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Biocatalytic Oxidation of 2-Methylquinoxaline to 2-Quinoxalinecarboxylic Acid

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Abstract:

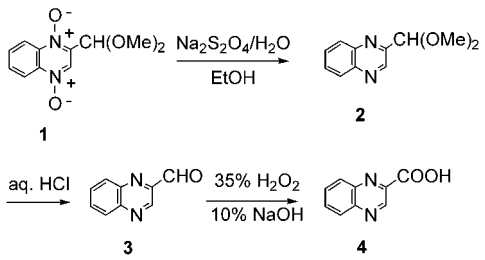
A microbial process using the fungus *Absidia repens* ATCC 14849 is described for the oxidation of 2-methylquinoxaline to 2-quinoxalinecarboxylic acid. A campaign consisting of three 14000-L runs produced 20.5 kg of the acid with a 28% overall yield. The bioconversion gave a lower yield compared with a three step chemical synthesis (35%), but was carried out in one pot, and avoided safety issues with a di-*N*-oxide intermediate. Although successfully scaled to produce kilograms of 2-quinoxalinecarboxylic acid for synthesis of a drug candidate, the *A. repens* bioconversion is unsuitable for further scale-up due to low product concentration (~1 g/L). A second microbial process using *Pseudomonas putida* ATCC 33015 is also described for the oxidation of 2-methylquinoxaline. The *P. putida* bioconversion gave an 86% in situ yield at 8-L scale and yielded a product concentration approximately 10-fold greater than that of the *A. repens* bioconversion.

Introduction

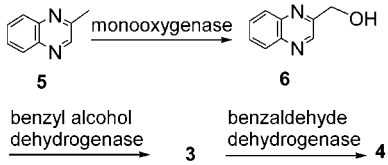
2-Quinoxalinecarboxylic acid **4** is used in the synthesis of a variety of biologically active compounds.[1] A recent project requiring kilograms of **4** prompted our investigation of scalable methods, since **4** was not commercially available in kilogram quantities at the time. Compound **4** is also a metabolite of Carbadox, a feed additive used to promote growth and control dysentery and bacterial enteritis in swine.[2] During investigations conducted in the 1970s, we prepared **4** from the di-*N*-oxide **1**, via the route shown in Scheme 1. While this route was demonstrated at 25 kg scale (35% overall yield), this synthesis was deemed unsuitable for further scale-up due to the mutagenic[3] and thermal[4] properties of **1**.

These safety issues prompted us to examine alternative chemical methods, none of which led to a suitable synthesis of **4**. A recent report describing the aerobic oxidation of methylpyridines to pyridinecarboxylic acids in the presence of Co(II), Mn(II), and a radical catalyst suggested that **4** could be produced by oxidation of 2-methylquinoxaline **5**.[5] This chemistry was not evaluated due to our lack of facilities for performing reactions in an oxygen atmosphere or under high pressure.

Scheme 1. **Chemical synthesis of 4**



Scheme 2. **Biocatalytic oxidation of 5 to 4**



In addition to synthetic approaches, we also examined biocatalytic methods for the oxidation of **5** to **4**. Biocatalytic oxidation of **5** would likely occur via the sequence shown in Scheme 2, based on known pathways for aromatic methyl group oxidation in bacterial and fungal systems. In the biosynthesis of patulin by the fungus *Penicillium griseofulvum*, transformation of *m*-cresol to *m*-hydroxybenzoic acid occurs in three steps, beginning with hydroxylation of the methyl group and followed by sequential oxidation of the alcohol and aldehyde intermediates.[6] The bacterium *Pseudomonas putida* ATCC 33015 can grow on a variety of methylated aromatic compounds, including toluene, *m*-xylene, and *p*-xylene, as sole carbon and energy source, by virtue of a degradative pathway that also begins with hydroxylation of an aromatic methyl group followed by oxidation to the carboxylic acid.[7] *P. putida* ATCC 33015 has also been shown to oxidize methyl groups in a variety of five- and six-membered heteroaromatic compounds, and

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Table 1. **Microbial conversions of 5 to 4 in test tube cultures**

microorganism	ATCC no.	**4** (%) 0.2 g/L **5**[a]	**4** (%) 1 g/L **5**[b]
Absidia glauca	22752	83	40
Absidia repens	14849	83	77
Aspergillus tamarii	16865	53	17
Alternaria solani	11078	53	1
Penicillium glabrum	11080	47	0
Diplodia gossypina	20575	31	24
Cunninghamella echinulata	8983	25	23

[a] In situ yields of **4** 4 days after treatment with **5** at 0.2 g/L. [b] In situ yields of **4** 12 days after treatment with **5** at 1 g/L.

is used in the commercial production of 5-methylpyrazine-2-carboxylic acid from 2,5-dimethylpyrazine.[8] To our knowledge, the biocatalytic oxidation of alkyl substituents on bicyclic heteroaromatic compounds such as **5** has not been reported. In this contribution, we report methods for the biocatalytic oxidation of **5** to **4** using bacterial and fungal whole cell systems.

Results and Discussion

We began our studies by evaluating the ability of *P. putida* ATCC 33015 to oxidize **5** to **4**. In experiments performed in flasks and 10-L fermentor cultures, we observed the production of **4** by *P. putida* cells grown in the presence of *p*-xylene and incubated with **5**. These preliminary experiments afforded in situ yields up to 81% starting from an initial substrate load of 1 g/L, thus demonstrating the ability of *P. putida* to perform the desired transformation. However, scale-up of a *P. putida*-based process requiring *p*-xylene induction was considered problematic for our fermentation pilot plant facility due to a potential explosion hazard from *p*-xylene. Thus, we initiated a parallel effort to identify an organism that could carry out the transformation without the addition of a potentially hazardous enzyme inducer. A screen was performed on over 200 microorganisms from our in-house culture collection including species of fungi and bacteria. Test tube cultures of individual organisms were grown for 2 days in Iowa medium and then incubated with **5** at an initial concentration of 0.2 g/L for 4 days. Extracts of culture broths were analyzed by HPLC revealing the presence of **4** in 32% of the cultures. While most of these organisms produced low yields of **4**, seven produced in situ yields of 25% or higher (Table 1). These organisms were re-tested at a substrate concentration of 1 g/L to assess their ability to produce higher product concentrations, a factor that affects production scale and product recovery and that is therefore a crucial requirement for commercial feasibility. While all of the organisms produced lower yields of **4** at the higher substrate concentration (Table 1), *Absidia repens* ATCC 14849 was selected for further evaluation since it produced similar yields of **4** at both substrate levels. Further experiments in test tube cultures of *A. repens* revealed no production of **4** at an initial substrate load of 3 g/L, suggesting toxicity of the substrate to the organism.

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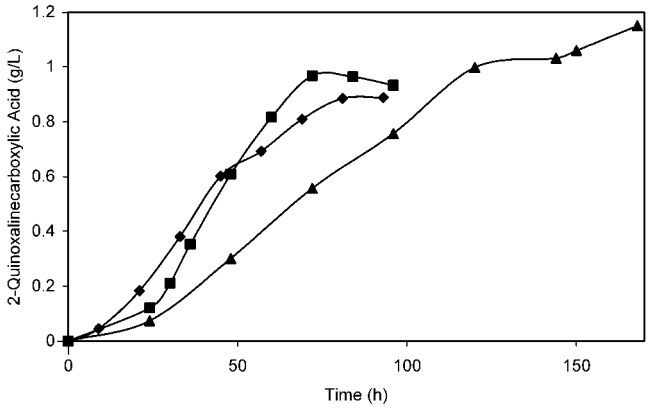

Figure 1. **Production of 4 in *A. repens* bioconversions at 8-L (◆), 100-L (■), and 14000-L (▲) scale.**

The next step in the development of the fermentation process was to evaluate the bioconversion in shake flask cultures. Flask cultures (300-mL Erlenmeyer flask; 25 mL of Iowa medium) of *A. repens* incubated with 1 g/L substrate produced **4** in 80% yield, but required 24 days of incubation at 29 °C and 210 rpm agitation. The extreme increase in reaction time observed in scaling from test tube to flask cultures was surprising since fermentation parameters, including inoculum ratio, were kept constant as much as possible. Although reaction time was a concern, the bioconversion of **5** to **4** in test tube and flask cultures of *A. repens* resulted in a product mixture that appeared clean by HPLC, with little or no starting material and no organism-related byproducts. Only one reaction intermediate, 2-hydroxymethylquinoxaline **6**, was observed in the early stages of the bioconversion. Aldehyde **3** was not detected in fermentation broth at any time during the bioconversions. Despite the potential limitations due to low substrate loading and long reaction times observed in test tube and flask experiments, 8-L fermentation trials were attempted to assess further scalability. Initially, bioconversions in 8-L fermentors were done using the same medium used in flask-scale runs with 1 g/L substrate added 2 days after inoculation. The results of these 8-L runs were poor, with in situ yields of 50% after 11 days. However, the process was greatly improved by changing to a glucose, corn steep solids medium, and adding a continuous nutrient feed containing glucose and yeast extract. Under these conditions, 1 g/L substrate was converted to **4** in 74% yield after 4 days (Figure 1). A further increase in scale to 100 L using the glucose, corn steep solids medium, gave even better results, reaching a maximum yield of 80% (0.97 g/L in broth concentration) after 3 days (Figure 1). While a product concentration of 0.97 g/L is unlikely to be suitable for a commercial process, it was satisfactory to meet our immediate needs. Thus, we proceeded to scale the process into 14000-L fermentors. Three 14000-L bioconversion runs were carried out using a two-stage fermentation process similar to the one described for the 100-L bioconversion, except that a total of 1.5 g/L substrate was added using a fed-batch protocol. This protocol resulted in a lower overall yield from **5** (57% average in situ yield) compared to the batch processes used at 8- and 100-L scale, but produced approximately the same product

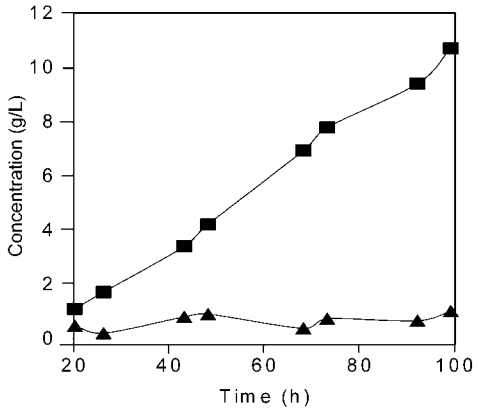

Figure 2. ***P. putida*** **bioconversion of 5 (▲) to 4 (■) at 8-L scale.**

concentration (1.0 g/L average concentration) and therefore maintained the volumetric productivity observed at lower scale. Production of **4** at 14000-L scale (Figure 1) was slower compared to that from the smaller-scale bioconversions, possibly due to a longer lag phase in the growth of the organism.

The isolation of **4** from the *A. repens* bioconversion was accomplished using the following process. The crude bioconversion broth was filtered to remove biomass, acidified to pH 3, and passed through a column packed with XAD-16 resin. Compound **4** was then eluted from the resin with phosphate buffer (pH 8.5) and extracted into methylene chloride. Four extractions were required to give a 90% yield for this step. Concentration of the methylene chloride extract and cooling to 5 °C afforded **4** as an off-white powder. This process recovered 49% of the **4** present in the crude bioconversion broth. Recovery of product from the three 14000-L runs yielded 20.5 kg of **4**. The overall yield of the *A. repens* bioconversion process at 14000 L was 28% based on a 57% bioconversion yield and a 49% recovery yield.

The *A. repens* bioconversion process gave a lower overall yield compared with the synthetic process, but was carried out in a single pot, and eliminated safety hazards associated with the chemical process. The major drawback of the bioconversion process is low product concentration, which limits production capacity and increases solvent consumption for recovery. Even with 70% solvent recovery, almost 26000 L of methylene chloride was used for the campaign, thus illustrating the challenge of recovering product from a dilute aqueous product stream. High product concentration is therefore a crucial requirement for a commercially feasible bioconversion process. With this goal in mind we reexamined the *P. putida* bioconversion. This time we directed efforts towards using an inducer that poses less of an explosion hazard than *p*-xylene and selected benzyl alcohol for evaluation due to its lower volatility. Trials at 8-L scale showed that *P. putida* could indeed catalyze the bioconversion of **5** using benzyl alcohol as inducer and sole carbon source, and produced greater than 10 g/L **4** (Figure 2). The *P. putida* bioconversion of **5** is sensitive to the accumulation of substrate and benzyl alcohol and performs poorly at substrate concentrations above 1.5 g/L and benzyl alcohol concentrations above 1 g/L. Therefore, carefully controlled additions of substrate and benzyl alcohol are very important for achieving a successful bioconversion. Further improvements to the process should be possible by implementing feedback controls for substrate and inducer additions.

Table 2. ***P. putida*** **bioconversion of bicyclic heteroatomatic compounds**

substrates	yield (%)[a]	substrates	yield (%)[a]
5	106	8-methylquinoline	17
2-methylquinoline	0	3-methylisoquinoline	21
3-methylquinoline	9	2-methylindole	1
4-methylquinoline	0	5-chloro-2-methylindole	0
6-methylquinoline	1		

[a] Average in situ yield of carboxylic acids from duplicate flasks after 45 h.

P. putida ATCC 33015 oxidizes methyl groups in a wide variety of methylated five- and six-membered heteroaromatic compounds, and in the case of mono- and dimethylated pyridine and piperazine analogues, methyl groups at all positions were susceptible to oxidation except those adjacent to another methyl group.[8] To examine the specificity of *P. putida* for methylated bicyclic heteroaromatic compounds, we incubated *P. putida* cells with methylated quinoline, isoquinoline, and indole derivatives. The results (Table 2) show that methyl groups on 3- and 8-methylquinoline and 3-methylisoquinoline were susceptible to oxidation, but with much lower yields compared to that from **5**. Methyl groups on 2-, 4-, and 6-methylquinoline were not oxidized, except for a trace of product from 6-methylquinoline. The lack of reaction for 2-methylquinoline was very surprising, considering the ability of *P. putida* to oxidize methyl groups on **5** and pyridine and piperazine derivatives as described above. Although these results do not provide a clear picture regarding the substrate specificity of *P. putida* for methyl groups on nitrogen-containing, bicyclic, heteroaromatic compounds, it appears that compounds with two nitrogens, such as quinoxaline and piperazine, are better substrates than quinoline derivatives.

Conclusions

In summary, we demonstrated two bioconversion processes for the oxidation of **5** to **4** that eliminated safety hazards associated with the synthetic process. A fungal process using *A. repens* was demonstrated at 14000-L scale, and while not commercially feasible due to low product concentration, yielded kilograms of material for the synthesis of a drug candidate. A second bioconversion process using *P. putida* ATCC 33015 was demonstrated at 8-L scale. The *P. putida* process yielded a product concentration greater than 10 g/L and is therefore more likely to be commercially feasible.

Experimental Section

Chemicals. Chemicals, reagents, and solvents were obtained from commercial sources and used without purification. All of the substrates for microbial bioconversions were purchased from Aldrich, as were samples of the corresponding carboxylic acids, except for 6-quinolinecarboxylic acid (Eastman Fine Chemicals). **1** was obtained from Farchemia.

Analytical Methods. The amounts of substrates and carboxylic acid products in broth samples from bioconversion experiments were determined using HPLC methods described below. These HPLC methods were calibrated using authentic samples of the substrates and carboxylic acids.

Method 1: Inertsil C8 column (4.6 mm × 250 mm, Column Engineering, Inc.) eluted isocratically with acetonitrile:0.05% TFA (1:4, v/v) at a flow rate of 1 mL/min with UV detection at 236 nm. Retention times for **4** and **5** were approximately 7.9 and 12.6 min, respectively.

Method 2: Symmetry C18 column (3.9 mm × 150 mm, Waters Corp.) eluted isocratically with acetonitrile: 0.05% TFA (1:9, v/v) at a flow rate of 1 mL/min with UV detection at 236 nm. Retention times for 2-methylquinoline, 2-quinolinecarboxylic acid, 3-methylquinoline, 3-quinolinecarboxylic acid, 4-methylquinoline, 4-quinolinecarboxylic acid, 6-methylquinoline, 6-quinolinecarboxylic acid, 3-methylisoquinoline, and 3-isoquinolinecarboxylic acid were approximately 2.4, 3.9, 3.1, 4.3, 3.0, 1.5, 3.9, 2.1, 3.0, and 2.5 min, respectively.

Method 3: Symmetry C18 column (3.9 mm × 150 mm) eluted isocratically with acetonitrile: 0.05% TFA (1:1, v/v) at a flow rate of 1 mL/min with UV detection at 236 nm. Retention times for 2-methylindole, 2-indolecarboxylic acid, 5-chloro-2-methylindole, and 5-chloro-2-indolecarboxylic acid were approximately 4.8, 2.1, 7.9, and 2.9 min, respectively.

Method 4: Discovery RPamide C16 column (4.6 mm × 150 mm, Supelco) eluted isocratically with acetonitrile: 0.05% TFA (1:9, v/v) at a flow rate of 1 mL/min with detection at 236 nm. Retention times for 8-methyquinoline and 8-quinolinecarboxylic acid were 2.4 and 2.9 min, respectively.

Preparation of 2-Quinoxalinecarboxylic Acid 4 via Scheme 1. 2-Quinoxalinecarboxaldehyde, Dimethylacetal 2. (**CAUTION**: Explosion hazard) An aqueous solution of sodium hydrosulfite (35.7 kg in 146 L of water) was added to 24.4 kg of **1** dissolved in 110 L of ethanol. This addition was completed within 0.5 h during which the reaction temperature increased from 20 to 50 °C. After 1 h, the reaction mixture was cooled to 35 °C and extracted with chloroform (5 × 69 L). The combined chloroform extracts were incubated with 2.7 kg of Darco KB for 0.5 h and filtered after the addition of 0.9 kg of Celite. The filtrate was concentrated to a volume of approximately 38 L and transferred to the next step without further purification.

2-Quinoxalinecarboxaldehyde 3. HCl (85 L, 4 N) was added to the crude 2-quinoxalinecarboxaldehyde, dimethylacetal **2**, and heated to between 50 and 60 °C for 0.5 h. The reaction mixture was cooled to 26 °C and extracted with chloroform (4 × 68 L). The combined chloroform extracts were incubated with 2.5 kg of Darco KB for 0.5 h, filtered, concentrated, and transferred to the next step without further purification.

2-Quinoxalinecarboxylic Acid 4. Water was added to the 2-quinoxalinecarboxaldehyde **3** to give a total volume of 363 L. This mixture was incubated with 40.9 L of 35% hydrogen peroxide followed by 73 L of 10% aqueous sodium hydroxide. The aqueous sodium hydroxide addition was completed in approximately 1 h at 17−26 °C. The reaction mixture was heated to 80 °C for 1 h and then cooled to 25 °C. During the cooling period, an additional 4.3 g of sodium hydrosulfite was added. The reaction mixture was then washed with chloroform (4 × 68 L) and the aqueous layer separated and incubated with 7.5 kg of Darco KB. The aqueous layer was filtered, adjusted to pH 2.8 with HCl, and cooled to 10 °C for 2 h. Filtration of this mixture gave 8.95 kg of a brown solid after drying. This material was dissolved in 227 L of *N*,*N*-dimethylacetamide and incubated with 2.7 kg of Darco KB for 1 h. The mixture was filtered, diluted with 210 L of water, stirred overnight, and then cooled to 10 °C for 1 h. Filtration of this mixture gave 6.25 kg (35%) of solids (mp 196−196.5 °C) after washing with water and drying.

Microbial Screen for Bioconversion of 5 to 4. Cultures of various microorganisms were obtained from the American Type Culture Collection (Manassas, VA) and stored as glycerol suspensions of spores or vegetative cells at −70 °C. Microbial cultures were grown in glass culture tubes (16 mm × 125 mm) containing 2.5 mL of Iowa medium (20 g/L glucose, 5 g/L nutrisoy flour, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L K_2HPO_4; adjusted to pH 7 before sterilization). Individual tubes were inoculated with spores or vegetative cells of various microorganisms and incubated at 29 °C on a rotary shaker (210 rpm) for 48 h. Each culture was then incubated with 0.5 mg of **5** (0.05 mL of 10 mg/mL DMSO solution) and incubated for an additional 4 days. The culture broths were then acidified to pH 2 with 4 N HCl, extracted with EtOAc, and analyzed by HPLC (method 1).

***A. repens* ATCC 14849 Bioconversion of 5 to 4.** *Procedure A (8-L Bioconversion).* A 14 L fermentor (New Brunswick Scientific, New Brunswick, NJ) containing 8 L of glucose−corn steep solids medium (20 g/L cornsteep solids, 20 g/L glucose; adjusted to pH 4.85 before sterilization) was autoclaved at 121 °C for 0.5 h. The fermentor was inoculated with 80 mL of a glycerol suspension of *A. repens* ATCC 14849 spores harvested from surface cultures grown on rice, and was operated at 29 °C with agitation at 600 rpm, aeration at 8 L/min, and pH below 7.5. The bioconversion was started 44 h after inoculation by the neat addition of 8 g of **5**. A feed consisting of glucose (160 g/L) and yeast extract (32 g/L) was started immediately after substrate addition and maintained at a rate of 8 mL/h for the duration of the fermentation. Aliquots of fermentation broth were removed at various times, extracted with methanol, and analyzed by HPLC to determine yields of **4** and **5**. The bioconversion reached a maximum concentration of 0.89 g/L **4** at 93 h after substrate addition with no residual substrate detected. This concentration corresponds to a 74% in situ yield.

Procedure B (100-L Bioconversion). An 8-L seed culture of *A. repens* was prepared in a 14 L fermentor as described in procedure A and operated for 23 h without substrate addition or nutrient feed. This seed culture was used to inoculate 100 L of glucose−corn steep solids medium containing 1 g/L polypropylene glycol P2000 (Dow). The

100-L fermentor was operated at 28 °C, 250−300 rpm agitation, 57 L/min aeration, and pH below 7.5. The bioconversion was initiated 21 h after inoculation by the addition of 100 g of **5** dissolved in 0.5 L of ethanol. A nutrient feed (100 mL/h) containing glucose (160 g/L) and yeast extract (32 g/L) was initiated 3 h after substrate addition and maintained throughout the bioconversion. Aliquots of fermentation broth were removed at various times, extracted with methanol, and analyzed by HPLC to determine yields of **4** and **5**. This bioconversion produced a maximum concentration of 0.97 g/L **4** (80% in situ yield) at 72 h after substrate addition. A small amount of **5** (0.02 g/L) was detected at 72 h, but not in later samples.

Procedure C (14000-L Bioconversion). A seed culture *of A. repens* was prepared in a 2000-L fermentor containing 1100 L of glucose−corn steep solids medium. This fermentor was inoculated with 100 mL of an *A. repens* spore suspension and operated at 28 °C, 550 L/min aeration, 175 rpm agitation, and pH 5.0 for 37 h. The seed culture was then transferred to a 20000-L airlift fermentor containing 14000 L of the glucose−corn steep solids medium. This transfer was conducted while the seed culture was in exponential growth with a respiration rate of 24 mmol/L/h. Aeration was set at 11200 L/min, and the temperature was controlled at 28 °C. The bioconversion was started 30 h after inoculation by the addition of 14 kg of **5**. An additional 7 kg of **5** was added 137 h after inoculation. A continuous nutrient feed containing 25 g/L ardamine PH and 125 g/L glucose was initiated along with the first substrate addition at a rate of 28 L/h. The bioconversion was stopped 168 h after the initial substrate addition with a final concentration of 1.15 g/L **4** and a residual concentration of 0.075 g/L **5**.

Recovery of 4 from 14 000-L *A. repens* Bioconversion. **4** was recovered from a 14000-L *A. repens* bioconversion using the following general procedure. The fermentation broth from a 14000-L bioconversion was filtered through a 0.2 μm tangential flow ceramic filter (U.S. Filter), diluted with 10 mM aqueous potassium phosphate (∼3500 L), and adjusted to pH 3 with phosphoric acid. The acidified broth filtrate was loaded onto a column (2.44 m × 0.61 m, i.d.) containing 743 L of XAD-16 resin. The resin was washed with three bed volumes of 10 mM potassium phosphate (pH 3) and then eluted with five bed volumes (∼3700 L) of 100 mM potassium phosphate (pH 8.5). The eluate was adjusted to pH 2 with phosphoric acid, charged with sodium chloride (360 kg) to produce a 10% solution, and extracted with methylene chloride (4 × 2580 L). The methylene chloride extract was concentrated to approximately 1000 L and cooled to 5 °C for 4 h, and the resulting solids were collected and dried to give **4** as an off-white solid. This process was carried out on three 14000-L bioconversion runs resulting in the recovery of 20.5 kg of **4** (28% yield).

***P. putida* ATCC 33015 Bioconversion of 5 to 4.** A two-stage fermentation process was used for the bioconversion of **5** to **4** by *P. putida*. The first-stage culture was prepared by inoculating three 2.8 L Fernbach flasks, each containing 200 mL of mineral salts medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.12 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 1 mL/L trace elements solution, and 15 mL/L FeEDTA solution; trace elements solution: 10 g/L di-sodium EDTA.$2H_2O$, 9 g/L $ZnSO_4 \cdot 7H_2O$, 4 g/L $MnCl_2 \cdot 4H_2O$, 2.7 g/L H_3BO_3, 1.8 g/L $CoCl_2 \cdot 6H_2O$, 1.1 g/L $CuCl_2 \cdot 2H_2O$, 0.18 g/L $NiCl_2 \cdot 6H_2O$, 0.2 g/L, $Na_2MoO_4 \cdot 2H_2O$; FeEDTA solution: 5 g/L di-sodium EDTA$\cdot 2H_2O$, 2 g/L $FeSO_4 \cdot 7H_2O$) and 0.2 mL of benzyl alcohol with a glycerol suspension of *P. putida* ATCC 33015 cells. After agitation on a rotary shaker (200 rpm) for 24 h at 30 °C, the contents of the three first stage cultures were combined, and 400 mL used to inoculate 8 L of mineral salts medium in a 14-L fermentor. The mineral salts medium used in the fermentor was identical to the first stage medium except for the addition of P2000 (1.5 mL/L) to prevent foaming. The fermentor was operated at pH 7−7.5, 29−30 °C, 8 L/min airflow, and 600−850 rpm agitation. During the bioconversion, $(NH4)_2SO_4$ (20 g) and NH_4Cl (20 g) were added at 25 and 75 h after inoculation, respectively. Immediately after inoculation, a continuous feed of benzyl alcohol was initiated at a rate of 0.58 g/h and increased incrementally to a rate of 4.5 g/h by 76 h after inoculation. A total of 258 g of benzyl alcohol was added over the course of the bioconversion. A total of 82 g of **5** was added to the fermentor in increments of 2, 2, 6, 4, 2, 8, 4, 8, 6, 6, 8, 4, 4, 8, 4, and 6 g at 2, 5, 11, 18, 23, 35, 42, 46, 51, 56, 60, 66, 75, 83, 92, and 96 h after inoculation, respectively. Aliquots of fermentation broth were removed at various times, extracted with methanol, and analyzed by HPLC to determine yields of **4** and **5**. The bioconversion reached a maximum concentration of 10.7 g/L **4** (86% in situ yield) after 99 h.

***P. putida* ATCC 33015 Oxidation of Bicyclic Heteroaromatic Compounds.** An 8-L fermentor culture *of P. putida* ATCC 33015 was prepared and inoculated essentially as described in the previous section and operated at pH 7, 29 °C, 8 L/min aeration, and 600 rpm agitation. A continuous feed of benzyl alcohol was initiated after inoculation at a rate of 0.55 g/h, and increased to 1.0, 1.8, and 2.5 g/h at 22, 30, and 47 h, respectively. Ammonium chloride (10 g) was added to the fermentor 31 h after inoculation. At 53 h after inoculation, 200-mL aliquots of the *P. putida* culture broth were transferred to 18 Erlenmeyer flasks (1 L). Duplicate flasks were then incubated with 0.1 g of **5**, 2-methylquinoline, 3-methylquinoline, 4-methylquinoline, 6-methylquinoline, 8-methylquinoline, 3-methylisoquinoline, 2-methylindole, and 5-chloro-2-methylindole, and agitated (210 rpm) for 45 h at 29 °C. Samples were then centrifuged to remove cells, diluted with methanol, and analyzed by HPLC using methods 1−4.

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