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# Synthesis of substituted catechols using nitroarene dioxygenases

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**Abstract**

The nitroarene dioxygenases are in the class of Rieske iron-containing oxygenases that incorporate atmospheric oxygen into substrates via electrophilic attack on the substrate. In their native role, the nitroarene dioxygenases start degradative pathways by hydroxylating nitro-substituted, and adjacent unsubstituted carbons of nitroaromatic compounds. The reaction yields the corresponding nitro-*cis*-cyclohexadienediol, which is unstable and spontaneously re-aromatizes to form a catechol and nitrite. In bacterial metabolism, the specificity of the hydroxylation determines subsequent steps in degradation pathways. Experiments were done to find whether the specificity could be exploited to direct the hydroxylation of multiply substituted aromatic substrates and thereby produce novel catechols. Recombinant strains carrying genes for nitroarene dioxygenases were used for transformation of various substituted nitroaromatic compounds. The reactions were analyzed using HPLC to track substrate consumption and product formation, then GC–MS and NMR to identify the reaction products. A number of substituted catechols were obtained using the recombinant biocatalysts. The nitro-substituted carbon was the primary site for dioxygenase hydroxylation. When substrates included nitro and halogen substituents, the halogen-substituted positions were also targeted, but less frequently than the nitro-substituted site. The production of catechols was limited in batch fermentations, likely due to toxicity of the quinones that result from air oxidation of catechols. The nitroarene dioxygenases will serve as catalysts for direct synthesis of highly substituted catechols, however, the reaction conditions must be engineered to overcome product toxicity and allow sustained accumulation of catecholic products.
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## 1. Introduction

Substituted catechols are used in the synthesis of pharmaceuticals and other industrial compounds, found as common constituents in biologically active natural products, and can serve as template molecules for organizing supramolecular complexes [1–4]. Several methods are used for chemical synthesis of substituted catechols [5,6]. Each has drawbacks such as; harsh acidic systems using metal halide catalysts, hazardous waste disposal issues, limited yields, and mixtures of products that require extensive purification to obtain the desired compound.

The regiospecific nature of biocatalytic reactions presents attractive alternative methods for synthesis of hydroxylated aromatic compounds. Oxygenase catalyzed production of catechols from phenolic or benzylic substrates has been well established on a laboratory scale [7–11]. Additional work demonstrated approaches for enhancing the efficiency of the biocatalytic synthesis of substituted catechols [4,12]. The increased productivity is necessary to compete with chemical methods and overcome perceived limitations of oxygenase-based biocatalysis [13].

The nitroarene dioxygenases are multicomponent, Rieske iron–sulfur containing dioxygenases [14,15]. Molecular genetic studies revealed that the nitroarene dioxygenases are closely related to naphthalene dioxygenases but their substrate preferences and catalytic specificities are quite different [16]. Nitroarene dioxygenases initiate bacterial pathways for degradation of nitroaromatic compounds by electrophilic attack on nitro-substituted and vicinyl unsubstituted carbons to yield corresponding cyclohexadienediols. The nitro-

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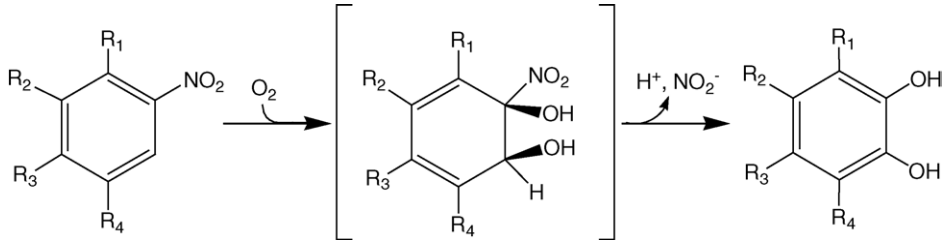


Fig. 1. Typical dioxygenation reaction of nitroarene dioxygenases. R-group substituents tested in the present work include combinations of H-, $CH_3$-, $NO_2$-, Cl-, Br-, $NH_2$-, and $CF_3$- (Table 1). The cyclohexadiene dihydrodiol intermediate shown in brackets was not isolated; the compounds are unstable and spontaneously rearomatize to form the corresponding catechol.

substituted diol is unstable and spontaneously rearomatizes to yield a catechol and nitrite ion [17]. The preference for directing hydroxylation at a nitro-substituted carbon can be exploited to obtain specific products (Fig. 1). By choosing an appropriate substrate, one can select the positions that will be hydroxylated to form the intermediate nitro-cyclohexadiene diol. The regioselectivity and spontaneous rearomatization in nitroarene catabolic pathways differs from other dioxygenase initiated pathways. In other pathways, the dioxygenases generally attack vicinyl un-substituted carbons to yield the corresponding cyclohexadiene-diols. Re-aromatization requires an NAD-dependent dehydrogenase to form a catecholic product [18]. The two characteristics of nitroarene dioxygenases (i) regiospecific attack at the nitro-substituted position and (ii) spontaneous rearomatization; support the potential use of the oxygenases as biocatalysts for catechol synthesis. The present work expands the understanding of substrate range and catalytic specificity for the nitrobenzene dioxygenase from *Comamonas* sp. strain JS765 [19] and the 2,4-dinitotoluene dioxygenase from *Burkholderia* sp. strain DNT [20] and also explores application of the nitrobenzene dioxygenases for synthesis of two chloromethylcatechol isomers from the corresponding chloronitrotoluenes.

## 2. Materials and methods

### 2.1. Strains, plasmids, and growth conditions

*Escherichia coli* JM109 {*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, Δ (*lac-proAB*) *relA1*, [F′, *traD36*, *proAB*+, *lacIqZ*ΔM15], $\lambda^-$} was used as cloning and expression host organism. Recombinant plasmid pJS1048 included genes for the 2,4-dinitrotoluene dioxygenase (24DDO) from *Burkholderia* sp. strain DNT [20]. The plasmid was derived by subcloning the 6.7-kb *Sac*I:*Sal*I restriction fragment from pJS48 [20] into cloning vector pK18 [21]. Recombinant plasmid pJS1927 includes the genes for the nitrobenzene dioxygenase (NBDO) from *Comamonas* sp. strain JS765 [19], the plasmid was obtained by subcloning the 4.7-kb *Sac*I:*Eco*RI restriction fragment from pJS927 into vector pK19 [21]. The dioxygenase genes were oriented to allow expression from the isopropylthio-β-D-galactoside (IPTG)-inducible *lac* promoter in the vector. *E. coli* strains were grown on Luria Bertani plates or broth containing ampicillin (100 mg $L^{-1}$) or kanamycin (25 mg $L^{-1}$) for plasmid maintenance as appropriate. Bacteria for substrate preference assays were cultivated in baffled shaker flasks containing LB broth; recombinant oxygenase synthesis was induced with addition of IPTG to culture broth. Bacteria for larger-scale transformations were cultivated using a 20-L stirred tank reactor (BiostatC, B-Braun Inc., Allentown, PA, USA) in buffered LB medium (potassium phosphate, 90 mM, pH 7.4). The medium was supplemented with glycerol (0.5 wt.%/v), Antifoam C (0.005% v/v) (Sigma–Aldrich, St. Louis, MO, USA) and kanamycin (25 mg $L^{-1}$). The reactor medium (15 L) was inoculated (5% v/v) with a mid-log-phase culture of *E. coli* JM109 (pJS1927). After 90 min incubation, IPTG (0.5 mM) was added to medium and the cultures incubated 13 h (30 °C, 250–400 rpm, 20 L air $min^{-1}$) prior to use in substrate biotransformations.

### 2.2. Analytical methods

Nitrite concentrations were measured using the diazotization method as described in [22]. Standard curves for nitrite provided from solutions of sodium nitrite in media identical to sample preparation, measurements were made with a microplate reader (Bio-Tek Instruments Model ELx808 (Winooski, VT)) at 562 nm.

HPLC analysis was done using a model 1100 chromatography system equipped with a diode array detector for monitoring the eluent (Agilent Inc. (Santa Clarita, CA)). Reaction compounds were separated on a Supelcosil LC-ABZ + Plus column (25 mm × 4.6 mm; Supelco Inc. Bellefonte, PA) using an acetonitrile:trifluoroacetic acid (0.1%) mobile phase. Mobile phase composition and flow rate were changed for various reactions to allow acceptable resolution of compounds and efficient run cycles. Eluent was monitored at 254, 280, and 310 nm for detection of substrates and products. Gas chromatography–mass spectroscopy (GC–MS) sample preparation and analysis done as described previously [19]. Reaction product identities were confirmed by comparison with authentic standards when available. Putative identification of other products made from interpretation of chromatographic and spectral data.

For NMR analyses the samples were dissolved in chloroform-d. NMR spectra were collected using a Varian

Inova spectrometer equipped with a 5 mm indirect detection probe operating at 499 Mhz for 1H and 126 MHz for 13C. Chemical shifts are reported in ppm relative to TMS. The $^{1}H$ and $^{13}C$ chemical shifts assignments were based on the $^{1}H$–$^{13}C$ one-bond and long-range correlations seen in the gradient heteronuclear multiple bond correlation (ghmbc) spectra. (NMR analysis and interpretation performed at University of Florida, Department of Chemistry, I. Ghiviriga.)

### 2.3. Substrate preference assays

Recombinant strains carrying nitroarene dioxygenase genes were grown to induce oxygenase synthesis, cells harvested by centrifugation, washed with potassium phosphate buffer (20 mM, pH 7.0) then used immediately in the biotransformation reaction. The reaction mixtures contained 5.0 mL buffered minimal media (MSB, pH 6.8) [23], substrates (100 μM) and the recombinant *E. coli* strains ($A_{600}$ − 2). The suspensions were incubated on a rotating drum shaker at 30 °C, samples were collected for analysis by reverse phase HPLC and colorimetric nitrite determination [24]. After 2 h incubation the cells were removed by centrifugation; the pH was lowered to 4.0 with hydrochloric acid, and ascorbic acid or sodium dithionite (2 mM) was added to limit air oxidation of products. The reaction mixture was extracted with ethyl acetate (3×, 0.25 vol.), the organic phases were combined and dried over anhydrous sodium sulfate, the solvent was removed under vacuum, and the residue was analyzed by using GC–MS.

### 2.4. Bulk transformation

Following cultivation, the cells (*E. coli* JM109 (pJS1927)) were harvested by using centrifugation, and then washed twice with one-fourth volume MSB (pH 6.8). The cells were resuspended in 4 L MSB (pH 6.8) and sparged with compressed air until use in biotransformation. The bulk transformations of substrates were done in a stepwise fashion in the 20 L reactor using the recombinant strain resuspended in MSB (pH 6.8) containing glucose (5 mM) and the chloronitrotoluene substrates. Samples were collected regularly and analyzed using HPLC to determine substrate and product levels. Substrate dissolved in dimethylformamide was added to the reaction as needed. When catechol accumulation rates slowed, the reactions were diluted with media and cell suspension added to resume the reaction. Total reaction times were 6–8 h.

### 2.5. Catechol purification

After the biotransformation reaction, the cells were removed from media using a tangential flow filtration system (Pellicon, Millipore Corp., Bedford, Mass, USA). The filtrate was transferred to glass carboys then acidified to pH 4.0 (conc. HCl) and treated with sodium dithionite (2 mM) to stabilize the catechols in aqueous solution. The solutions were stored at 4 °C and were stable at least 5 days under these conditions. The catechol was concentrated from the filtrates by binding to DPA-6 resin (Supelco Inc., Bellefonte, Penn, USA). The media was loaded onto packed resin beds (25 or 50 g in 90 or 110 mm Buchner funnels) and allowed to flow by gravity through the matrix. Samples of the eluent were monitored for product breakthrough and the resin replaced when binding capacity attained. The chloromethylcatechols were eluted from the resin with 100 ml aliquots of methanol then analyzed by reverse phase HPLC. Aliquots containing individual chloromethylcatechols were combined and the methanol removed by vacuum distillation. Following distillation, a tan to brown residue including the catechol remained in the flask. The residue was dissolved in ethyl acetate, passed over anhydrous sodium sulfate to remove remaining water, then the solvent removed by vacuum distillation. The residue was then collected and placed in a desiccator under vacuum for 24–72 h to continue drying the product. Final purification was done using vacuum sublimation; crystals that formed on the apparatus condensation point were collected and stored desiccated in amber vials at −20 °C.

### 2.6. Chemicals

Nitroaromatic substrates, available product standards, and other chemicals were purchased from Sigma–Aldrich or ChemServices Inc. (Westchester, PA). Antibiotics were from Sigma–Aldrich. Molecular biology reagents were purchased from Roche Applied Science (Indianapolis, IN).

## 3. Results

### 3.1. Substrate preferences-catalytic specificity of NBDO and 24DDO

The NBDO demonstrated a significantly broader substrate preference than the 24DDO (Table 1). Dioxygenase attack measured by nitrite evolution was detected in just 6 of the 23 substrates tested with 24DDO, nitrite evolution was found with 20 of 23 substrates incubated with NBDO. The result with NBDO corroborate previous experiments which showed that NBDO has a relatively relaxed substrate preference [19].

Results from GC–MS analysis revealed that the methyl group, unsubstituted, and halogen-substituted carbons were targeted in addition to the nitro substituted carbon. Catalytic specificities differed for the two nitroarene dioxygenases; a few examples are described next. When mononitrotoluenes were tested as substrates, mixtures of the corresponding methylcatechols and nitrobenzylalcohols resulted from dioxygenation and monooxygenation of the substrates, respectively [19]. GC–MS analysis revealed that 24DDO only hydroxylated the methyl group of the mononitrotoluenes, no nitrite was detectable (Table 1) and no methylcatechol isomers were identified in GC–MS results. Another contrast in catalytic specificity was found

Table 1
Dioxygenation of substrates by nitrobenzene- and 2,4-dinitrotoluene dioxygenase

| Substrate | Relative activity | |
|---|---|---|
| | NBDO | 24DDO |
| Nitrobenzene | 1.00 | – |
| 2-Nitrotoluene | 0.59[a] | – |
| 3-Nitrotoluene | 2.21[a] | – |
| 4-Nitrotoluene | 0.42[a] | – |
| 2,3-Dinitrotoluene | 0.05 | 0.01 |
| 2,4-Dinitrotoluene | 0.06 | 1.00 |
| 2,6-Dinitrotoluene | 0.40 | 0.05 |
| 3,4-Dinitrotoluene | 0.03 | – |
| 1,3-Dinitrobenzene | 0.89[a] | 0.15 |
| 2-Bromo-3-nitrotoluene | 1.01 | – |
| 2-Chloro-3-nitrotoluene | 1.09 | – |
| 2-Chloro-4-nitrotoluene | 0.61 | 0.56 |
| 2-Chloro-6-nitrotoluene | 1.12 | 0.02 |
| 4-Chloro-3-nitrotoluene | 0.11 | – |
| 4-Bromo-3-nitrotoluene | 0.16 | – |
| 2,5-Dichloronitrobenzene | – | – |
| 2,4-Diamino-6-nitrotoluene | 0.11 | – |
| 4-Amino-3,5-dinitrotoluene | 0.13 | – |
| 2-Amino-6-nitrotoluene | 0.07 | – |
| 4-Amino-2-nitrotoluene | 0.30 | – |
| 4-Amino-3-nitrotoluene | – | – |
| 2,6-Dinitroaniline | 0.83 | – |
| 4-Nitro-ααα-trifluorotoluene | – | – |

Values are mean rate of triplicate trials for each substrate/oxygenase combination relative to the transformation of nitrobenzene or 2,4-dinitrotoluene. No nitrite detectable by sulfanilamide assay NBDO: nitrobenzene dioxygenase (*E. coli* JM109 (pJS1927)) 24DDO: 2,4-DNT dioxygenase (*E. coli* JM109 (pJS1048)).

[a] From Lessner et al. Appl. Environ. Microbiol. 2002;68:634.

with 2-chloro-4-nitrotoluene as the substrate. The relative dioxygenase activities with 2-chloro-4-nitrotoluene were similar for both tested nitroarene dioxygenases, but the product distribution differed. Results from GC–MS analysis showed that 24DDO only catalyzed dioxygenase attack of the nitro-substituted and adjacent carbon, NBDO catalyzed dioxygenation of the ring as well as monooxygenation of the methyl group on 2-chloro-4-nitrotoluene [19].

### 3.2. Laboratory scale transformation of two chloronitrotoluene isomers

Two chloronitrotoluene isomers were selected for gram-scale biotransformation reactions with NBDO as the oxygenase catalyst. The predicted products from the transformations were two chloromethylcatechol isomers, substituted at the substituted at the 3,4-positions of the ring. The larger scale reactions using NBDO provided sufficient amounts of purified chloromethylcatechol isomers for unequivocal identification of the compounds as well as insight about the application of NBDO in a synthetic process.

The recombinant cells transformed 2-chloro-3-nitrotoluene (2Cl3NT) and 2-chloro-6-nitrotoluene (2Cl6NT) as in the substrate preference screens; a single catechol isomer was formed from 2Cl3NT, and two catechols from

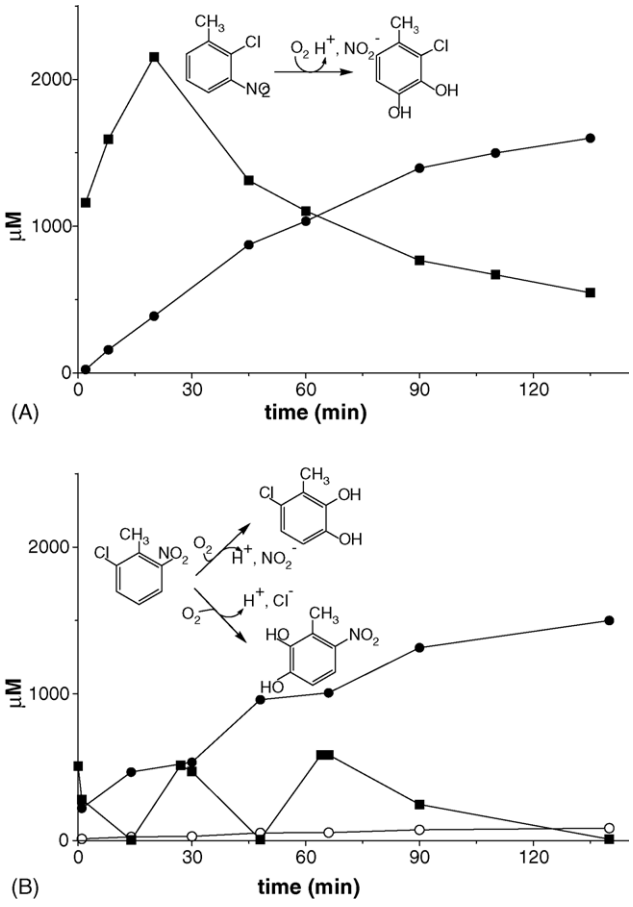


Fig. 2. Initial stages in gram scale transformation of 2-chloro-3-nitrotoluene and 2-chloro-6-nitrotoluene by NBDO. (A) 2-chloro-3-nitrotoluene reaction. (■) 2-chloro-3-nitrotoluene, (●) 3-chloro-4-methylcatechol. Initial increases in chloronitrotoluene concentration are due to slow dissolution of compound. (B) 2-chloro-6-nitrotoluene reaction. (■) 2-chloro-6-nitrotoluene, (●) 4-chloro-3-methylcatechol, (◯) 3-methyl-4-nitrocatechol.

2Cl6NT (Fig. 2). The products were initially noted as polar peaks that accumulated through the reaction. 3-Methyl-4-nitrocatechol was identified by comparison to a chemical standard [25]. The identity of the 3-chloro-4-methylcatechol (3Cl4MC) and 4-chloro-3-metylcatechol (4Cl3MC) peaks were subsequently confirmed from product isolation and characterization (below). The nitrobenzene dioxygenases showed good catalytic specificity. The nitro-substituted carbon was the exclusive site for the dioxygenase with 2Cl3NT, and favored 18:1 over the chloro-substituted carbon with 2Cl6NT. No evidence for monooxygenation of the methyl group of either substrate was detectable. The initial catechol production rates were comparable for the two reactions (17.6 nmol $min^{-1}$ (mg protein)$^{-1}$ (4Cl3MC) and 14.0 nmol $min^{-1}$ (mg protein)$^{-1}$ (3Cl4MC)). The conversion efficiencies (chloromethylcatechol in supernatant/chloronitrotoluene disappearance) differed significantly at reaction end; 0.83 (3.49 g 4Cl3MC/4.23 g 2Cl6NTol) and 0.46 (3.59 g 3Cl4MC/7.88 g 2Cl3NTol). The conversion was limited by three factors. Substrate was lost

to volatilization in both reactions. During the transformation of 2Cl6NT reaction, there was a detectable level of misdirected hydroxylation of the chloro-substituted carbon (Fig. 2b). Additional losses occurred during the reaction from air-oxidation of catechols to form semi- and ortho-benzoquinones [26].

Product toxicity is commonly encountered during biocatalytic catechol synthesis [13]. In the 2Cl3NT transformation reaction, catalysis stalled as the product concentration approached 1500 μM, but dilution of the reaction mixture restored activity. The observation suggests inhibition by the chloromethylcatechol. The phenomenon was not examined further in the present work, but the putative toxicity was considered in the subsequent reaction with 2Cl6NT. During the 2Cl6NT transformation (Fig. 2b) the substrate concentrations and additions were controlled to keep product levels below the apparent 1500 μM inhibitory threshold.

### 3.3. Analysis of chloromethylcatechol isomers

The accumulation of nitrite in the reaction media provided presumptive evidence of chloromethylcatechol formation [19] and preliminary GC–MS results of the trimethylsilane-derivatized products were also consistent with chloromethylcatechol isomers. In order to confirm the structures, the products were isolated from the two reaction mixtures, purified (>95% by GC and HPLC analysis), then characterized using additional chemical analyses. Product yields from reaction supernatants were 17% for 3Cl4MC and 10% for 4Cl3MC.

Vacuum sublimation provided bright yellow, flaked crystals (mp 73–76 °C) from the 2-chloro-3-nitrotoluene reaction product. LC–MS and GC–MS analysis gave a molecular ion ($M$) of 158 AMU, as expected for chloromethylcatechol. The mass spectrum showed an $M$:$M+2$ ratio of 3:1 along with a principal fragment at $M-35$; both are characteristic of a single chloride atom. The aromatic fragment at 77 AMU was also evident in the product mass spectrum. Proton and $^{13}$C NMR supported the presumptive identification of the structure as 3-chloro-4-methylcatechol [H1 NMR (499 MHz, $CDCl_3$) $\delta$ 2.28 (s, 3H), 5.42 (s, 2H), 6.70 (d, $J$ = 8.4 Hz, 1H), 6.75 (d, $J$ = 8.4 Hz, 1H) – C13 NMR (126 MHz, $CDCl_3$) $\delta$ 19.5, 113.6, 120.3, 121.9, 127.8, 139.3, 142.6]. The spectra are in agreement with those of the 3-chloro-4-methylcatechol previously identified as a minor product from 2-chlorotoluene transformation by tetrachlorobenzene dioxygenase [27].

White, needle-shaped crystals (mp 67 °C) were obtained from vacuum sublimation of the putative chloromethylcatechol produced from 2Cl6NT transformation. The mass spectrum was consistent with chloromethylcatechol ($M-158$; $M$:$M+2$ of 3:1; strong $M-35$ peak; and the aromatic peak at 77 amu). Proton and $^{13}$C NMR were in agreement with the structure assignment as 4-chloro-3-methylcatechol [H1 NMR (499 MHz, $CDCl_3$) $\delta$ 2.29 (s, 3H), 5.15 (s, 1H), 5.35 (s, 1H), 6.64 (d, $J$ = 8.7 Hz, 1H), 6.80 (d, $J$ = 8.7 Hz, 1H) – C13 NMR (126 MHz, $CDCl_3$) $\delta$ 13.0, 113.4, 120.6, 123.3, 127.0, 141.7, 143.3]. Spectral characteristics are consistent with those previously reported for 4-chloro-3-methylcatechol, an intermediate in the 3-chloro-2-methylbenzoate degradation pathway [28].

## 4. Discussion

The nitroarene dioxygenases expand possible synthetic routes for substituted catechols; the enzymes provide a single-step biocatalytic route to the products. The dehydrogenase-catalyzed step that follows hydroxylation in other dioxygenase-based processes [7,8] is not required because nitrite elimination leads to spontaneous rearomatization of the ring. The oxygenase preference for the nitro-substituted carbon provides a strategy to direct regiospecific hydroxylation of the substrate. The availability or cost of nitroarenes as starting materials will be a consideration in process design. Although acid-catalyzed nitration of aromatic compounds occurs quite readily, there are instances where nitration of specific carbons in the ring is a low-yield reaction, which complicates obtaining the necessary substrate. In a case where the appropriate nitroarene is not available, alternate biocatalysts might transform other benzylic substrates to yield the product. Another potential limitation of the approach is reduction of the nitro substituents by host enzymes. Gratuitous reduction of nitroaromatic compounds is common in microorganisms and catalyzed by a number of enzymes [29,30]. The unwanted host-catalyzed reactions must be guarded against in any application.

The substrate/catalyst screening carried out in the present work highlights the advantages in evaluating a number of oxygenases for a given transformation. The terminal oxygenase components of NBDO and 24DDO share more than 87% amino acid sequence identity [19], yet their substrate preferences and catalytic specificities are very different. The results support creating a bank of recombinant nitroarene dioxygenases from degradation pathways that could be screened for specific transformations. Additional catalytic range for 24DDO has been reported through mutagenesis of the oxygenase [31]. The molecular genetic approach could provide an almost unlimited source of enzyme variants and the appropriate screens will uncover desired oxygenase specificity.

The present study demonstrates the application of nitroarene dioxygenases for synthesis of two chloromethylcatechol isomers. We are currently extending the strategy to produce catechols from other substituted nitroarenes. Process modifications will improve efficiency of the transformation. Membrane oxygenation would reduce substrate losses due to volatilization [32] Two-phase reaction media [3] and in situ product recovery [12] could be used to limit the contact of catalyst and product, overcoming anticipated toxicity problems. The changes, along with medium and

catalyst optimization could be used in combination to provide practical product yields.

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## References

[1] Albrecht M, Napp M, Schneider M, Weis P, Frohlich R. Kinetic versus thermodynamic control of the self-assembly of isomeric double-stranded dinuclear titanium(IV) complexes from a phenylalanine-bridged dicatechol ligand. Chem Commun 2001;2001:409–10.
[2] Bui VP, Hudlicky T, Hansen TV, Stenstrom Y, Mander LN, Williams CM. Direct biooxidation of arenes to corresponding catechols with E. coli JM109 (pDTG602). Application to synthesis of combretastatins A-1 and B-1. Tetrahedron Lett 2002;43:2839–41.
[3] Held M, Schmid A, Kohler HPE, Suske W, Witholt B, Wubbolts MG. An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. Biotechnol Bioeng 1999;62:641–8.
[4] Meyer A, Held M, Schmid A, Kohler HP, Witholt B. Synthesis of 3-*tert*-butylcatechol by an engineered monooxygenase. Biotechnol Bioeng 2003;81:518–24.
[5] Bui VP, Hansen TV, Stenstrom Y, Hudlicky T. Direct biocatalytic synthesis of functionalized catechols: a green alternative to traditional methods with high effective mass yield. Green Chem 2000;2:263–5.
[6] Olah G, Reddy V, Prakesh G. In: Howe-Grant M, editor. Kirk-Othmer Encyclopedia of chemical technology, vol. 11. New York: Wiley; 1994.
[7] Endoma MA, Bui VP, Hansen J, Hudlicky T. Medium-scale preparation of useful metabolites of aromatic compounds via whole-cell fermentation with recombinant organisms. Org Process Res Dev 2002;6:525–32.
[8] Gennaro PD, Galli E, Orsini F, Pelizzoni F, Sello G, Bestetti G. Development of biocatalysts carrying naphthalene dioxygenase and dihydrodiol dehydrogenase genes inducible in aerobic and anaerobic conditions. Res Microbiol 2000;151:383–91.
[9] Held M, Suske W, Schmid A, Engesser K, Kohler H, Witholt B, Wubbolts M. Preparative scale production of 3-substituted catechols using a novel monooxygenase from Pseudomonas azelaica HBP 1. J Mol Catal B Enzyme 1998;5:87–93.
[10] Johnston JB, Renganathan V. Production of substituted catechols from substituted benzenes by a *Pseudomonas* sp. Enzyme Microb Technol 1987;9:706–8.
[11] Lynch RM, Woodley JM, Lilly MD. Process design for the oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida*. J Biotechnol 1997;58:167–75.
[12] Husken LE, Dalm MC, Tramper J, Wery J, de Bont JA, Beeftink R. Integrated bioproduction and extraction of 3-methylcatechol. J Biotechnol 2001;88:11–9.
[13] van Beilen JB, Duetz WA, Schmid A, Witholt B. Practical issues in the application of oxygenases. Trends Biotechnol 2003;21:170–7.
[14] Mason JR, Cammack R. The electron transport proteins of hydroxylating bacterial dioxygenases. Annu Rev Microbiol 1992;46:277–305.
[15] Wackett LP. Mechanism and applications of Rieske non-heme iron dioxygenases. Enzyme Microb Technol 2002;31:577–87.
[16] Parales RE. In: Spain JC, Hughes EJ, Knackmuss H-J, editors. Biodegradation of nitroaromatic compounds and explosives. Boca Raton, Florida: Lewis Publishing; 2000. p. 63–90.
[17] Spanggord RJ, Spain JC, Nishino SF, Mortelmans KE. Biodegradation of 2, 4-dinitrotoluene by a *Pseudomonas* sp. Appl Environ Microbiol 1991;57:3200–5.
[18] Gibson DT. In: Hagedorn SR, Hanson RS, Kunz DA, editors. Microbial metabolism and the carbon cycle. Chur, Switzerland: Harwood Academic Publishers; 1988. p. 43–52.
[19] Lessner DJ, Johnson GR, Parales RE, Spain JC, Gibson DT. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl Environ Microbiol 2002;68:634–41.
[20] Suen W-C, Haigler BE, Spain JC. 2, 4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J Bacteriol 1996;178:4926–34.
[21] Pridmore RD. New and versatile cloning vectors with a kanamycin-resistance marker. Gene 1987;56:309–12.
[22] Smibert RM, Krieg NR. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. Methods for general and molecular bacteriology. Washington, DC: American Society for Microbiology; 1994. p. 607–54.
[23] Stanier RY, Palleroni NJ, Doudoroff M. The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 1966;43:159–271.
[24] Daniels L, Hanson RS, Phillips JA. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. Methods for general and molecular bacteriology. Washington, DC: American Society for Microbiology; 1994. p. 607–54.
[25] Nishino SF, Paoli G, Spain JC. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl Environ Microbiol 2000;66:2139–47.
[26] Schweigert N, Zehnder AJB, Eggen RIL. Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. Environ Microbiol 2001;3:81–91.
[27] Pollman K, Wray V, Hecht H-J, Piper D. Rational engineering of the regioselectivity of TecA tetrachlorobenzene dioxygenase for the transformation of chlorinated toluenes. Microbiology 2003;149:903–13.
[28] Higson FK, Focht DD. Utilization of 3-chloro-2-methylbenzoic acid by *Pseudomonas cepacia* through the meta fission pathway. Appl Environ Microbiol 1992;58:2501–4.
[29] Riefler RG, Smets BF. Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: kinetics linked to one-electron redox potentials. Environ Sci Technol 2000;34:3900–6.
[30] Esteve-Nuñez A, Caballero A, Ramos JL. Biological degradation of 2,4,6-trinitrotoluene. Microbiol Mol Biol Rev 2001;65:335–52.
[31] Keenan BG, Leungsakul T, Smets BF, Wood TK. Saturation mutagenesis of *Burkholderia cepacia* R34 2,4-dinitrotoluene dioxygenase at DntAc valine 350 for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone. Appl Environ Microbiol 2004;70:3222–1.
[32] Husken LE, Oomes M, Schroen K, Tramper J, de Bont JA, Beeftink R. Membrane-facilitated bioproduction of 3-methylcatechol in an octanol/water two-phase system. J Biotechnol 2002;96:281–9.