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A High-Throughput *in Vitro* Assay System for the Detection of the Enzymatic Dihydroxylation of Aliphatic Olefins

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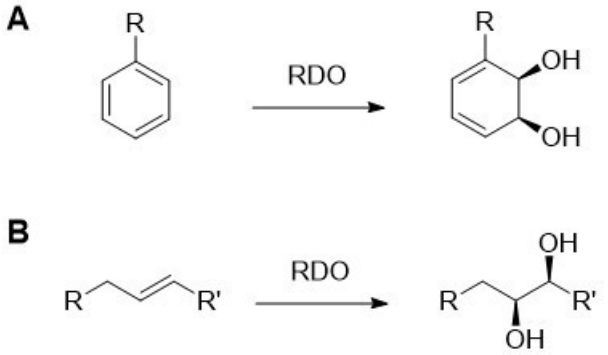
Abstract: Rieske dioxygenase enzymes can perform the *cis*-dihydroxylation of aliphatic olefins, representing a potential green alternative to established methods of performing this important transformation. However, the activity of the natural enzymes in this context is low relative to their more well-known activity in the *cis*-dihydroxylation of aromatics. To enable the engineering of dioxygenase enzymes for improved activity in the dihydroxylation of aliphatic olefins, we have developed an assay system to detect the relevant diol metabolites produced from whole-cell fermentation cultures. Optimization studies were carried out to maximize the sensitivity of the assay system, and its utility in the *in vitro* screening of enzyme variant libraries was demonstrated. The assay system was utilized in screening studies that identified Rieske dioxygenase variants with significantly improved activity in the dihydroxylation of aliphatic olefins relative to the wild-type enzyme.

Keywords: Enzyme catalysis; Dihydroxylation; High-throughput screening; Protein engineering

The enantioselective dihydroxylation of aliphatic olefins is an extremely important chemical transformation that has been widely applied in the synthesis of valuable compounds.[1] Due to the toxicity of transition metal catalysts applied in the traditional methods used to perform this transformation, as well as the potential for the production of unwanted byproducts,[1,2] recent work has aimed to develop more sustainable means of performing this transformation, including osmium-free[3] and enzymatic methods.[4] Studies aimed at developing reliable enzymatic means of performing this transformation have utilized natural Rieske dioxygenases (RDOs) as templates for the engineering of improved dihydroxylation catalysts.[4]

RDOs are a class of non-heme iron-containing metalloenzymes that play an important role in the metabolism of aromatic compounds by soil bacteria.[5] These enzymes are most well known for their ability to catalyze the oxidative dearomatization of aromatics to produce *cis*-diene diol metabolites (Scheme 1A).[5] In this context, RDOs have been widely applied as biocatalysts in the synthesis of valuable compounds.[6] In addition to oxidative dearomatization, however, RDOs can also catalyze other reactions including sulfoxidation, C–H amination, desaturation, monohydroxylation, and the asymmetric dihydroxylation of aliphatic olefins (Scheme 1B).[4,7–10] These enzymes represent a promising biocatalytic alternative to the traditional means of performing the asymmetric dihydroxylation of aliphatic olefins due to their broad substrate scopes and their unique ability to stereoselectively introduce two hydroxyl groups in a single enzymatic step.[11,12] The utility of natural RDOs in this



Scheme 1. Enantioselective dihydroxylation of aromatics (A) and aliphatic olefins (B) catalyzed by Rieske dioxygenases (RDOs).[4,5]

context, however, has been limited by low activity and poor enantioselectivity, particularly in the context of the dihydroxylation of linear aliphatic olefins.[13]

To overcome these limitations on the practical utility of RDOs as catalysts for the enantioselective dihydroxylation of aliphatic olefins, Hauer, *et al.* have developed variants of cumene dioxygenase (CDO) and naphthalene dioxygenase (NDO) with improved activity and stereoselectivity.[4] These studies also demonstrated the capability of enzyme engineering to alter the reaction specificity and regioselectivity of RDOs.[4] To expand on these pioneering studies and to engineer new RDO variants with improved activity across a broad range of aliphatic olefins, a high-throughput assay system is required that will afford the capability of screening thousands of RDO variants in a relatively short time. Recently, our laboratory has reported the (meta)periodate fluorescent *cis*-diol assay system (MPFCD), an assay system that can be used to screen for RDO activity in the *cis*-dihydroxylation of aromatics,[14] which we have used to engineer improved RDO variants in this context.[15] Exploratory studies indicated that the MPFCD assay system was insufficiently sensitive to the presence of metabolites produced from the dihydroxylation of aliphatic olefins to be used in this context (Figure S1). Therefore, it was necessary to develop a novel assay system to enable the expansion of our RDO engineering efforts.

As our established assay system for the detection and quantification of RDO metabolites relies on the conversion of the *cis*-diol functionality to a corresponding dialdehyde analyte,[14] we envisioned a similar system that would make use of an alternate probe capable of detecting and quantifying aliphatic aldehydes. 2-amino-benzamidoxime (ABAO) derivatives have been reported as probes for detecting and quantifying aldehydes, both in the context of small-molecule aldehydes produced by enzymes,[16] and of aldehyde-tagged proteins.[17] Further, these probes were shown to be applicable for use in high-throughput assays and the detection of a wide variety of aldehyde classes.[16] To facilitate exploratory studies, multiple diol compounds were produced on a preparative scale through the Sharpless asymmetric dihydroxylation procedure,[1b] including the cyclohexane-1,2-diol used for exploratory and optimization studies. As previous studies have shown that 5-methoxy-2-amino-benzamidoxime (5-MeO-ABAO) affords the lowest aldehyde detection limit among 2-amino-benzamidoxime derivatives, this probe was selected and synthesized from 5-methoxy-2-aminobenzonitrile.[16] Initial tests demonstrated a concentration-dependent increase in absorbance when the dialdehyde produced from cyclohexane-1,2-diol was treated with the 5-MeO-ABAO probe (Figure 1B).

To maximize the sensitivity of the assay system, optimization studies were carried out. Assay parameters including the buffer system employed, the final concentration of the probe in the assay mixture, the final concentration of sodium (meta)periodate ($NaIO_4$) in the assay mixture, the final pH at which the reaction of the aldehyde analyte and the probe was performed, etc. were varied to maximize the absorbance response of the assay system (Figure 2). These studies indicated the maximum assay sensitivity could be achieved using the HEPES buffer system, a final probe concentration of 5 mM, a final $NaIO_4$ concentration of 2 mM, and a pH of 3.5 (Figure 2). Under these optimized conditions, increases in absorbance could reliably be detected in response to diol concentrations of <20 μM.

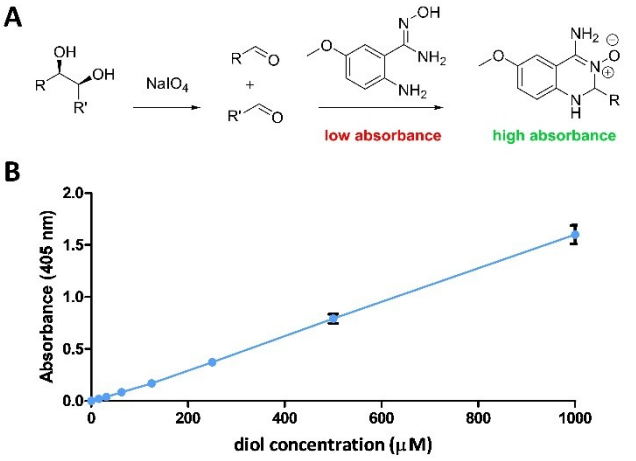


Figure 1. (A) Schematic representation of the coupled reactions employed by the absorbance-based assay system for the detection of aliphatic *cis*-diol metabolites; (B) Concentration-dependent absorbance response of the assay system to the presence of cyclohexane-1,2-diol. All studies were performed in triplicate, with absorbance levels normalized to the absorbance of the negative control (0 mM cyclohexane-1,2-diol). Data points with error bars not visible demonstrated S. D. $<5\%$.

One challenge posed by the coupled reactions employed in this assay system that became apparent through these optimization studies was an unwanted side reaction between 5-MeO-ABAO and $NaIO_4$ that generated a solid byproduct that obscured absorbance readings. The production of this byproduct was likely caused by chelation of the nucleophilic functional groups of 5-MeO-ABAO to $NaIO_4$, although attempts to isolate and characterize the byproduct were unsuccessful owing to the instability of the compound. Optimization studies showed that the production of the byproduct was suppressed by the use of HEPES buffer relative to phosphate or acetate buffer systems, and that high $NaIO_4$ concentrations or extended reaction times led to increased byproduct production (Figure S6). This unwanted side reaction was eliminated

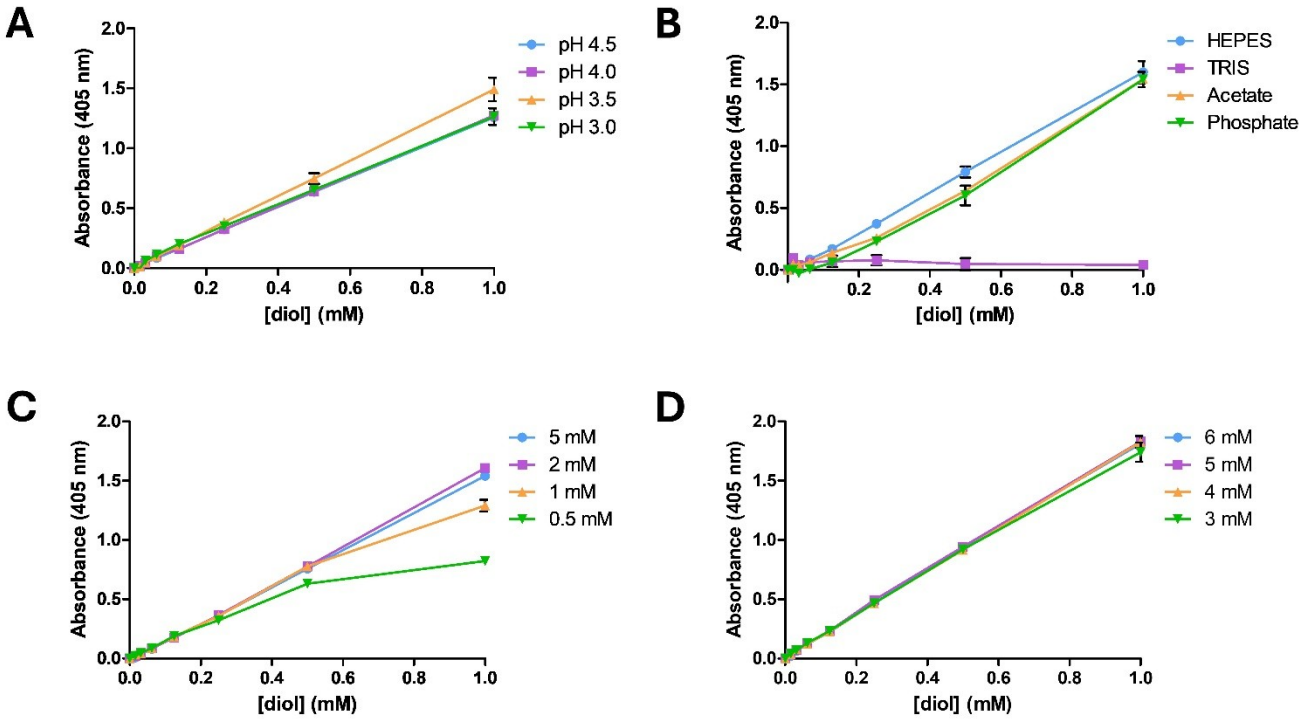


Figure 2. Assay Optimization Studies; (A) Absorbance response at varied pH levels (0.1 M HEPES, 5 mM 5-MeO-ABAO, 2 mM sodium metaperiodate) (B) Absorbance response in varied buffer systems (0.1 M, 2 mM $NaIO_4$, 5 mM 5-MeO-ABAO, pH 3.5) (C) Absorbance response at varied sodium (meta)periodate concentrations (0.1 M HEPES buffer, 5 mM 5-MeO-ABAO, pH 3.5); (D) Absorbance response at varied probe (5-MeO-ABAO) concentrations (0.1 M HEPES, 2 mM $NaIO_4$, pH 3.5). All experiments were performed in triplicate. Data points with error bars not visible demonstrated S. D. $<5\%$. All absorbance values normalized to the mean absorbance of the negative controls.

by the use of HEPES buffer, low concentrations of $NaIO_4$ (2 mM), and short reaction times (<5 min).

To confirm the utility of the assay system in detecting/quantifying diol metabolites produced from diverse classes of alkenes, the response of the assay system to increasing concentrations of a cyclic diol (cyclohexene-1,2-diol), an exocyclic diol (1-cyclohexylethane-1,2-diol), and to terminal and internal linear diols (octane-1,2-diol and octane-3,4-diol) was tested (Figure 3). These studies confirmed that the assay system was responsive to the presence of all of these classes of diol metabolites (Figure 3). Henceforth, the described optimized assay system is referred to as the metaperiodate 2-amino-benzamidoxime diol assay (MPAD).

Having established the utility of MPAD for detecting/quantifying diol metabolites in buffered aqueous solutions, it remained to determine whether this system could detect the diol metabolites produced from the dihydroxylation of aliphatic olefins by live, respiring cells expressing RDOs. Exploratory tests indicated that this application would be complicated by a reaction between the 5-MeO-ABAO probe and excess glucose present in the growth media used for bacterial culture. This side reaction, which is not observed in the fluorescence-based MPFCD assay,[14] was confirmed by studies on the absorbance response of the assay system to the presence of glucose (Figure S7). To overcome this issue, a resting cell growth/biotransformation protocol was utilized, wherein cells expressing Rieske dioxygenases are treated with potential olefinic substrates only after being grown to confluence and resuspended in a HEPES buffer solution free from

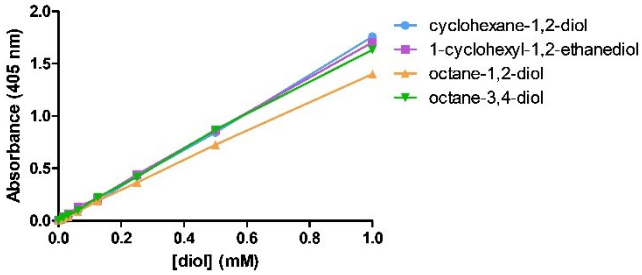


Figure 3. Absorbance response to varied diol compounds (0.1 M HEPES, 5 mM 5-MeO-ABAO, 2 mM $NaIO_4$, pH 3.5). All experiments were performed in triplicate. Data points with error bars not visible demonstrated S. D. $<5\%$. All absorbance values normalized to the mean absorbance of the negative controls.

glucose. To identify the optimal dioxygenase enzyme with which to carry out further whole-cell assay studies, expression systems for four wild-type RDOs developed in our laboratory (toluene dioxygenase (TDO) from *P. putida* F1,[5e] naphthalene dioxygenase (NDO) from *P. putida* G7,[18] cumene dioxygenase (CDO) from *P. fluorescens* IP01,[19] and biphenyl dioxygenase (BPDO) from *Rhodococcus* strain sp. RHA1[20]) were utilized. These four wild-type RDOs were tested for their activity in the dihydroxylation of cyclohexene and 1-hexene using the MPAD assay system (Figure S8). From these studies, it was shown that TDO possessed the greatest native activity for the dihydroxylation of both cyclohexene and 1-hexene (Figure S8), therefore all further studies employing whole-cell biotransformations utilized this enzyme system or variants thereof.

Despite TDO demonstrating the greatest activity in the dihydroxylation of 1-hexene and cyclohexene among the RDOs tested in this study, the activity of TDO in this context, and therefore the resultant absorbance responses from the MPAD assay system, was relatively low. In this light, it was determined that to properly investigate the potential utility of the MPAD assay system, an RDO variant with greater activity in the dihydroxylation of aliphatic olefins was required. To this end, a series of TDO variants bearing active site mutations that have previously demonstrated improved activity in other contexts[15] were screened for their activity in the dihydroxylation of 1-hexene and cyclohexene (Figure S9). Among these, TDO V309G, bearing an active site valine-to-glycine mutation, demonstrated improved activity over the wild-type enzyme in the dihydroxylation of both substrates (Figure S9). Using this TDO V309G variant, a checkerboard assay was performed, wherein a 96-well plate was alternately inoculated with single colonies of *E. coli* (BL21 (DE3)) expressing TDO V309G, and a negative control (*E. coli* (BL21 (DE3) pCP-01))[14] (Figure 4). The cultures were then grown according to the described resting cell protocol, and treated with a solution of a model olefinic substrate (1-hexene). After a period of incubation with the substrate, the cells were pelleted and the buffered aqueous media was transferred to new, clear-bottom 96-well plates for treatment under the MPAD assay conditions (Figure 4A). The significant increase in the absorbance response observed from cultures expressing TDO V309G over the negative control (Figure 4B) demonstrates the power of this assay system to detect the diol metabolites produced from the dihydroxylation of aliphatic olefins by respiring cells.

As described, the intended application of the MPAD assay system is in the high-throughput screening of large libraries of RDO variants for their activity in the dihydroxylation of aliphatic olefins. Having demonstrated the value of the assay system in detecting the relevant metabolites produced by respiring cells, the utility of the system in high-throughput screening could be assessed. To this end, saturation mutagenesis was applied to produce a library of TDO variants at the valine-309 position through the procedure of Liu and Naismith.[21] Given that the TDO V309G variant had been shown to possess improved activity over the wild-type enzyme for the dihydroxylation of multiple aliphatic olefins, it was expected that a functioning assay system should identify this variant from a larger library in screening studies. The plasmid library bearing mutations at the valine-309 position was transformed into *E. coli* (BL21 (DE3)) and single colonies were inoculated into a 96-well plate along with the wild-type enzyme (*E. coli* (BL21 (DE3)) pCP-02)[14] and negative controls enzyme (*E. coli* (BL21 (DE3)) pCP-01).[14] The cultures were grown to confluence and treated with a model olefinic substrate (*cis*-2-octene), with the biotransformation being performed using the described resting cell protocol. After a period of incubation with the substrate, the cells were pelleted and the buffered aqueous media was transferred to new 96-well plates and treated under the MPAD assay conditions (Figure 5A). Upon measuring the absorbance from each culture produced by the MPAD assay system, it was clear that several cultures demonstrated increased absorbance over the wild-type

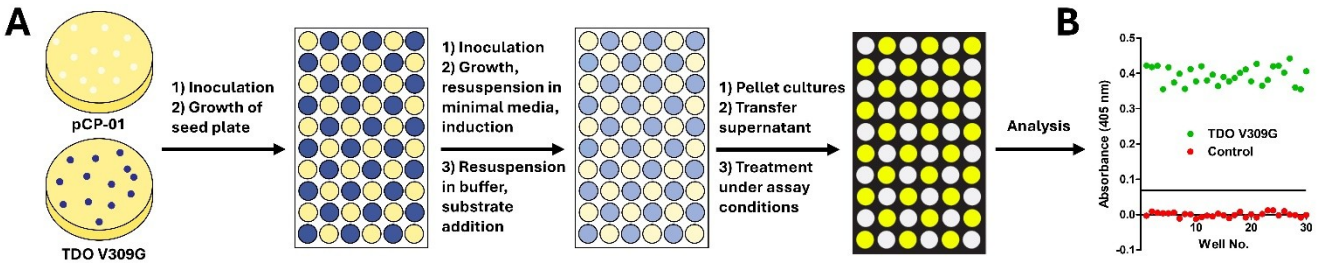


Figure 4. (A) Schematic overview of the procedure for the whole-cell checkerboard assay; (B) Normalized absorbance data for checkerboard assay with 3σ threshold indicated (0.074). The statistical effect size (Z') for this assay was calculated as 0.76. Absorbance of cultures expressing toluene dioxygenase V309G (*E. coli* BL21 (DE3)) ($n = 30$) were normalized to the mean absorbance of the negative controls (*E. coli* BL21 (DE3) pCP-01)[14] ($n = 30$).

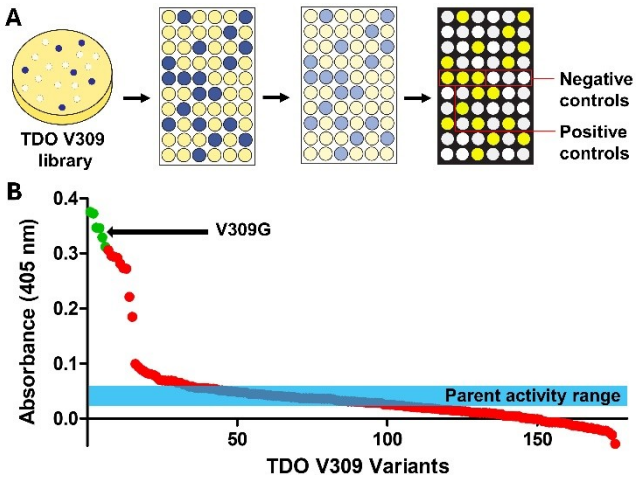


Figure 5. (A) Schematic overview of the procedure for the whole-cell toluene dioxygenase (TDO) V309 variant screening assay; (B) Activity of TDO V309 variants in the dihydroxylation of *cis*-2-octene as detected by the MPAD assay system ($n = 172$). Variants for which activity was validated through secondary screening and which were determined through sequencing to bear V309G mutations are indicated (green). Absorbance response of each variant was normalized to the mean absorbance of the negative controls (*E. coli* BL21 (DE3) pCP-01).[14] Parent activity range was determined from positive controls expressing wild-type TDO (*E. coli* BL21 (DE3) pCP-02),[14] with the absorbance response normalized to the mean absorbance of the negative controls (*E. coli* BL21 (DE3) pCP-01).[14]

enzyme, indicating that these TDO variants possessed improved dihydroxylation activity for *cis*-2-octene (Figure 5B). To confirm the activity observed among these variants in the primary screen, the plasmid DNA encoding the TDO variants was isolated and re-transformed into *E. coli* (BL21 (DE3)). The cells expressing the TDO variants were then carried through the growth/biotransformation/MPAD assay protocol a second time to validate their activity. Following the secondary screen, variants with confirmed activity were submitted for sequencing analysis, which revealed that each of the improved variants identified from this proof of concept screening study carried the V309G active site mutation. In this way, the practical utility of the MPAD assay system in the screening of large enzyme variant libraries, and its ability to identify improved enzyme variants, was conclusively demonstrated.

In summary, a sensitive high-throughput assay system has been developed that is capable of detecting/quantifying the diol metabolites produced from the enzymatic dihydroxylation of alkyl olefins. This system has been named as the metaperiodate 2-aminobenzamidoxime diol (MPAD) assay. Assay optimization studies were carried out to identify the conditions under which the sensitivity of the assay system can be maximized and under which any unwanted side reactions can be eliminated. The utility of the assay system in the whole-cell assays was also demonstrated when a resting cell protocol was employed. Proof of concept screening studies were also carried out, in which the MPAD assay system was able to identify members of a large enzyme variant library with greater dihydroxylation activity than the wild type enzyme. These studies led to the identification of TDO V309G as an improved enzymatic catalyst for the dihydroxylation of *cis*-2-octene. Because the MPAD assay relies on relatively cheap and readily available materials and does not require the use of specialized equipment to carry out, this assay system represents an accessible option for the high-throughput screening of dioxygenase enzymes, or any other system that generates vicinal diol moieties. In future studies, the MPAD assay will be employed to engineer improved Rieske dioxygenase variants with enhanced and expanded reactivity in the enantioselective dihydroxylation of aliphatic olefins. The availability of such enzymes will provide a sustainable alternative for performing this valuable synthetic transformation to the chemical community.

Experimental Section

General Experimental

E. coli BL21 (DE3) competent cells were obtained from ThermoFisher. Plasmid isolation/purification was performed using the New England Biolabs Monarch® miniprep kit. Transformations of electrocompetent cells were performed on an Eppendorf Eporator®. Whole-cell assay cultures were grown in Greiner Bio-One polystyrene clear, round-bottom 96-well plates. All cultures were incubated in a Barnstead MaxQ 4000 Digital Orbital Incubator Shaker equipped with an Enzyscreen universal clamp system unless otherwise stated. Absorbance analyses were performed using a Biotek® Synergy™ H1 monochromator-based multi-mode plate reader, using Nunclon® polystyrene clear, flat-bottom 96-well plates. All reagents were obtained from MilliporeSigma unless otherwise stated. Media were made at pH 7.2 and streptomycin was added at 50 $\mu g\,mL^{-1}$. All *E. coli* cultures were maintained at 37 °C unless otherwise stated. NMR analyses were performed using a Jeol ECZ 400S (400 MHz) instrument.

Synthesis of 5-Methoxy-2-amino-benzamidoxime (5-MeO-ABAO)[16]

1.18 g of 5-methoxy-2-aminobenzonitrile (8 mmol, 1 eq.) was dissolved in 16 mL EtOH in a 50 mL round-bottom flask by stirring at room temperature. 0.61 g (8.8 mmol, 1.1 eq.) hydroxylamine hydrochloride was added to the stirring mixture, followed by 0.81 g sodium bicarbonate (9.6 mmol, 1.2 eq.) dissolved in 6.4 mL H_2O. The reaction mixture was stirred at room temperature for 15 min before being heated to reflux and stirred for 96 h. The reaction mixture was then diluted with 16 mL H_2O and concentrated under reduced pressure. The

resultant crystalline product was obtained through vacuum filtration and washed with DCM. The product was obtained as a light brown crystalline solid (1.10 g, 76% yield).

Preparative-Scale Synthesis of Diols for Assay Studies[1b]

1.4 g of AD-mix-α ($(DHQ)_2PHAL$ 0.0032 eq.; K_2CO_3 1.0 eq.; $K_3[Fe(CN)_6]$ 1.0 eq.; $K_2OsO_4 \cdot 2H_2O$ 0.0014 eq.) was dissolved in 10 mL of *t*-BuOH/H_2O (50:50) by stirring at room temperature in a 25 mL round-bottom flask. 95 mg (1 mmol, 1 eq.) methane sulfonamide was added for 1,2-disubstituted olefinic substrates. The reaction mixture was then cooled to 0 °C in an ice bath. Olefinic substrates (1 mmol, 1 eq.) were added to the stirring mixture and the reaction was warmed to room temperature overnight (~16 h). The reaction was quenched by the addition of 1.5 g sodium sulfite and stirred at room temperature for 1 h. The reaction mixture was extracted with 3×15 mL EtOAc. The combined organic extracts were washed with 2 M aq. KOH in cases where methane sulfonamide was used. The combined organic extracts were dried with anhydrous $MgSO_4$ and concentrated under reduced pressure. Crude reaction mixtures were purified using silica gel chromatography (EtOAc/Hex) to afford analytically pure diol products.

Assay Protocol for Optimization Studies

Stock solutions of *cis*-diols were prepared in 0.1 M specified buffer systems. Stock solutions of $NaIO_4$ were freshly prepared immediately before use in the assay. Stock solutions of 5-MeO-ABAO were prepared in 0.1 M specified buffer systems. 100 µL of *cis*-diol stock solutions were transferred in 96-well clear, flat-bottom assay plates. Negative controls were included for each assay plate (buffer media without *cis*-diol was used, negative controls were otherwise treated identically). Oxidative cleavage was initiated by adding 40 µL of $NaIO_4$ stock solution to each well, and the assay plate was incubated with shaking at room temperature for 30 min. Aldehyde detection was performed by adding 60 µL of 5-MeO-ABAO stock solution to each well. The absorbance of each well at 405 nm was analyzed and normalized to the mean absorbance of the negative controls.

Whole-Cell Fermentation 96 Well-Plate Assay Protocol[14]

E. coli (BL21 (DE3)) electrocompetent cells were transformed with isolated pCP-02 plasmids expressing toluene dioxygenase (parent and/or mutant libraries), and with isolated pCP-01 plasmids as negative controls.[14] The transformation cultures were selected on LB + streptomycin plates overnight. Single colonies were inoculated into 160 µL LB + streptomycin media with 0.3% glucose in a 96-well round bottom seed plate and incubated with shaking at 37 °C overnight. Seed plates were used to inoculate 5 µL into 145 µL LB media containing streptomycin in a fresh 96-well round bottom assay plate, and the cultures were incubated with shaking at 37 °C for 2 h 50 min. The assay plates were then pelleted, and the supernatant was discarded. Cultures were resuspended in 145 µL minimal media (KH_2PO_4-7.5 g L^{-1}; citric acid-2 g L^{-1}; $MgSO_4 \cdot 7H_2O$-5 g L^{-1}; trace metal solution-2 mL L^{-1} [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]; conc. H_2SO_4-1.2 mL L^{-1}; ferric ammonium citrate-0.3 g L^{-1}; glucose-4 g L^{-1}; thiamine-0.034 g L^{-1}; pH 7.2) containing streptomycin and incubated for a 1 h recovery period at 37 °C. Following this, the cultures were induced to a final concentration of 0.5 mM IPTG, and the incubation temperature was reduced to 30 °C. After a 2 h induction period, the cultures were pelleted, and the supernatant was discarded. The cultures were resuspended in 120 µL of HEPES buffer (0.1 M, pH 7.2) and the substrate(s) were added as 50 mM stock solutions in DMSO to a final concentration of 2 mM. Cultures were incubated with substrates overnight at 30 °C, after which the cultures were pelleted. A 100 µL portion of supernatant from each well was transferred to 96-well clear, flat-bottom assay plates. The reaction was initiated by adding 40 µL of $NaIO_4$ stock solution to each well to a final concentration of 2 mM, and the assay plates were incubated with shaking at room temperature for 30 min. Cleaved diols were detected by adding 60 µL of 5-MeO-ABAO stock solution (prepared with 15.7 µL conc. HCl (11.65 M)/1 mL 5-MeO-ABAO solution) to each well to a final concentration of 5 mM. The absorbance of each well at 405 nm was analyzed and normalized to the mean absorbance of the negative controls.

Mutant Library Generation

The pCP-02 expression system was used as the template for toluene dioxygenase mutant library generation.[14] Saturation mutagenesis was performed following the procedure of Liu and Naismith.[21] Amplification was performed using an ABI GeneAmp® 9700 Thermal Cycler. Mutagenic primers were designed according to the procedure of Liu and Naismith[21] (TDO V309 forward primer-GAAACTCATG**NNK**GAGCA-CATGACCGTCTTCCCCACGTGTTCCTTC; TDO V309 reverse primer-CATGTGCTC**MNN**CATGAGTTTCGAGCCGCGCTCCACG-CTACCCAG).[15a] Sequencing analyses were performed by Eurofins Genomics© (Louisville, KY).

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