Reider, P. J. *Tetrahedron Lett.* **1995**, *36*, 7619. (b) Davies, I. W.; Reider P. J. *Chem. Ind. (London)* **1996**, 412.
(50) Johnson, C. R.; Ple, P. A.; Adams, J. P. *J. Chem. Soc., Chem. Commun.* **1991**, 1006.
(51) Johnson, C. R.; Adams, J. P.; Collins, M. A. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1.
(52) (a) Mandel, M.; Hudlicky, T. *J. Chem. Soc., Perkin Trans. 1* **1993**, *13*, 1537. (b) Hudlicky, T.; Restrepo-Sanchez, N.; Kary, P. D.; Jaramillo-Gomez, L. M. *Carbohydr. Res.* **2000**, *324*, 200.
(53) (a) Ensley, B. D.; Ratzkin, B. J.; Osslund, T. D.; Simon, M. J.; Wackett, L. P.; Gibson D. T. *Science* **1983**, *222*, 167. (b)Whited, G. Presented at Biotrans '95, University of Warwick, 1995.

Table 4. **Use of *cis*-dienediols in synthesis**

A. Asymmetric syntheses

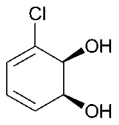
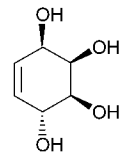
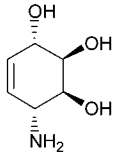
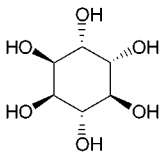
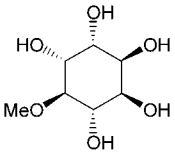
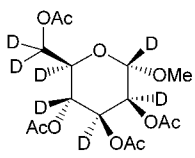
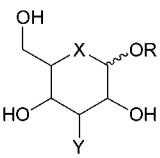
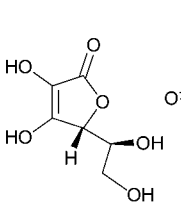
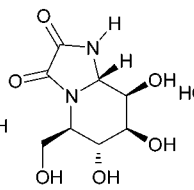
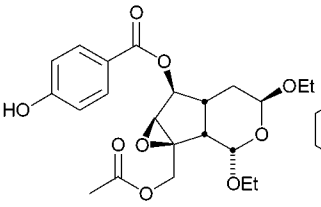
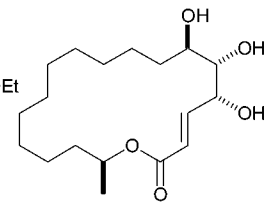
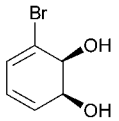
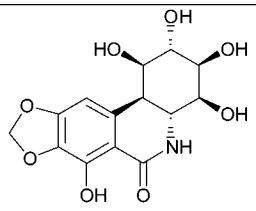
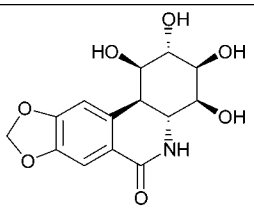
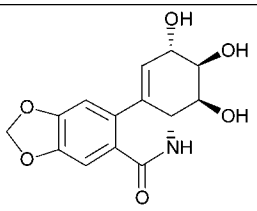
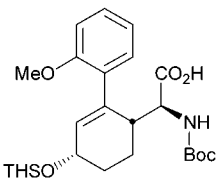
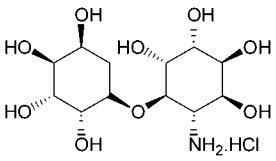
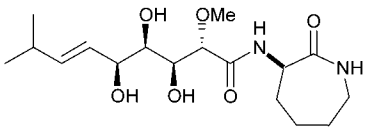
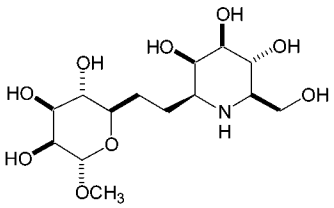
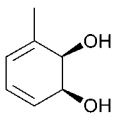
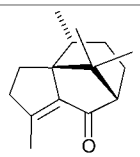
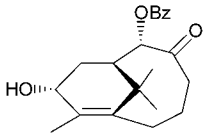
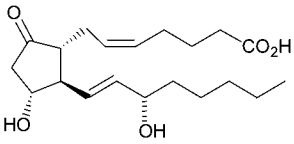
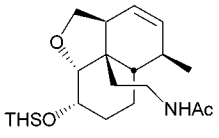
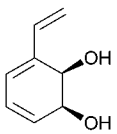
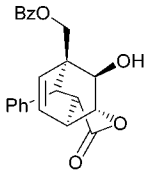
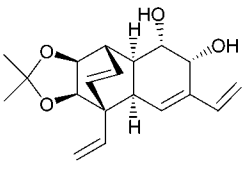
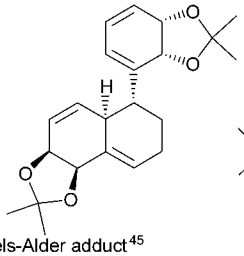
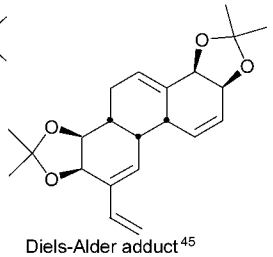
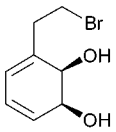
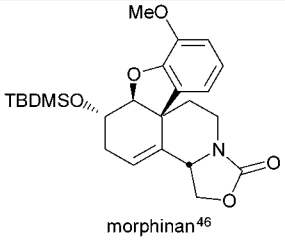
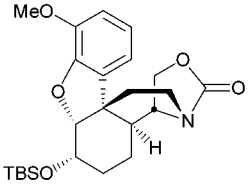
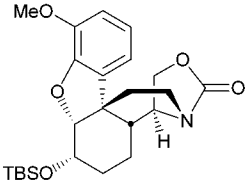
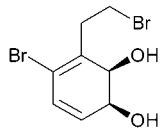
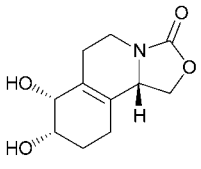
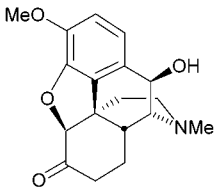
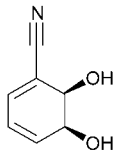
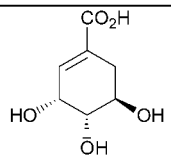
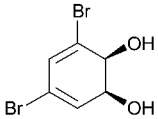
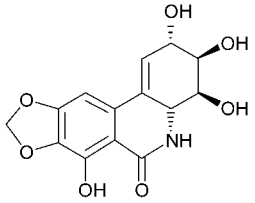
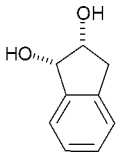
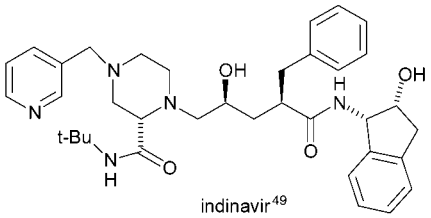
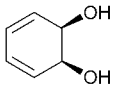
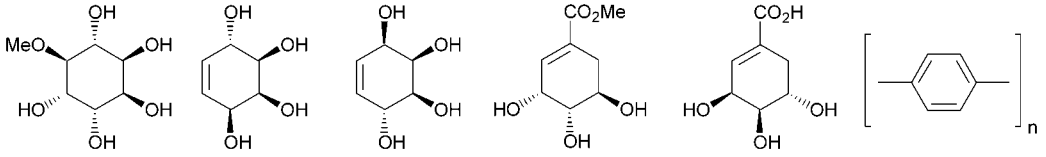
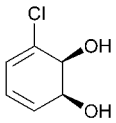
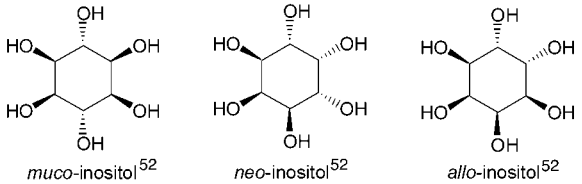
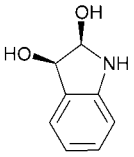
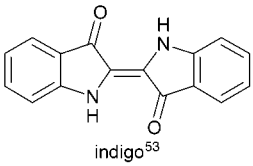
Diols	Product
	 conduritol C[25]
	 conduramine A-1[26]
	 D-*chiro*-inositol[27]
	 (-)-pinitol[25,28]
	 deuterated hexose[29]
	 X = CH_2, NH, O Y = NH, OH hexoses[5e] aminohexoses[5e] azahexoses[5e] carbasugar[5e]
	 L-ascorbic acid[30]
	 kifunensine[31]
	 specionin acetate[32]
	 (+)-aspicilin[33]
	 pancratistatin[34]
	 7-deoxypancratistatin[34b,35]
	 lycoricidine[36]
	 morphinan[37]
	 amino-inositol dimer[38]
	 *ent*-Bengamide E[39]
	 aza-C-disaccharide[40]
	 (-)-patchoulenone[41]
	 *ent*-taxoids AB-ring system[42]
	 $PGE_{2\alpha}$[4]
	 morphinan[43]
	 zeylena[44]
	 Diels-Alder adduct[45]
	 Diels-Alder adduct[45]
	 Diels-Alder adduct[45]
	 morphinan[46]
	 morphinan[47]
	 morphinan[47]

Table 4. (Continued)

A. Asymmetric syntheses

Diols	Product
	 morphinan[47b]  *ent*-morphinan[47b]
	 shikimic acid[48]
	 narciclasine[19a]
	 indinavir[49]

B. Non-asymmetric syntheses[a]

Diols	Product
	 (±)-pinitol[3] (+)-conduritol C[50] (−)-conduritol C[50] (−)-methyl shikimate[51] (+)-methyl shikimate[51] polyphenylene[2]
	 *muco*-inositol[52] *neo*-inositol[52] *allo*-inositol[52]
	 indigo[53]

[a] Syntheses in which either the asymmetry of the arene *cis*-diol has been destroyed or, if present in the product, was introduced by other means to a *meso*-diol.

broth is harvested and centrifuged, and then the supernatant is extracted with ethyl acetate. Removal of solvent from the combined extracts provides relatively pure diol, which can be further purified by recrystallization. The residue of cell material is autoclaved before disposal.

Medium-Scale Fermentation with *E. coli* JM 109 (pDTG601)

Plate Preparation. Agar plates consist of bactotryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1}), agar (15 g L^{-1}), and ampicillin (200 mg L^{-1}). Previously thawed *E. coli* JM 109 (pDTG601)[8] cells in cryovials are streaked onto a plate. The streaked plate is incubated at 35 °C for 2 d. Single-cell colonies, which have a dark tinge, are chosen for the preparation of preculture.

Preculture Preparation and Inoculation. A mineral salts broth (MSB, 600 mL) containing K_2HPO_4 (9.6 g), KH_2PO_4 (8.4 g), $(NH_4)_2SO_4$ (3 g), yeast extract (9 g), glucose (18 g), and $MgSO_4 \cdot 7\ H_2O$ (1.2 g) is divided into two 2.8-L Fernbach flasks and sterilized. After the broth is cooled to room temperature, ampicillin (60 mg in 6 mL of autoclaved water) is added, and each flask is inoculated with a single colony of *E. coli* JM 109 (pDTG601) from a fully grown plate. These preculture flasks are placed in an orbital shaker (35 °C, 150 rpm) for 12 h.

Sterilization of Fermentor. The production medium in the fermentor consists of an aqueous solution of KH_2PO_4 (60 g), citric acid (16 g), $MgSO_4 \cdot 7H_2O$ (40 g), trace metal solution [16 mL: Na_2SO_4 (1 g L^{-1}), $MnSO_4$ (2 g L^{-1}), $ZnCl_2$ (2 g L^{-1}), $CoCl_2 \cdot 6H_2O$ (2 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.3 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (10 g L^{-1}), pH 1.0], concentrated H_2SO_4 (9.6 mL), and ferric ammonium citrate (9.6 mL, 270 g L^{-1}). The fermentor containing these ingredients and approximately 8 L of water is sterilized at 120 °C for 20 min. The fermentor is allowed to cool to 35 °C, and air is passed through the fermentor at a flow rate of 3 L/min. The stirrer is set at 500 rpm while the pH is gradually adjusted to 6.8 by means of automatic addition of ammonium hydroxide. Once the desired pH is reached, thiamine hydrochloride (2.69 g in 8 mL of autoclaved water) and ampicillin (800 mg in 8 mL of autoclaved water) are added to the fermentor.

Transfer of Precultures to Fermentor. On day two, the pre-cultures are transferred to the fermentor, and stirring is reduced to 300 rpm. A sample is taken from the fermentor immediately after adding the precultures to serve as a blank for monitoring the increase in optical density (OD) of the fermentor medium. UV absorbance at 640 nm (1 to 100 dilution) is measured at 2 h intervals after transfer. During this time, the dissolved oxygen content gradually decreases until it reaches a minimum and then sharply increases, which usually occurs 4 h after the addition of precultures. Glucose (720 g L^{-1}) is then introduced to the medium. The rate of glucose addition is increased exponentially as the cells multiply, and the rate is critical to the growth of the bacteria. The optimum rate of glucose addition is determined by the amount of dissolved oxygen in the medium (measured by oxygen electrode) and rate of stirring. As glucose is added and the cells grow and divide, more oxygen is consumed. The amount of dissolved oxygen is maintained at a constant level (optimum at 20%) by increasing the rate of stirring. Hence, as the OD of the medium increases with time, which means the density of bacteria increases, the rate of glucose addition is also increased. Correspondingly, as glucose is metabolized by the bacteria, the amount of dissolved oxygen decreases, and to maintain a steady amount of dissolved oxygen, stirring is increased. The rate of addition of glucose and stirring rate must be balanced to maintain optimum conditions for bacterial growth. If glucose is added too rapidly oxygen consumption will be too large and no reasonable amount of stirring could adequately compensate. Also in this case, the addition of glucose is not optimum because the cells are subjected to excess glucose. Likewise, if glucose is added too slowly the bacteria will divide too slowly. When the turbidity or OD of the medium has reached 15 times that of the blank as measured by UV absorbance, IPTG (80 mg) is added to induce the production of toluene dioxygenase.

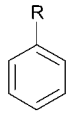
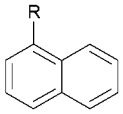
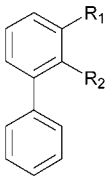
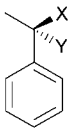
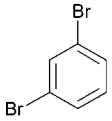
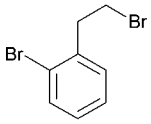
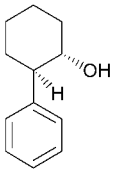
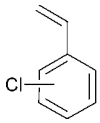
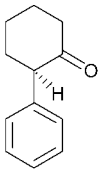
Feeding of Substrate. On day three, the pH of the fermentor medium is adjusted to 7.0 prior to the addition of substrate. A sample is taken before the aromatic substrate is introduced. This sample gives the value of the OD for this particular fermentation batch. Also, this sample serves as the blank for the measurement of absorbance of diol chromophore in the UV−vis region (usually between 265 and 275 nm). The rate of feeding of the substrate mainly depends on the type of substrate. In the case of bromobenzene the rate is 35 g h^{-1}. If the substrate is added too rapidly cells begin to die. When this occurs foam begins to form. Samples are taken every 30 min to assess if there is an increase in the amount of diol produced over time. When the UV absorbance due to the diol levels off, substrate addition is stopped, and the broth that contains the diol metabolite is harvested. In the case of bromobenzene, the amount of substrate added ranges from 90 to 120 g or about 3 h of biotransformation. The rates of addition for other substrates are listed in Table 5.

Harvesting of Culture and Metabolite. The pH of the medium in the fermentor is adjusted to 7.6 after the biotransformation. The broth is centrifuged at 7000 rpm and 5 °C for 20 min. The supernatant liquid is decanted and saved for extraction; the residue of cell material is collected and autoclaved at 120 °C for 20 min prior to disposal.

Isolation of Metabolite. The supernatant liquid is extracted three times with one-third its volume of ethyl acetate. The combined organic extracts are dried over Na_2SO_4, filtered, and evaporated without heating to dryness to afford the crude diol, which is essentially pure. Further purification can be achieved by recrystallization from ethyl acetate (Na_2CO_3-washed)−hexanes mixtures. In the case of bromobenzene, typical yields are in the range of 90−120 g of diol for 8−10 L of culture medium.

SAFETY NOTE. The extraction of diol metabolites must be done with base-washed solvents. Trace amounts of acid, for example phenols, have been known to autocatalyze a rapid and extremely exothermic dehydration and aromatization with a serious potential for a flame-up or explosion, especially on large scales. In one instance ~80 g of a diol

Table 5. **Rate of substrate addition in g h^{-1}**

Substrate	Rate of addition in gh^{-1}	Substrate	Rate of addition in gh^{-1}
			
R=Br	30-40		
Cl	40-45		
I	30-40		
Me	40-50		
Ph	5-10		
OPh	5-10	R = H	20-25
$CH=CH_2$	30-35	Br	20-25
CH=CHBr	15-20		
CH_2CH_2Br	20-30		
CH_2CH_2CN	15-20		
$CH_2CH_2N_3$	15-20		
CH_2CH_2OAc	15-20		
	5-10	$R_1=R_2=H$	5-10
	5-10	$R_1=H$, $R_2=OMe$,	5-10
	5-10	$R_1=OMe$, $R_2=OMe$	1-5
	10-15		
	10-15	X = H, Y = OH	35-40
		X = OH, Y = H	30-35
	15-20		9-15
	10-15		5-10

spontaneously dehydrated with complete obliteration of the container. The diols are best stored in crystalline form (extracted and crystallized from acid-free solvents), resuspended in the phosphate buffer, and frozen. Extraction and immediate use is recommended on small- to medium-scale (1−10 g). It is absolutley essential that all traces of acids (including phenols) be removed from the diols when these are in the crystalline and dry state and handled at room temperature. The diols are stable indefinitely in crystalline state when acid-free and stored at low temperature.

Medium-Scale Fermentation with *E. coli* JM109 (pDTG602). The process of generating catechols from the corresponding arenes using *E. coli* JM109 (pDTG602)[7] is carried out in similar fashion as described for *E. coli* JM 109 (pDTG601). The metabolism of substrate is much more limited in comparison to that of *E. coli* JM 109 (pDTG601). In a typical experiment with bromobenzene, the yields are in the range of 9−12 g of catechol for 8−10 L of culture medium.

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