J Ind Microbiol Biotechnol (2012) 39:1789–1799
DOI 10.1007/s10295-012-1189-7

BIOCATALYSIS

Microbial transformation of 2-amino-4-methyl-3-nitropyridine

Thomas Tully · Mark Liu · Yande Huang · Qingmei Ye · Ramesh N. Patel · Animesh Goswami

Received: 25 June 2012 / Accepted: 11 August 2012 / Published online: 29 August 2012

Abstract Biotransformation of the highly substituted pyridine derivative 2-amino-4-methyl-3-nitropyridine by *Cunninghamella elegans* ATCC 26269 yielded three products each with a molecular weight of 169 Da which were identified as 2-amino-5-hydroxy-4-methyl-3-nitropyridine, 2-amino-4-hydroxymethyl-3-nitropyridine, and 2-amino-4-methyl-3-nitropyridine-1-oxide. Biotransformation by *Streptomyces antibioticus* ATCC 14890 gave two different products each with a molecular weight of 169 Da; one was acid labile and converted to the other stable product under acidic conditions. The structure of the stable product was established as 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone, and that of the less stable product was assigned as its tautomer 2-amino-6-hydroxy-4-methyl-3-nitropyridine. Four of the five biotransformation products are new compounds. Several strains of *Aspergillus* also converted the same substrate to the lactam 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone. Microbial hydroxylation by *C. elegans* was found to be inhibited by sulfate ion. In order to improve the yield and productivity of the 5-hydroxylation reaction by *C. elegans*, critical process parameters were determined and Design of Experiments (DOE) analyses were performed. Biotransformation by *C. elegans* was scaled up to 15-l fermentors providing 2-amino-5-hydroxy-4-methyl-3-nitropyridine at ca. 13 % yield in multi-gram levels. A simple isolation process not requiring chromatography was developed to provide purified 2-amino-5-hydroxy-4-methyl-3-nitropyridine of excellent quality.

Keywords *Cunninghamella elegans* · *Streptomyces antibioticus* · Biotransformation · Hydroxylation · DOE

T. Tully (✉) · M. Liu · Y. Huang · Q. Ye · R. N. Patel · A. Goswami
Chemical Development, Bristol-Myers Squibb, One Squibb Drive, New Brunswick, NJ 08903, USA
e-mail: thomas.tully@bms.com

Introduction

Microbial hydroxylation is an excellent way of regioselectively hydroxylating aromatic compounds to produce phenol derivatives. Aromatic compounds with aliphatic side chains can be hydroxylated at that side chain also generating the corresponding alcohol [1]. Microbial hydroxylation of pyridine derivatives often leads to the addition of a hydroxyl group next to the ring nitrogen [8, 10] with the product often reported to be in the lactam form [5, 6]. Hydroxylation of the alkyl side chain of alkylpyridines has also been observed, and the microbial biotransformation of picolines has resulted in hydroxylation of the methyl group to yield the corresponding hydroxymethylated products [13]. The presence of a hydroxyl group at the 4-position of pyridine has facilitated introduction of a new hydroxyl group at the adjacent 3-position by microbial hydroxylation [11]. In contrast, microbial transformation of 3-hydroxypyridine has been demonstrated to yield two dihydroxy compounds with the addition of a new hydroxyl group at the 2- or 4-position, as well as N-oxide and 1,2-dihydro-2,3-dihydroxypyridine [12]. Introduction of a hydroxyl group at other positions in pyridine requires the presence of an adjacent hydroxyl group in the substrate. The present work describes the results of microbial hydroxylation of a highly substituted pyridine derivative, 2-amino-4-methyl-3-nitropyridine.

Materials and methods

The substrate 2-amino-4-methyl-3-nitropyridine was purchased from Aldrich.

Analytical

HPLC

HPLC method A utilized an isocratic mixture of 90 % solvent A (0.05 % trifluoroacetic acid in water) and 10 % solvent B (acetonitrile) on a YMC Pack ODS-AM S-5 μ 4.6 × 150 mm column at ambient temperature with a flow rate of 1 ml/min and total run time of 30 min. Substrate and products were monitored by UV detection at 220 and 254 nm. The retention time for the starting material 2-amino-4-methyl-3-nitropyridine was 7.1 min under these conditions. Method A was used during initial screening.

HPLC method B incorporated a gradient mixture of solvent A (0.05 % trifluoroacetic acid in water) and solvent B (acetonitrile) on a YMC Pack ODS-A S-3 μ 4.6 × 150 mm column at a flow rate of 1 ml/min at ambient temperature and UV detection at 220 or 254 nm for a total run time of 30 min. The gradient conditions were 0–8 min 5 % B, 8–30 min 5 to 20 % B. The retention time for the starting material 2-amino-4-methyl-3-nitropyridine (**I**) was 14.8 min. Retention times for the various products were as follows: 6.8 min for 2-amino-4-hydroxymethyl-3-nitropyridine (**III**), 12.4 min for 2-amino-4-methyl-3-nitropyridine-1-oxide (**IV**), 20.0 min for 2-amino-5-hydroxy -4-methyl-3-nitropyridine (**II**), 20.9 min for 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**), and 22.0 min for 2-amino-6-hydroxy-4-methyl-3-nitropyridine (**V**). Method B was developed to demonstrate the relative retention times of substrate and products in a single chromatogram.

HPLC method C employed an isocratic mixture of 85 % solvent A (0.05 % trifluoroacetic acid in water) and 15 % solvent B (acetonitrile) on a YMC Pro C18 3 μ 4.6 × 150 mm column at a flow rate of 1 ml/min at ambient temperature and UV detection at 254 nm for a total run time of 10 min. The retention time for the starting material 2-amino-4-methyl-3-nitropyridine (**I**) is 5.6 min under these conditions. Method C was developed for monitoring preparative biotransformation by *C. elegans* ATCC 26269 at the 15-l scale.

Preparative HPLC method D used an isocratic mixture of 90 % solvent A (0.05 % trifluoroacetic acid in water) and 10 % solvent B (acetonitrile) on a YMC S5 ODS 5 μ 20 × 100 mm column at a flow rate of 20 ml/min at ambient temperature and UV detection at 220 nm with a run time of 8.5 min. The retention time for the starting material 2-amino-4-methyl-3-nitropyridine (**I**) was 8.9 min. Retention times for the three products were as follows: 4.1 min for 2-amino-4-hydroxymethyl-3-nitropyridine (**III**), 6.8 min for 2-amino-4-methyl-3-nitropyridine-1-oxide (**IV**), and 12.9 min for 2-amino-5-hydroxy-4-methyl-3-nitropyridine (**II**). Method D was used for preparative isolation of compounds generated in flasks from *C. elegans* ATCC 26269.

Preparative HPLC method E used an isocratic mixture of 85 % solvent A (0.05 % trifluoroacetic acid in water) and 15 % solvent B (acetonitrile) on a YMC S5 ODS-A 20 × 250 mm column at a flow rate of 20 ml/min at ambient temperature and UV detection at 220 nm and a run time of 12 min. Retention times for the two products were as follows: 13.7 min for 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**) and 15.0 min for 2-amino-6-hydroxy-4-methyl-3-nitropyridine (**V**). Method E was used for preparative isolation of compounds generated in flasks from *S. antibioticus* ATCC 14890.

NMR spectroscopy

NMR spectra were recorded at 25 °C using DMSO-d_6 as solvent on a Bruker DRX 400 MHz instrument equipped with a 3 mm Nalrac inverse probe with the exception of ^{13}C spectrum which was collected with a dual probe. The ^{1}H and ^{13}C chemical shift values were reported relative to DMSO-d_6 ($\delta = 2.50$ ppm and $\delta = 39.5$ ppm for ^{1}H and ^{13}C, respectively) as the internal standard.

LC–MS

The molecular weights of the isolated products were measured by LC–MS using a short isocratic method with a mobile phase of 0.05 % trifluoroacetic acid in water and methanol (80:20 v/v) and a Phenomenex Synergi Hydro-RP 4 μm 4.6 × 50 mm column. The run time was 3 min with a flow rate of 3 ml/min. The mass spectra were obtained on a Micromass ZQ (Single Quadrupole) mass spectrometer operated under positive electrospray ionization mode. The source temperature was held at 125 °C. The desolvation temperature was 275 °C; the capillary voltage was 3.3 kV; cone voltage was 30 V; cone gas flow was 0 l/h; desolvation gas flow was 550 l/h.

Culture media

For the preliminary studies, several fungi (primarily *Aspergillus* and *Cunninghamella*) were tested in flasks, while multiple strains of bacteria were screened in flasks or multi-well plates. The following media were used during these studies.

F7 medium 2.0 % glucose, 1.0 % yeast extract (Difco), 0.1 % peptone (Difco), 1.0 % malt extract (Difco).

F7-M1 medium 2.2 % glucose monohydrate, 1.0 % yeast extract (Tastone 154, Sensient Bionutrients), 0.1 % soy hydrolysate (Hy-Soy, Quest), 1.0 % rice hydrolysate (Hy-Rice, Quest).

K45-M medium 1.5 % glucose monohydrate, 0.5 % yeast extract (Tastone 154), 0.5 % soy hydrolysate (Hy-Soy, Quest), 1.0 % soy flour (Toasted Nutrisoy, ADM), 0.5 % pea hydrolysate (Hy-Pea, Quest), 1.5 % glycerol.

SG medium 2.0 % glucose, 0.5 % yeast extract (Difco), 0.5 % soy flour (Toasted Nutrisoy, ADM), 0.5 % K_2HPO_4.

SG-M medium 2.2 % glucose monohydrate, 0.5 % yeast extract (Tastone 154), 0.5 % soy flour, 0.5 % K_2HPO_4.

SG-M1A medium 3.0 % glucose, 0.4 % yeast extract (Difco), 0.2 % soy flour, 0.5 % K_2HPO_4.

SG-M1B medium 3.3 % glucose monohydrate, 0.4 % yeast extract (Difco), 0.2 % soy flour, 0.5 % K_2HPO_4.

SG-M1B medium for 15-l fermentors 3.3 % glucose monohydrate, 0.4 % yeast extract (Difco), 0.2 % soy flour, 0.5 % K_2HPO_4, 0.04 % Dow Corning AF Emulsion, 0.04 % Ucon LB625 Antifoam (Dow).

A109 rice medium 15 g long grain rice, 12 ml water per 500-ml flask.

PDB Potato Dextrose Broth (Difco).

All media (except A109 rice) were adjusted to pH 7.0 and sterilized at 121 °C for 20–30 min in flasks and 30 min in 15-l fermentors.

Microbial screening

The substrate, 2-amino-4-methyl-3-nitropyridine (**I**), was prepared as a 50 mg/ml stock solution in DMSO and added to a final level of 0.05 % in the biotransformation medium containing the selected microorganisms. To initiate testing of fungi, inoculum (e.g., 5 ml of culture broth) was transferred to 500-ml flasks containing 100 ml of F7 medium. Following incubation at 28 °C and 250 rpm for 3 days, a 10 % transfer was made to 50-ml flasks containing 9 ml of SG medium. After an additional 18 h incubation, 100 µl of substrate solution was added. One day later, 1.0 ml was removed from each flask, diluted with 1.0 ml acetonitrile, and samples were assayed by HPLC. In some cases, additional assays were performed at daily intervals.

For bacteria, most of the screening incorporated the use of 24-well plates containing 1 ml SG medium per well or alternatively, 50-ml flasks containing 10 ml medium. Inoculated plates were incubated at room temperature by gentle agitation for 3 days followed by transfer to freshly prepared plates containing 1 ml SG medium per well. For flask screening, cultures were inoculated into 250-ml flasks containing 50 ml F7 medium and similarly grown for 3 days at 28 °C and 250 rpm. A 2 % transfer was then made to 50-ml flasks containing 10 ml SG medium. After an additional 24 h incubation of the second stage, substrate solution was added to either the plates or flasks, followed by incubation for 1–3 more days. Transfers and extractions using the 24-well plates were performed via a Gilson Liquid Handler.

Selected strains of interest from initial screening were scaled to 500-ml flasks containing 100 ml medium. Microbes were grown from vial to stage 1, and then to stage 2, as described above. Substrate solution was added to 18–24 h stage 2 cultures and the biotransformation was conducted at 28 °C and 250 rpm. Assays were performed by withdrawing a sample of whole broth at various intervals, adjusting to pH 7–8, and extracting with an equal volume of ethyl acetate. The ethyl acetate solution was evaporated, the residue dissolved in acetonitrile, and the acetonitrile solution was filtered through a 0.45-µm filter and analyzed by HPLC.

Biotransformation by *Cunninghamella elegans* ATCC 26269 in flasks

Cunninghamella elegans ATCC 26269 inoculum for biotransformation studies was generated by inoculating A109 rice medium with spores or viable cells and allowing sufficient time for sporulation (minimum of 1–2 weeks). A spore suspension was then prepared in water and used to inoculate F7 medium. The inoculum flasks (F1 stage) were typically incubated for ca. 18–30 h before transfer to biotransformation flasks (F2 stage) containing SG medium. Alternatively, a single stage consisting of only SG medium was also used. Substrate **I** was then added to a 0.05 % input 16–24 h later. Standard incubation conditions were 250 rpm at 28 °C. Assays were performed 1–5 days following substrate addition as described above. The ratio of substrate to the different products was estimated from HPLC area at 254 nm (or 220 nm where products were not present at sufficient levels to be detectable at the higher wavelength). Experiments employing pH control in flasks were run utilizing the DASGIP Fedbatch-Pro (Julich, Germany) fermentation system, which permitted up to 16 flasks to be run in parallel and evaluated at the same time.

Biotransformation by *C. elegans* and isolation of products by preparative HPLC

For the first growth stage, a 3-ml aliquot of *C. elegans* ATCC 26269 culture was inoculated into a 250-ml flask

containing 50 ml F7 medium and incubated at 28 °C and 250 rpm for 72 h. The broth was then blended aseptically using a Waring blender and 5 ml was transferred into a 500-ml flask containing 100 ml SG medium. After 20 h of incubation of this second stage, 1 ml of a 50 mg/ml solution of substrate **I** in DMSO was added to the flask. The biotransformation was monitored by HPLC and generated peak area ratios after 67 h of substrate **I**, 1.0; product **II**, 0.14; **III**, 0.64; and **IV**, 0.86. The whole broth was adjusted to pH 7.8 and extracted with 100 ml of ethyl acetate. The solvent was removed under vacuum and the residue was dissolved in acetonitrile. A small portion of the acetonitrile solution was used for HPLC assay and the remainder was again taken to dryness under vacuum to yield a brown solid. The crude solid was then dissolved in water (25 ml) and filtered through a 0.45-μm PTFE membrane filter. The aqueous solution was subjected to preparative HPLC method D and the relevant fractions were combined and lyophilized to afford yellowish powders for NMR analysis. Three pure products were obtained from three different fractions: 2-amino-5-hydroxy-4-methyl-3-nitropyridine (**II**), 2-amino-4-hydroxymethyl-3-nitropyridine (**III**), and 2-amino-4-methyl-3-nitropyridine-1-oxide (**IV**). LC–MS (ESI(+) *m*/*z* 170) showed the same molecular weight of 169 Da for each. The structures of **II**, **III**, and **IV** were determined by 1D and 2D NMR homo- and heteronuclear correlation experiments (COSY, HMQC, and HMBC). Compound **III** has recently been synthesized as an intermediate in the preparation of a drug development candidate [7].

2-Amino-5-hydroxy-4-methyl-3-nitropyridine (**II**) ^{1}H NMR: δ 2.16 (s, 3H), 7.85 (s, 1H). ^{13}C NMR: δ 12.2, 130.2, 131.9, 139.2, 143.8, 146.6.

2-Amino-4-hydroxymethyl-3-nitropyridine (**III**) ^{1}H NMR: δ 4.70 (s, 2H), 6.96 (d, 1H), 8.22 (d, 1H). ^{13}C NMR: δ 60.3, 110.2, 127.4, 150.9, 153.5.

2-Amino-4-methyl-3-nitropyridine-1-oxide (**IV**) ^{1}H NMR: δ 2.36 (s, 3H), 6.65 (d, 1H), 7.76 (bs, 2H), 8.31 (d, 1H). ^{13}C NMR: δ 19.2, 112.7, 131.7, 133.1, 138.7, 146.6.

Biotransformation by *S. antibioticus* and isolation of products by preparative HPLC

For the first growth stage, a 1-ml aliquot of *S. antibioticus* ATCC 14890 culture was inoculated into a 250-ml flask containing 50 ml F7 medium and incubated at 28 °C and 250 rpm for 48 h. A 2-ml transfer was then made into a second stage 500-ml flask containing 100 ml SG medium. After 17 h, 1 ml of a 50 mg/ml substrate solution in DMSO was added to the flask. The biotransformation was monitored by HPLC and yielded the following peak area ratios after 96 h: substrate **I**, 1.0; product **V**, 4.25; **VI**, 6.81. The whole broth (pH 7.5) was extracted with an equal volume of ethyl acetate and the organic phase was concentrated to dryness under vacuum to yield a brown solid (15 mg) designated as sample 1. The remaining aqueous layer was lyophilized, dissolved in an equal volume of acetonitrile (i.e., a volume equal to the starting broth), and concentrated to dryness to provide sample 2 as a red oil (450 mg).

Sample 1 (containing **I** and **VI**) was dissolved in acetonitrile/water (1:1, 8 ml) while sample 2 (containing **V**) was dissolved in water (14 ml). The solutions were separately filtered through 0.45-μm PTFE membrane filters and each was subjected to preparative HPLC method E. Relevant fractions from preparative HPLC were combined and freeze-dried to yield yellowish powders. Both compounds **V** and **VI** showed a molecular weight of 169 Da (ESI(+) *m*/*z* 170). The later-eluting product **V** (RT = 15.0 min) was found to be acid labile, with approximately half being converted to the stable product **VI** (RT = 13.7 min) in under 6 h (mobile phase solution, ca. pH 3) at room temperature.

Only the structure of the stable product was established by 1D and 2D NMR to be 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**).

2-Amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**) ^{1}H NMR: δ 2.37 (s, 3H), 5.59 (s, 1H), 7.98 (bs, 2H), 11.01 (s, 1H). ^{13}C NMR: δ 23.6, 109.6, 115.6, 149.1, 152.5, 160.2.

The less stable product was identified as 2-amino-6-hydroxy-4-methyl-3-nitropyridine (**V**), a tautomer of **VI**, on the basis of its chemical behavior.

Biotransformation using *C. elegans* ATCC 26269 in a 15-l fermentor

A 500-ml A109 flask was inoculated with *C. elegans* ATCC 26269 spores or mycelium and incubated by standing at ambient temperature for a minimum of 10 days until sporulation occurred. At this time, 100 ml sterile water was added to the flask. Spores were scraped from the solid growth mass and the flask was shaken at 150 rpm for 1 h. Approximately 80 ml of the resulting spore suspension was then transferred to a 4-l flask containing 1 l of F7 medium. The flask was incubated at 28 °C for 16–24 h at 250 rpm. At this time, ca. 300–1,000 ml broth was transferred to a fermentor containing a working volume of 15 l SG-M1B medium. Fermentation operating parameters were airflow 1.0 vvm, temperature 28 °C, and agitation 500 rpm (tip speed 130 m/min) with a back pressure of 690 mbar. Following incubation at 28 °C and 250 rpm for ca. 18–24 h, substrate **I** was added with constant mixing to the desired concentration (0.5 g/l or 1.0 g/l) from a 100× substrate slurry prepared in either 30 % ethanol (not sterilized) or 50 % propylene glycol in water (autoclaved). The latter was ultimately selected for routine use based on the

fact that the substrate solution was stable to autoclaving and found to significantly reduce the frequency of contamination. Foaming was controlled by addition of Ucon LB625 antifoam on demand.

The time course of all biotransformations was monitored over the course of 3–8 days post-substrate addition, with samples taken once daily for assay. When assays had determined that the biotransformation had peaked, the entire broth was harvested by centrifugation. The supernatant containing final product was then recovered for downstream processing. Isolation of product using the direct solvent extraction process is described in further detail below.

While early isolation methods utilized a direct solvent extraction of the product-containing supernatant, it was subsequently found that adsorption to XAD-16 resin was a more efficient process for product isolation. For XAD-16 adsorption, the supernatant was transferred to an appropriate tank and the pH was adjusted to 7.0 with NaOH. Next, 2.0 % (w/v) XAD-16 (300 g per 15 l supernatant) was added to the tank and the mixture was agitated at 200 rpm and 25 °C for 2 h. Samples were taken for assay every hour. An additional aliquot of resin (initially 1.0 %, then 0.4 % for later runs) was added after 2 h, and mixing continued until it was determined that most of the product was adsorbed on the resin and only a small amount (<10 %) remained in solution. The resin was then recovered by filtration and processed for isolation of products similar to the process described below.

Preparative isolation of product from the 15-l-scale biotransformation

A 26-l combined aliquot from two early batches of biotransformation supernatant containing 1.7 g **II** was extracted with an equal volume of ethyl acetate. The concentrated organic extract was partitioned in 1.2 l of acetonitrile/heptane (1:1 v/v) and the acetonitrile phase was concentrated to obtain 13.9 g crude solid containing 1.65 g **II**.

The solid (12.9 g) was chromatographed on a silica gel column and the product was eluted with a mobile phase of heptane/ethyl acetate/acetone with shallow step gradients. Three major fractions A, B, and C were recovered. Fraction A, a red solid, exhibited a purity of >99 AP for **II** by HPLC method C. Product **II** in fraction B was contaminated with **III**. Fraction B was subsequently dissolved in 1 N NaOH and extracted with MTBE for the removal of **III**. The aqueous layer was neutralized and back extraction in MTBE provided **II** with >99 AP. Fraction C also contained both **II** and **III**. Application of the same procedure as described for fraction B yielded **II** of 97 AP which was further purified by recrystallization from dichloromethane–methanol to provide II with >99 AP. Product **II** obtained from the three purified fractions of A, B, and C were combined, giving 1.13 g of **II** as an orange-red solid with >99 AP and ^{1}H and ^{13}C NMR consistent with the structure shown.

DOE analysis

Two Design of Experiments (DOE) studies were performed: one 2^3 full factorial with 4 center points, and one 2-factor central composite. The 2-level factorial design was analyzed using a set of in-house custom designed Excel spreadsheets with all 8 factorial points replicated twice, with an additional four center points yielding a total of 20 runs or data points. The design was able to estimate all of the main effects as well as all three two-way interactions. Macros were used to construct Main Effect and Two-Way Interaction Plots for all significant terms. The central composite design was analyzed using PROC GLM of SAS® (version 8.2) and consisted of 4 factorial points (each replicated twice), 4 axial points, and 4 center points for a total of 16 runs. Each continuous variable was set at 5 levels. This allowed a full quadratic polynomial regression model to be fit to the data. For these analyses, p values less than 0.05 were considered significant, whereas those between 0.05 and 0.10 were considered marginally significant. Center points for the factorial design were used to test for overall curvature as well as the replication error for testing main and two-way interaction effects. Plots for the central composite design were constructed using PROC G3D and PROC CONTOUR of SAS.

For the factorial study, *C. elegans* ATCC 26269 product yields were expressed as HPLC area at 220 nm, whereas for the central composite study, a wavelength of 254 nm was used. Both studies utilized a 50 mg/ml substrate **I** solution prepared in DMSO. The 'factorial' flasks were fed substrate 24 h after inoculation of each flask with a 40-h broth initiated from a spore suspension. Product **II** yields were determined following a 72-h biotransformation.

For the 'central composite' flasks, substrate was added 24 h after direct inoculation of each flask with an ATCC 26269 spore suspension. Product **II** yields in this case were determined following a 98-h biotransformation. This experiment was designed with a center point of 2 % glucose and 0.3 % yeast extract such that both the (3 % glucose/0.2 % yeast extract) and (1 % glucose/0.2 % yeast extract) combinations (the best ratios from the previous DOE study) were represented as factorials.

Results and discussion

Screening and product identification

Over 100 bacterial and fungal strains from our culture collection with the potential to effect hydroxylation reactions were screened for the hydroxylation of 2-amino-4-methyl-3-nitropyridine (**I**). These included the genera *Pseudomonas* (21 strains), *Streptomyces* (14), *Aspergillus* (13), *Rhodococcus* (12), *Bacillus* (11), *Nocardia* (4), *Corynebacterium* (4), and *Cunninghamella* (3). Since no product markers were available, generation of any new peaks in HPLC indicated biotransformation and those with an increase of 16 AMU in MW over the starting material suggested the formation of a hydroxylated product. Samples of greatest interest based on the generation of new HPLC peaks were derived from strains of *Cunninghamella*, *Streptomyces*, and *Aspergillus*. Such samples were then analyzed by LC–MS, where up to five small peaks with molecular weights corresponding to the addition of an oxygen (M + 16) were observed. Cultures yielding such peaks were scaled up into larger flasks and biotransformation products were isolated for structural elucidation.

Cunninghamella elegans ATCC 26269 afforded three products with the same molecular weight of 169 Da (16 AMU higher than starting material). The products were isolated by preparative HPLC. The structures of three products were established by 1D and 2D NMR homo- and heteronuclear correlation experiments (COSY, HMQC, and HMBC): 2-amino-5-hydroxy-4-methyl-3-nitropyridine (**II**), 2-amino-4-hydroxymethyl-3-nitropyridine (**III**), and 2-amino-4-methyl-3-nitropyridine-1-oxide (**IV**). The structure of **II** was also confirmed by single-crystal x-ray crystallography analysis. Biotransformation products **II** and **IV** are new compounds. This microorganism has the ability to carry out oxidation and insertion of oxygen at aromatic and aliphatic positions as well as on the heterocyclic nitrogen atom. Whether these reactions are catalyzed by different enzymes or by the same enzyme is not known at this time.

One key observation was that the highest yields were obtained during those flask and tank runs where mycelial pelleting was limited (i.e., dispersed growth was maximized). For this reason, the use of spore suspensions and a limited number of inoculum stages was required, because each successive transfer stage resulted in heavier pelleting despite attempts to minimize it. Such efforts included the use of various impeller designs and high agitation rates. The problematic pelleting of *C. elegans* ATCC 26269 during growth could be minimized by using a spore suspension derived from A109 rice medium, and such spore suspensions were routinely used to initiate the first inoculum (F1) stage, with F7 the preferred medium. A spore suspension could also be successfully used to inoculate the biotransformation flask directly.

Two products with a molecular weight of 169 Da were isolated from the biotransformation mixture of *Streptomyces antibioticus* ATCC 14890 by preparative HPLC. However, the late-eluting product (**V**) was acid labile. It was converted to the other product (**VI**) under acidic conditions (pH $\sim$3) after isolation. The structure of the stable product was established as 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**) by 1D and 2D ^{1}H and ^{13}C NMR spectra. The less stable product was identified as 2-amino-6-hydroxy-4-methyl-3-nitropyridine (**V**), a tautomer of **VI**, on the basis of its chemical behavior. Approximately half of the tautomer **V** was converted to **VI** in less than 6 h in solution at room temperature. These two biotransformation products **V** and **VI** are also new compounds. The structures of the substrate and all five biotransformation products are shown in Fig. 1.

The chromatogram shown in Fig. 2 illustrates a *combined* extract from these two organisms, ATCC 26269 and ATCC 14890, with the relative retention times of all of the compounds shown. The *C. elegans* ATCC 26269 strain was the only one to exhibit hydroxylation at the 5-position during the initial studies. On the basis of this observation, additional *Cunninghamella* strains were tested, and although several produced a similar product profile, none demonstrated an improved yield. Similarly, only *S. antibioticus* ATCC 14890 appeared to produce the 6-hydroxy compound **V**. In contrast, several organisms, on the basis of HPLC retention time, produced the corresponding lactam **VI**. These included multiple *Streptomyces* and *Aspergillus* strains. *Aspergillus* in particular appeared to produce 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**) very efficiently as evidenced from the corresponding HPLC peak, with some exhibiting nearly quantitative conversion of **I** to **VI** within 48 h. Four of the five biotransformation products (**II**, **IV**, **V**, and **VI**) are novel compounds, and microbial hydroxylation of pyridine at a remote center without the assistance of an adjacent hydroxy group, e.g., biotransformation of **I** to **II**, has not been previously reported.

The biotransformation products (**II** to **VI**) containing highly substituted and functionalized pyridine rings are attractive intermediates for the synthesis of pharmaceuticals and other chemicals. Compound **III** has recently been reported as an intermediate in the synthesis of a drug development candidate [7]. We were interested in compound **II** as a synthetic intermediate, also for a drug development candidate.

Biotransformation by *Cunninghamella elegans*

After establishment of the structure of the biotransformation products, further efforts focused on improving the

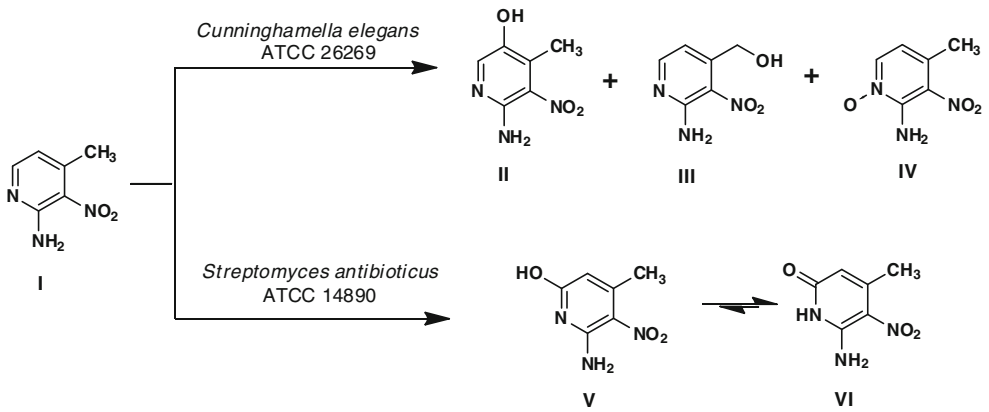

Fig. 1 Biotransformation of substrate **I** to five products by two organisms, *Cunninghamella elegans* ATCC 26269 and *Streptomyces antibioticus* ATCC 14890

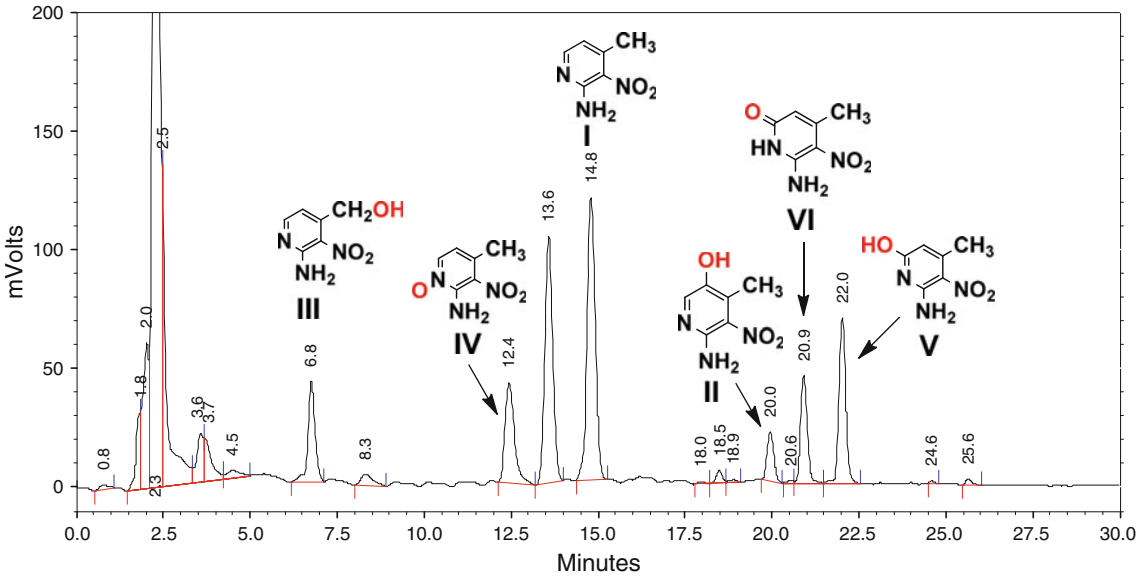

Fig. 2 HPLC method B chromatogram at 220 nm of the substrate **I** and combined extracts from ATCC 26269 (**II**, **III**, and **IV**) and ATCC 14890 (**V** and **VI**), demonstrating HPLC relative retention times. The identity of the HPLC peak at RT = 13.6 min (from *S. antibioticus*) was not determined

yield and productivity of microbial hydroxylation of **I** to **II**. While production yields of the other hydroxylation products appeared to be significantly greater and more reproducible under a variety of conditions, the production of **II** was more problematic.

An initial comparison of multiple alternative biotransformation media with the standard SG formulation revealed only two additional media that yielded the desired peak: Difco Potato Dextrose Broth and K45-M. However, neither was superior to the SG medium. Additional studies revealed that formation of the desired product appeared to be extremely sensitive to either the inoculum and/or biotransformation medium, as demonstrated by the observation that employing F7 medium at the F1 inoculum stage was successful, whereas using F7-M1 was not. Conversely, the use of SG-M (versus SG) biotransformation medium also failed to yield the desired product. Results of four different inoculum media and three biotransformation media are shown in Table 1.

On the basis of the composition of SG-M, it appeared that the presence of Tastone-154 and/or lack of Difco YE was the critical factor in the poor showing by SG-M medium. Similarly, the use of F7-M1 inoculum also yielded minimal product **II** formation, further suggesting that the Tastone-154 was inhibitory. Thus, yeast extract from two different sources appeared to yield different outcomes in this case: while the Tastone 154 was inhibitory, the Difco product showed good biotransformation. At this point, it was determined that only the standard F7 inoculum and SG biotransformation media—both of which contained Difco yeast extract—would be used.

Table 1 Effect of inoculum and biotransformation medium upon production of compound **II**

Inoculum medium	Biotransformation medium	Product **II** area count (64 h)
F7	SG	2.94
	SG-M	ND
	PDB	2.44
F7-M1	SG	ND
	SG-M	ND
	PDB	0.73
SG	SG	2.38
	SG-M	ND
	PDB	1.77
PDB	SG	2.24
	SG-M	ND
	PDB	1.40

Yields are expressed as HPLC area counts ($\times 10^5$) at 220 nm

ND not detected

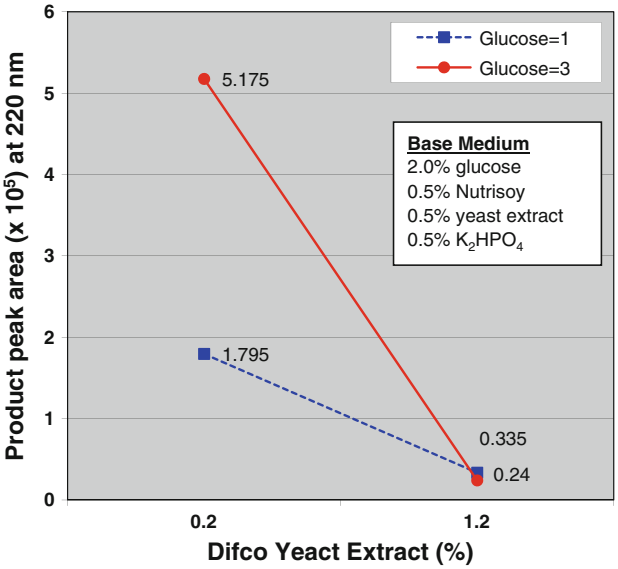

Fig. 3 Product **II** peak area interaction means ($N = 4$) for a three-variable two-level factorial demonstrating a highly significant two-way interaction ($P < 0.0001$) for glucose and yeast extract. All concentrations are expressed in units of percent

DOE studies

The principles of statistical analysis have been utilized for process optimization of many secondary metabolites over the years [2–4], and DOE has found great utility in our laboratories [9]. Similar experimental design principles were employed for the current biotransformation studies. Using SG medium, a three-variable two-level factorial study was performed to determine the critical medium components and interactions for biotransformation of **I** to product **II**. The three components investigated were soy protein (Nutrisoy), glucose, and yeast extract (Difco). It was clear that yeast extract (P 4.5504E−11) and glucose (P 5.4642E−08) had significant main effects as well as the strongest two-way interaction (P 3.0443E−08) as shown in Fig. 3. The two-way interaction between glucose and soy protein was also significant (P 1.7681E−06), as was that between yeast extract and soy protein (P 0.0406581).

A two-factor central composite design was then utilized to further study the effects of glucose and yeast extract upon production of **II**. The optimal concentration of soy protein tested (0.2 %) was held constant, which along with K_2HPO_4 at 0.5 % comprised the basal medium for the study described in Fig. 4. The best data, both actual and predicted, were obtained with 3.0–3.7 % glucose and 0.4–0.47 % yeast extract. The lower concentration of each component was selected for further studies and the medium was designated SG-M1A. Overall yield for the desired product **II** was improved by ca. 23 % versus the starting medium, and the results were the basis for scale-up of the reaction from flasks into larger fermentors as discussed below.

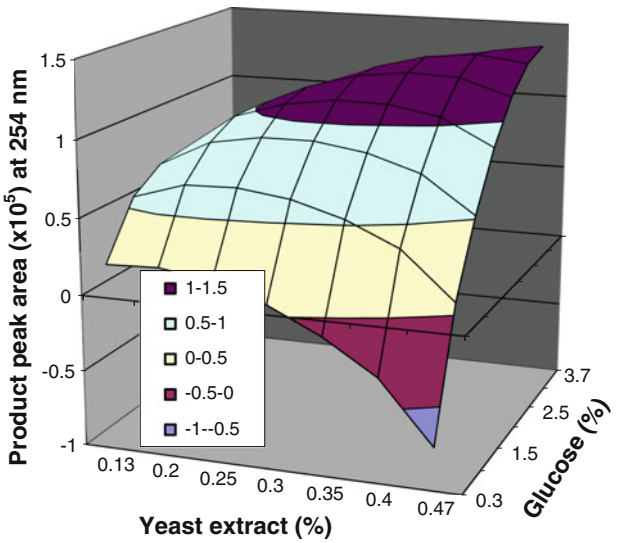

Fig. 4 Product **II** response surface plot of a two-factor central composite DOE for Difco Bacto yeast extract versus glucose in a base medium composed of 0.2 % Toasted Nutrisoy and 0.5 % K_2HPO_4. All concentrations are expressed in units of percent

The critical nature of the yeast extract demonstrated by both the DOE and earlier studies was confirmed in the experiment shown in Fig. 5 directly comparing yeast extracts from several sources. Noteworthy was the fact that Tastone-154 once again appeared to completely inhibit the formation of **II**. Tastone either contains an inhibitory component or lacks a critical component required for product formation. Difco Bacto yeast extract offered the best overall product yield. Yeast extract is a complex

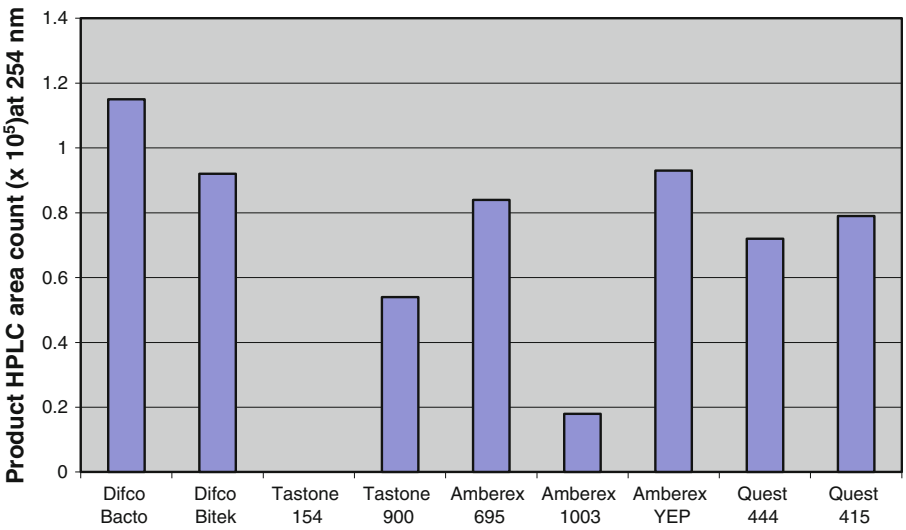

Fig. 5 Effect of various yeast extracts upon production of **II**. Yields were determined from single flasks following a 96-h biotransformation

mixture, and determination of the root cause for inhibition and enhancement of this particular hydroxylation activity down to a specific component (or components) was not attempted at this time.

Additional factors were also evaluated for their possible effects upon product formation, including carbon and nitrogen feeds as well as supplementation with multiple trace metals. All resulted in no improvement in product yield.

Nature of acid used for pH control during the C. elegans biotransformation

During the flask studies, medium pH was adjusted by HCl prior to autoclaving. However, on the basis of poor compatibility with stainless steel fermentors, the acid for pH adjustment was switched to H_2SO_4 when the biotransformation experiments were initiated at the 15-l fermentor scale. It was surprising to find minimal or no yield of product **II**. Accordingly, a series of flask and 15-l tank experiments were conducted to determine the effect of various factors, e.g., the type of acid used (HCl vs. H_2SO_4) for pre-sterilization pH adjustment, as well as control during the biotransformation. It was subsequently found that product **II** formation was inhibited when H_2SO_4 was used for either initial pH adjustment or control of pH during biotransformation. The data from one flask study where the medium pH was pre-adjusted to 5.0 with HCl, H_2SO_4, or H_3PO_4 is shown in Table 2. A separate unidentified product exhibiting an earlier retention time of 6.5 min. (area $= 4.10 \times 10^5$) was observed in those flasks receiving sulfuric acid, but no product **II** peak was seen by HPLC. Similarly, inhibition was seen when ammonium sulfate, but not ammonium chloride, was used to supplement the medium (data not shown). The results confirmed

Table 2 Effect of pH adjustment with different acids upon production of compound **II**

Acid used	Product **II** area count	
	Day 2	Day 5
HCl	1.96	2.47
H_2SO_4	ND	ND
H_3PO_4	1.98	2.49

Yields are expressed as HPLC area counts ($\times 10^5$) at 254 nm

ND not detected

that sulfate inhibited production of **II**. As far as we know, this is the first reported case of inhibition of microbial hydroxylation by sulfate ion. The mechanistic basis for the inhibition of microbial hydroxylation by sulfate is unknown at this time. This surprising result demonstrated the importance of controlling every aspect of a microbial hydroxylation process. Clearly, even a seemingly unimportant component such as the acid used for pH control can significantly influence the outcome. The extent of bioconversion was also improved by up to 24 % when medium pH was pre-adjusted from 7.0 to 5.0. For all subsequent experiments, pH adjustment was limited to the use of phosphoric acid and a starting value of 5.0 prior to sterilization. This applied to both flasks and preparative tanks. Furthermore, no pH control was employed during all fermentor runs.

Substrate addition for 15-l-scale biotransformation

The substrate **I** was added as a 50 mg/ml solution in DMSO for flask-scale studies to a final substrate input of 0.5 g/l in the biotransformation mixture. In 15-l fermentors, the desired final substrate input was 0.5–1 g/l which

would have required a substantial amount of DMSO. The use of such large quantities of this solvent at the pilot scale was not desirable for several reasons (e.g., smell, absorption through skin). Solutions of the substrate **I** in 30 % ethanol–water or 50 % propylene glycol–water were tested. The 50 % propylene glycol–water preparation had the added advantage of being heat sterilizable, thereby significantly reducing the potential for contamination. A sterilized 50 % propylene glycol (50–100 g/l) solution was therefore used to provide the final substrate input of 0.5–1 g/l in the 15-l-scale fermentor biotransformations.

Biotransformation by C. elegans in 15-l fermentors

The hydroxylation was successfully scaled up to 15-l fermentors using the SG-M1B medium developed from the DOE optimization studies. The microbial hydroxylation was found to be quite reproducible. The typical time course of hydroxylation is shown in Fig. 6. The desired product **II** reached a steady state concentration after ca. 70 h. Several multiple 15-l-scale biotransformations were carried out and multi-gram quantities of product **II** were isolated. The profile of substrate **I** and product **II** in three representative 15-l-scale biotransformations is shown in Fig. 7. The solution yield for the biotransformation of substrate **I** to compound **II** in tanks was typically 13 %.

As described in the "Materials and methods", isolation and purification of **II** from the biotransformation broth were achieved by ethyl acetate extraction of the reaction mixture followed by silica gel chromatography. The procedure was quite laborious, however, requiring a large amount of solvent, and was not suitable for scale-up. In search of an alternative process, it was observed that most (>90 %) of the biotransformation products remained in the

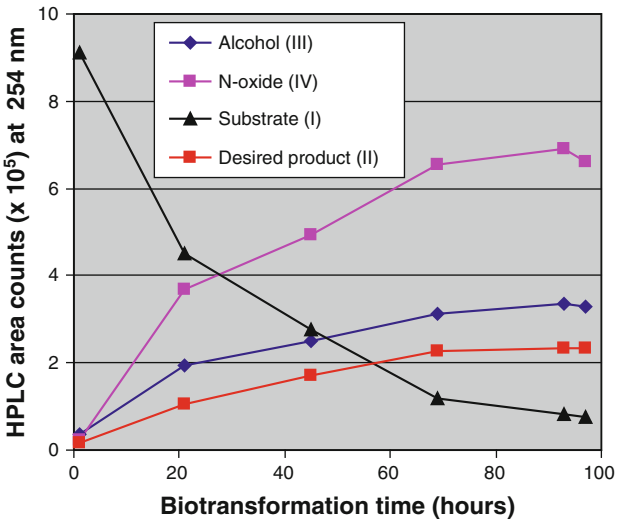

Fig. 6 Time course profile of a representative 15-l preparative batch

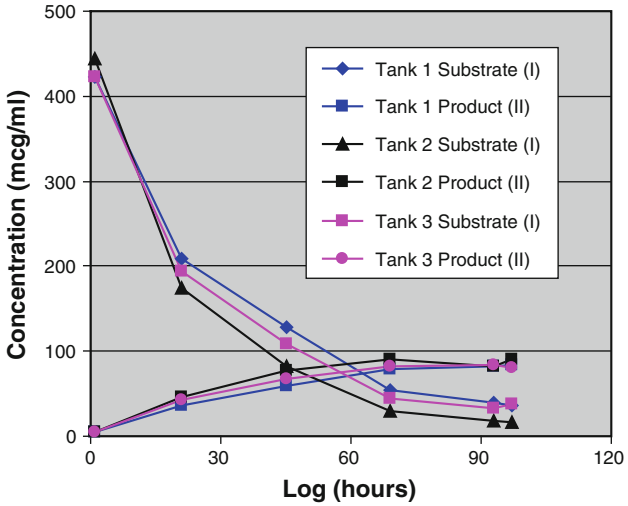

Fig. 7 Time course profile of three representative 15-l preparative batches

supernatant after separation of cells by centrifugation and could be adsorbed efficiently from the solution by polymeric XAD-16 resin. The desired products could then be readily eluted from the resin with a solvent such as MTBE and concentrated to a solid which could be partitioned in heptane/acetonitrile. It is believed that further simplification of the isolation process can be achieved by acid/base extractions that take advantage of the acidic and basic natures of products **II** and **III**, respectively. Isolation of purified **II** (AP 97–99) with high recovery yield (85–95 %) in early experiments suggested that a chromatography-free method yielding purified product of excellent quality could readily be developed.

Conclusions

Several microorganisms were found to convert the substituted pyridine derivative 2-amino-4-methyl-3-nitropyridine (**I**) to multiple biotransformation products via introduction of oxygen at the 5 and 6 aromatic carbons, the aliphatic methyl group, and heterocyclic nitrogen. A total of five biotransformation products were obtained. Four (**II**, **IV**, **V**, and **VI**) of the five are new compounds, and all are novel biotransformation products. The structures of five biotransformation products were established as 2-amino-5-hydroxy-4-methyl-3-nitropyridine (**II**), 2-amino-4-hydroxymethyl-3-nitropyridine (**III**), 2-amino-4-methyl-3-nitropyridine-1-oxide (**IV**), 2-amino-6-hydroxy-4-methyl-3-nitropyridine (**V**), and 2-amino-4-methyl-3-nitro-6(1*H*)-pyridinone (**VI**). *Streptomyces* and several strains of *Aspergillus* were capable of producing the lactam **VI** as well. Only *Cunninghamella elegans* afforded the 5-hydroxylated product **II**. The 5-hydroxy compound **II** was by far the most difficult to

produce with initial conversion yields under 5 %. A series of studies including DOE analysis ultimately improved the yield and a reproducible and robust biotransformation process was subsequently developed. It was noteworthy that the microbial hydroxylation of **I** to **II** was found to be inhibited by sulfate ion and impacted by the source of yeast extract. The conversion was scaled up to generate multi-gram quantities in 15-l fermentors providing **II** in ca. 13 % yield.

References

1. Archelas A, Furstoss R, Pohl T, Waldmann H (1995) Oxidation reactions. In: Drauz K, Wladmann H (eds) Enzyme catalysis in organic synthesis. VCH, New York, pp 667–704
2. Box GEP, Behnken DW (1960) Some new three-level designs for the study of quantitative variables. Technometrics 2:455–475
3. Box GEP, Draper NR (1987) Empirical model-building and response surfaces. Wiley, New York
4. Box GEP, Hunter WG, Hunter JS (1978) Statistics for experimenters: an introduction to design, data analysis, and model building. Wiley, New York. (2nd edition: Statistics for experimenters: design, innovation and discovery, 2005)
5. Ishikawa T, Maeda K, Hayakawa K, Kojima T (1996) Regiospecific hydroxylation of 3-(methylaminomethyl)pyridine to 5-(methylaminomethyl)-2(1H)-pyridinone by *Arthrobacter ureafaciens*. J Mol Catal B 1:173–179
6. Kiener A, Gloeckler R, Heinzmann K (1993) Preparation of 6-oxo-1,6-dihydropyridine-2-carboxylic acid by microbial hydroxylation of pyridine-2-carboxylic acid. J Chem Soc Perkin Trans I:1201–1202
7. Murray PJ, Onions ST, Williams JG, Joly K (2011) Preparation of pyrido[2,3-b]pyrazine compounds for use in drug formulations for treating inflammation, respiratory disorders, and viral infections. PCT Int Appl WO11158044
8. Tinschert A, Tschech A, Heinzmann K, Kiener A (2000) Novel regioselective hydroxylations of pyridine carboxylic acids at position C2 and pyrazine carboxylic acids at position C3. Appl Microbiol Biotechnol 53:185–195
9. Tully TP, Bergum JS, Schwarz SR, Durand SC, Howell JM, Patel RN, Cino PM (2007) Improvement of sordarin production through process optimization: combining traditional approaches with DOE. J Ind Microbiol Biotechnol 34:193–202
10. Uchida A, Yoshida T, Ogawa M, Nagasawa T (2003) Regioselective hydroxylation of quinolinic acid, lutidinic acid and isocinchomeronic acid by resting cells of pyridine dicarboxylic acid-degrading microorganisms. Appl Microbiol Biotechnol 62:337–341
11. Watson GK, Houghton C, Cain RB (1974) Microbial metabolism of the pyridine ring. The hydroxylation of 4-hydroxypyridine to pyridine-3,4-diol (3,4-dihydroxypyridine) by 4-hydroxypyridine-3-hydroxylase. Biochem J 140:265–276
12. Zefirov NS, Modyanova LV, Ouyuntsetseg A, Piskunkova NF, Terent'ev PB, Vagrov VV, Ovcharenko VV (1993) Transformation of 3-hydroxypyridine by *Pseudomonas fluorescens* and *Rhodococcus opacus* strains. Chem Heterocycl Compd 29(6): 730–731
13. Zefirov NS, Terent'ev PB, Modyanova LV, Dovgilevich EV (1993) Regio- and stereoselective hydroxylation of some nitrogen heterocyclic compounds by microorganisms. Indian J Chem Sect B 32B:54–57