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Metabolite Generation via Microbial Biotransformations with Actinomycetes: Rapid Screening for Active Strains and Biosynthesis of Important Human Metabolites of Two Development-Stage Compounds, 5-[(5*S*,9*R*)-9-(4-Cyanophenyl)-3-(3,5-dichlorophenyl)-1-methyl-2,4-dioxo-1,3,7-triazaspiro[4.4]non7-yl-methyl]-3-thiophenecarboxylic Acid (BMS-587101) and Dasatinib

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ABSTRACT:

The enzymes present in many microbial strains are capable of carrying out a variety of biotransformations when presented with drug-like molecules. Although the enzymes responsible for the biotransformations are not well characterized, microbial strains can often be found that produce metabolites identical to those found in mammalian systems. However, traditional screening for microbial strains that produce metabolites of interest is done with many labor intensive steps that include multiple shake flasks and many manual manipulations, which hinder the application of these techniques in drug metabolite preparation. A 24-well microtiter plate screening system was developed for rapid screening of actinomycetes strains for their ability to selectively produce metabolites of interest. The utility of this system was first demonstrated with the well characterized cytochrome P450 substrate diclofenac. Subsequently, the use of this system allowed the rapid identification of several actinomycetes strains that were capable of converting two drug candidates under development, 5-[(5*S*,9*R*)-9-(4-cyanophenyl)-3-(3,5-dichlorophenyl)-1-methyl-2,4-dioxo-1,3,7-triazaspiro[4.4]non7-yl-methyl]-3-thiophenecarboxylic acid and *N*-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl)]-2-methyl-4-pyrimidinyl]amino)]-1,3-thiazole-5-carboxamide (dasatinib, Sprycel, BMS-345825), to mammalian metabolites of interest. Milligram quantities of the metabolites were then prepared by scaling-up the microbial biotransformation reactions. These quantities were sufficient for initial characterization, such as testing for pharmacological activity and use as analytical standards, prior to the availability of authentic chemically synthesized compounds.

An integral part of the drug discovery and development process involves characterization of the metabolites of a drug candidate. The major purposes of these characterizations are to: 1) help ensure that human metabolites are adequately tested in toxicology species, 2) determine whether any of the pharmacological activity of the drug is due to active metabolites, and 3) aid in the determination of the mechanism of metabolic clearance of the parent drug (Baillie et al., 2002; http://www.fda.gov/cder/guidance). Standard testing paradigms for ensuring the safety of drug metabolites have been recently proposed, and aspects of these proposals were recently reviewed (Davis-Bruno and Atrakchi, 2006; Guengerich, 2006; Humphreys and Unger, 2006; Smith and Obach, 2006; http://www.fda.gov/cder/guidance). Considerable effort is often required to prepare sufficient quantities of key mammalian metabolites of drug candidates for biological activity evaluation or for use as analytical standards. Metabolite biosynthesis methods using mammalian systems (microsomes, S9, hepatocytes, in vivo, etc.) are useful for generating limited quantities of metabolites for structure elucidation by LC/MS/MS and NMR analysis. Chemical synthesis is the preferred method for larger scale metabolite preparation, but it is often a resource-intensive exercise, and certain metabolites present particularly difficult synthetic challenges. In many cases, neither mammalian biosynthesis nor chemical synthesis is particularly effective for making quantities of metabolite useful for initial characterizations (typical early characterization studies require 1–50 mg). Larger quantities can be prepared by chemical synthesis; however, it is often not an efficient use of resources to embark on a multistep synthesis before gathering any information about the metabolite of interest.

The concept of microbial models of mammalian metabolism has

Portions of this work were presented as *Metabolite Generation and Characterization via Microbial Biotransformation with Actinomycetes* (Li W, Josephs JL, and Humphreys WG) at the 13th North American ISSX Meeting, Oct. 23–27, 2005, Maui, Hawaii (abstract 351).

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ABBREVIATIONS: LC/MS, liquid chromatography/mass spectrometry; MS, mass spectrometry; BMS, Bristol-Myers Squibb Co.; P450, cytochrome P450; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; RLM, rat liver microsomes; HLM, human liver microsomes; DMSO, dimethyl sulfoxide; MTBE, methyl-t-butyl ether; ESI, electrospray ionization; HRMS, high-resolution mass spectrometry.

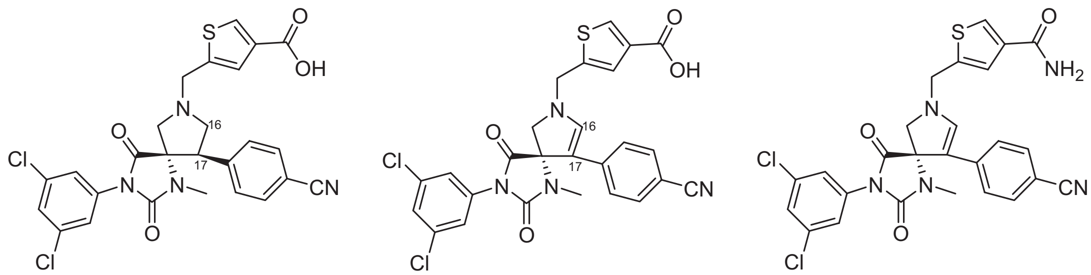


FIG. 1. Structures of BMS-587101, metabolite M6, and amide of M6.

been well established (Smith and Rosazza, 1974). Work in this area has been reviewed (Abourashed et al., 1999; Azerad, 1999), and there has been an increasing number of reports describing the use of microbial systems to mimic or predict mammalian metabolite formation and to prepare larger quantities of mammalian metabolites (Moody et al., 1999, 2002; Xie et al., 2005; Zhang et al., 2006; Zmijewski et al., 2006). The traditional microbial screening methods using shake flasks are labor intensive, which has likely limited the widespread use of microbial biotransformation in drug metabolism studies. Found mainly in soil, actinomycetes are Gram-positive mycelia bacteria known to produce a diversity of natural products and perform a wide variety of metabolic conversions on molecules with a range of physicochemical properties. These metabolic conversions include oxidative biotransformations that are similar to those catalyzed by mammalian P450 enzymes. There are more than one hundred known genera of actinomycetes, including *Streptomyces* sp., *Actinoplanes* sp., and *Nocardia* sp. Actinomycetes culturing conditions are amenable to a well-plate-format fermentation, which makes them attractive bioreactors for metabolite biosynthesis.

A preliminary study examining the ability of a variety of actinomycetes strains to metabolize marketed drugs and produce their respective mammalian metabolites has been previously reported (Li et al., 2005). In this manuscript, the application of an actinomycetes screening system that led to the rapid synthesis of three mammalian oxidative metabolites of drugs under development, BMS-587101 (Fig. 1) and dasatinib (Sprycel, BMS-345825) (Fig. 2), is described. The three metabolites, (*S*)-5-((9-(4-cyanophenyl)-3-(3,5-dichlorophenyl)-1-methyl-2,4-dioxo-1,3,7-triazaspiro[4.4]non8-en-7-yl)methyl)thiophene-3-carboxylic acid (M6; a dehydrogenated metabolite of BMS-587101), M20, and M24 (hydroxylated metabolites of dasatinib) (D. Cui, W. Li, L. Chistopher, A. Barros, V. Arora, H. Zhang, L. Wang, D. Zhang, J. A. Manning, K. He, A. M. Fletcher, M. Ogan, M. Lago, S. J. Bonacorsi, W. G. Humphreys, and R. A. Iyer, submitted manuscript), were the major circulating human metabolites that were difficult to synthesize chemically, which made them ideal candidates for microbial biosynthesis.

Materials and Methods

Materials. BMS-587101 [5-[(5*S*,9*R*)-9-(4-cyanophenyl)-3-(3,5-dichlorophenyl)-1-methyl-2,4-dioxo-1,3,7-triazaspiro[4.4]non7-yl-methyl]-3-thiophenecarboxylic acid] and dasatinib [*N*-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl)]-2-methyl-4-pyrimidinyl]amino)]-1,3-thiazole-5-carboxamide] were synthesized at BMS. All other organic solvents and the reagents were of HPLC or reagent grade. Diclofenac (HCl salt), 4′-hydroxydiclofenac, pig liver esterase, and catalase were obtained from Sigma Co. (St. Louis, MO). Rat liver microsomes were obtained from Xenotech LLC (Lenexa, KS). The microbial strains were acquired from American Type Culture Collection (with ATCC numbers) or from BMS in-house collections (with SC numbers).

Fermentation. Malt extract medium used in the fermentation was prepared as follows: 20 g dextrose (EM Science, Gibbstown, NJ), 10 g malt extract (Difco, Detroit, MI), 10 g yeast extract (Difco), and 1 g peptone (Difco) were dissolved in one liter deionized water. The pH of the solution was adjusted to approximately 7 with 1 N NaOH or 1 N HCl. The solution was dispensed into containers and autoclaved at 121°C for 30 min. Fermentation was performed at 28°C on a New Brunswick Scientific (Edison, NJ) Innova 4500 environmental rotary shaker with a throw of 2 inches. Shaking speed was 250 to 275 rpm for microtiter plates and 200 to 250 rpm for Erlenmeyer flasks.

NMR Analysis. NMR analyses of BMS-587101 metabolites were preformed in methanol-d_4 at 25°C on a Bruker DRX 500 MHz spectrometer equipped with a 5-mm TXI cryo probe (Bruker BioSpin Corporation, Billerica, MA) operated at 500.13 MHz for proton and 125.76 MHz for carbon, respectively. NMR analyses of dasatinib metabolites were preformed in DMSO-d_6 at 30°C on a Bruker Avance 600 MHz NMR spectrometer equipped with a 5-mm TCI cryo probe and a Bruker Avance 700 MHz NMR spectrometer equipped with a 5-mm cryo triple resonance probe.

The proton and carbon chemical assignments were based on one-dimensional and two-dimensional NMR, including ^{1}H-^{1}H-gCOSY (gradient-selected correlation spectroscopy), ^{1}H-^{13}C-HSQC (heteronuclear single-quantum coherence), ^{1}H-^{13}C-HMBC (heteronuclear multiple bond correlation), and ^{1}H-^{15}*N*-HMBC. ^{13}C chemical shift data were deduced from ^{1}H-^{13}C-HMBC and ^{1}H-^{13}C-HSQC spectra. ^{15}N chemical shift data were deduced from ^{1}H-^{15}*N*-HMBC spectra. The proton and carbon chemical shifts were referenced to solvents, methanol-d_4 (proton, δ3.30 ppm; ^{13}C, δ49.0 ppm), and DMSO-d_6 (proton, δ2.50 ppm; ^{13}C, δ39.5 ppm). The nitrogen chemical shifts were referenced to $^{15}NH_4{}^{15}NO_3$ at δ20.7 and δ376.3 ppm, respectively. The patterns of peaks were reported as singlet (s), doublet (d), triplet (t), or broad (b).

MS Analysis. Accurate mass analysis was performed on a Finnigan MAT 900 high-resolution mass spectrometer (Thermo Finnigan, San Jose, CA). LC/MS analysis was performed with a Finnigan LCQ or LTQ ion trap mass spectrometer.

Microbial Screening Plate. Twenty actinomycetes strains were used (Table 1). A frozen vial (approximately 2 ml) of each selected strain was used to inoculate a 500-ml flask containing 100 ml of malt extract medium. The culture was incubated for 3 days at 28°C on a rotary shaker operating at 250 rpm. The resulting culture (0.1 ml) was transferred into a well of a UNIPLATE (24 well, 10 ml, irradiated; Whatman, Clifton, NJ). Multiple copies of the screening plates, containing one well each of the 20 selected actinomycetes strains, were made and stored at −78°C.

Screening of Microbial Strains for Formation of Diclofenac Metabolites. One frozen screening plate was thawed at room temperature, and 1 ml of malt extract medium was added to each well. The plate was incubated for 2 days at 28°C on a rotary shaker operating at 275 rpm. Diclofenac (10 μl of 25 mM solution in 1:1 v/v methanol/water) was added to each well, and then the plate was incubated with shaking for an additional 24 h at 28°C. One ml of methanol was added to each well, and the plate was shaken at room temperature for 1 h and centrifuged at 3000 rpm for 15 min. The supernatant (10 μl) was analyzed by HPLC/MS on an Agilent 1100 series HPLC system (binary pump, autosampler, and a Photodiode Array UV detector; Agilent Technologies, Wilmington, DE), which was coupled to a Thermo Finnigan LTQ ion trap mass spectrometer. Samples were injected onto a YMC ProC18 column (2.0 ×

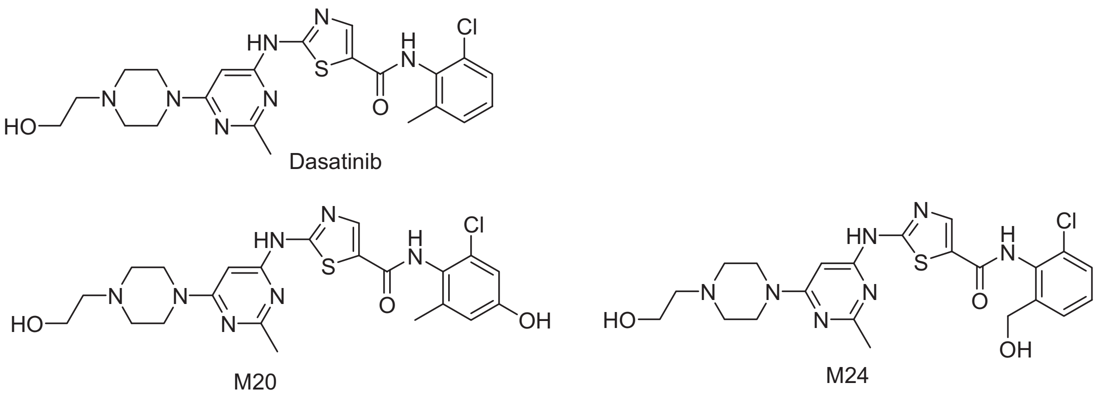


FIG. 2. Structures of dasatinib and metabolites M20 and M24.

TABLE 1

Microbial strains contained in the screening plate

Position in the Screening Plate	SC Number	ATCC Number	Strain	Reference
A1		53771	*Actinoplanes* sp.	Chen et al., 1992
A2		35204	*Nocardia autotrophica*	Okazaki et al., 1983
A3		35203	*N. autotrophica*	Okazaki et al., 1983
A4	15761		*Streptomyces* sp.	
A5		13400	*Streptomyces roseochromogenus*	Ferrer et al., 1990
B1		31560	*Streptomyces violascens*	Tombo et al., 1989
B2		25453	*Streptomyces flocculus*	Smith et al., 1983
B3	15847	PTA-1043	*Amycolatopsis orientalis*	Li et al., 2004
B4	15848		*Actinomycetes* sp.	
B5	15849		*Actinomycetes* sp.	
C1	15850		*Actinomycetes* sp.	
C2	15851		*Actinomycetes* sp.	
C3	15852		*Actinomycetes* sp.	
C4	15853		*Actinomycetes* sp.	
C5	15837		*Actinomycetes* sp.	
D1	15838		*Actinomycetes* sp.	
D2	15839		*Actinomycetes* sp.	
D3	15840		*Actinomycetes* sp.	
D4		10137	*Streptomyces griseus*	Smith et al., 1983
D5		13273	*Streptomyces griseus*	Trower et al., 1992

50 mm, S5; Waters Corporation, Milford, MA) and separated at a flow rate of 0.2 ml/min using mobile phases consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The solvent gradient was as follows: 20% B for 2 min, 20 to 90% B in 20 min. The elution was monitored with UV detection at 295 nm. Metabolites of diclofenac were detected based on their MS and product ion spectra obtained by positive ion electrospray with data-dependent MS/MS. The identity of 4′-hydroxy-diclofenac (Stierlin et al., 1979) was confirmed by comparison with an authentic standard.

Biosynthesis of BMS-587101 M6 Metabolite with Rat Liver Microsomes. The incubation contained 100 mM potassium phosphate buffer (pH 7.5), 360 μM BMS-587101, 1.2 mg/ml catalase, 2 mg/ml rat liver microsomal protein, and 2 mM NADPH in a total volume of 50 ml. The reaction mixture was extracted twice with ethyl acetate (70 ml each time). The ethyl acetate extracts were combined, and solvent was removed with a rotary evaporator. The residue was dissolved in 0.6 ml of methanol and subjected to semipreparative HPLC separation with a YMC ODS AQ 20 × 150 mm, S5 column (Waters Corporation). The mobile phases were 1 mM HCl in water (A) and acetonitrile (B). The gradient used was: 20% B for 5 min, 20 to 50% B in 2 min, 50 to 75% B in 36 min. The flow rate was 10 ml/min with UV detection at 230 nm. Under these conditions, M6 eluted at approximately 31 min. Fractions containing M6 were pooled and lyophilized. Approximately 20 μg of M6 were obtained as an HCl salt.

Characterization: LC/MS, −ESI $[M\text{-}H]^-$: *m/z* 551; MS2: *m/z* 551 → *m/z* 507. 1H NMR (500 MHz, CD_3OD): δ ppm 2.81 (s, 3H), 3.76 (d, *J* = 12.4 Hz, 1H), 3.81 (d, *J* = 12.4 Hz, 1H), 4.53 (d, *J* = 15.4 Hz, 1H), 4.68 (d, *J* = 15.4 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.45 (s, 1H), 7.50 (t, *J* = 1.8 Hz, 1H), 7.53 (d, *J* = 1.8 Hz, 2H), 7.55 (d, *J* = 8.6 Hz, 2H), 7.62 (s, 1H), 8.13 (s, 1H).

Screening of Microbial Strains for Formation of BMS-587101 Metabolite M6. One frozen screening plate was thawed at room temperature, and 1 ml of malt extract medium was added to each well. The plate was incubated for 2 days at 28°C on a rotary shaker operating at 250 rpm. BMS-587101 (10 μl of 18 mM solution in DMSO) was added to each well, and then the plate was incubated with shaking for an additional 18 h at 28°C. One ml of methyl-t-butyl ether (MTBE) was added to each well, the plate was shaken at room temperature for 30 min and centrifuged at 3000 rpm for 15 min, and then the supernatant from each well was transferred to an HPLC vial, and solvent was removed under a stream of nitrogen gas. The residue in the vial was then dissolved in ethanol (200 μl) and analyzed by HPLC. HPLC analyses were performed on an Agilent 1100 series HPLC system. Samples were injected onto a YMC ODS AQ column (4.6 × 150 mm, S3; Waters Corporation) and separated in 15 min at a flow rate of 1 ml/min using mobile phases consisting of 1 mM HCl in water (solvent A) and acetonitrile (solvent B). The solvent gradient was as follows: 30% B for 2 min, 30 to 55% B in 1 min, 55 to 80% B in 8 min, 80 to 90% B in 1 min, 90% for 2 min, 90 to 30% B in 1 min. The elution was monitored with UV detection at 240 nm.

Preparation of BMS-587101 Metabolite M6 by Microbial Biotransformation. From the frozen stock culture of *Actinomycetes* sp. SC15850, 2 ml was used to inoculate 100 ml of malt extract medium. The culture was incubated for 3 days at 28°C on a rotary shaker operated at 250 rpm. Two ml of this culture was used to inoculate each of two 500-ml flasks containing 100 ml of malt extract medium. The flasks were incubated at 28°C on a rotary shaker operated at 250 rpm for 24 h. Ten milligrams of BMS-587101 in 1 ml DMSO was added to each flask, and the culture was returned to the shaker and incubated for an additional 12 h at 28°C and 220 rpm. At the end of the incubation, cultures from the two flasks were pooled and extracted with 100 ml

MTBE. The MTBE extract was completely evaporated with a rotary evaporator. The residue was dissolved in 0.6 ml of methanol and subjected to semipreparative HPLC separation with a YMC ODS AQ 20 × 150 mm, S5 column. The mobile phases were 1 mM HCl in water (A) and acetonitrile (B). The gradient used was: 20% B for 5 min, 20 to 45% B in 15 min, 45% B for 30 min, 45 to 53% B in 20 min, 53% B in 20 min. The flow rate was 10 ml/min with UV detection at 230 nm. The fractions containing M6 and an amide derivative of M6 eluted at 76 and 56 min, respectively. The pure M6 and M6-amide fractions were pooled accordingly and lyophilized. M6 (HCl salt, 2.5 mg) [HRMS (ESI) calculated for $C_{26}H_{18}Cl_2N_4O_4S$ [M-H]$^-$: 551.0348; found: 551.0354; LC/MS/MS and proton NMR data were consistent with M6 isolated from the RLM incubation] and M6 amide (HCl salt, 3.5 mg) [HRMS (ESI) calculated for $C_{26}H_{19}Cl_2N_5O_3S$ [M+H]$^+$: 552.0664; found: 552.0659] were obtained as white solids.

Preparation of BMS-587101 Metabolite M6 via Hydrolysis of the Amide. A 500-ml flask containing sodium phosphate buffer (0.1 M, pH 8.0), pig liver esterase (176 U), methanol (2 ml), and amide of M6 (HCl salt, 27 mg) was incubated for 4 days at 37°C on a rotary shaker operating at 180 rpm. At the end of the incubation, the reaction mixture was extracted with MTBE (400 ml). The MTBE was removed from the extract with a rotary evaporator. The residue was dissolved in 1 ml of methanol and subjected to semipreparative HPLC separation with the following conditions: column, YMC ODS AQ 20 × 150 mm, S5; mobile phases, 1 mM HCl in water (A)/acetonitrile (B). The gradient used was: 20% B for 5 min, 20 to 45% B in 5 min, 45% B for 80 min with a flow rate of 10 ml/min and UV detection at 230 nm. The fractions containing pure M6 were pooled and lyophilized. M6 (HCl salt, 9.1 mg) [HRMS (ESI) calculated for $C_{26}H_{18}Cl_2N_4O_4S$ [M-H]$^-$: 551.0348; found: 551.0356; LC/MS/MS and NMR (proton, ^{1}H-^{1}H-gCOSY, ^{1}H-^{13}C-HSQC, ^{1}H-^{13}C-HMBC) data were consistent with M6 isolated from the RLM incubation] was obtained as a white solid.

Screening of Microbial Strains for Formation of Dasatinib Metabolites M20 and M24. One frozen screening plate was thawed at room temperature, and then 1 ml of malt extract medium was added to each well. The plate was incubated for 2 days at 28°C on a rotary shaker operated at 275 rpm. Dasatinib (2 μl of a 100-mM solution in DMSO) was added to each well, and the plate was incubated for an additional 23 h at 28°C on a rotary shaker operating at 275 rpm. One ml of methanol was added to each well, and then the plate was incubated at 28°C at 150 rpm for 10 min and centrifuged at 3000 rpm for 15 min. The supernatant was analyzed by HPLC/MS. HPLC/MS analysis was performed on an Agilent 1100 series HPLC system, which was coupled to a Thermo Finnigan LTQ ion trap mass spectrometer. Samples were injected onto a Phenomenex Synergy Polar-RP 2.0 × 150 mm (Phenomenex, Torrance, CA) and separated in 28 min at a flow rate of 0.2 ml/min using mobile phases consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The solvent gradient was as follows: 20% B for 1 min, 20 to 22.1% B in 5 min, 22.1 to 90% B in 1 min, 90% B for 2 min, 90 to 20% B in 1 min, 20% B for 3 min. The elution was monitored with UV detection at 320 nm and MS detection (dasatinib HPLC method 1).

An HPLC/UV method was used to separate M20 and M24 with a YMC ODS AQ column (4.6 × 150 mm, S3) using mobile phases consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 1 ml/min. The solvent gradient was as follows: 20% B for 1 min, 20 to 25% B in 13 min, 28 to 80% B in 1 min, 80% B for 3 min, 80 to 20% B in 12 min, 20% B for 3 min. The elution was monitored with UV detection at 320 nm and MS detection (dasatinib HPLC method 2).

Preparation of Dasatinib Metabolite M20 by Microbial Biotransformation. From the frozen stock culture of *Streptomyces* sp. strain SC15761, 2 ml was used to inoculate a 500-ml flask containing 100 ml of the malt extract medium. The flask was incubated for 3 days at 28°C on a rotary shaker operated at 250 rpm. One ml of the resulting culture was added to each of eleven 500-ml flasks containing 100 ml of the malt extract broth. The cultures were incubated at 28°C and 250 rpm for 47 h. Dasatinib solution in DMSO (200 μl of a 48.9 mg/ml solution) was then added to each of the eleven flasks. The flasks were returned to the shaker and incubated for an additional 27 h at 28°C and 250 rpm. The reaction cultures were pooled and extracted twice with 1000 ml and 500 ml of ethyl acetate, respectively. The combined ethyl acetate extract was evaporated to dryness in vacuo. The residue was then dissolved in 2 ml of DMSO. A portion of the DMSO solution (1 ml) was subjected to semipreparative HPLC with the following conditions: column, YMC ODS-AQ 20 × 150 mm, S5; mobile phase, 0.1% formic acid in water (A)/methanol (B); gradient, 10 to 28% B in 5 min, 28% B for 30 min. The flow rate was 10 ml/min with UV detection at 240 nm. Fractions containing pure M20 were pooled and evaporated to a small volume with a rotary evaporator. After lyophilization, 23 mg of M20 (formic acid salt) was obtained as a white powder.

Characterization was as follows: LC/MS: +ESI [M+H]$^+$: m/z 504; MS2: m/z 504 → m/z 417; MS3: m/z 504 → m/z 417 → m/z 381, 232, 260. ^{1}H NMR (700 MHz, *DMSO-d*$_6$) δ ppm 2.13 (s, 3H), 2.40 (s, 3H), 2.42 (t, J = 6.19 Hz, 2H), 2.48 (t, J = 5.05 Hz, 4H), 3.50 (t, *broad*, 4H), 3.53 (t, J = 6.19 Hz, 2H), 6.05 (s, 1H), 6.67 (d, J = 2.65 Hz, 1H), 6.76 (d, J = 2.65 Hz, 1H), 8.17 (s, 1H), 9.61 (s, 1H), 11.44 (s, 1H).

Preparation of Dasatinib Metabolite M24 by Microbial Biotransformation. From the frozen stock culture of *Streptomyces griseus* ATCC 10137, 2 ml was used to inoculate a 500-ml flask containing 100 ml of the malt extract medium. The flask was incubated for 3 days at 28°C on a rotary shaker operated at 250 rpm. One ml of the resulting culture was added to each of eleven 500-ml flasks containing 100 ml of the malt extract broth. The cultures were incubated at 28°C and 250 rpm for 47 h. Dasatinib solution in DMSO (200 μl of a 48.9 mg/ml solution) was then added to each of the eleven flasks. The flasks were returned to the shaker and incubated for an additional 27 h at 28°C and 250 rpm. The reaction cultures were pooled and extracted twice with 500 ml and 250 ml of ethyl acetate, respectively. The combined ethyl acetate extract was evaporated to dryness in vacuo. The residue was then dissolved in 1.3 ml of DMSO. A portion of the DMSO solution (0.4 ml) was subjected to semipreparative HPLC with the following conditions: column, YMC ProC18 20 × 250 mm, S5; mobile phase, 0.1% formic acid in water (A)/methanol (B); gradient, 15 to 27% B in 3 min, 27% B for 45 min. The flow rate was 10 ml/min with UV detection at 240 nm. Fractions containing pure M24 were pooled and evaporated in vacuo to a small volume with a rotary evaporator. After lyophilization, 2.8 mg of M24 (formic acid salt) was obtained as a white powder.

Characterization was as follows: LC/MS: +ESI [M+H]$^+$: m/z 504; MS2: m/z 504 → m/z 486, 347; MS3: m/z 504 → m/z 486 → m/z 399, 347, 263. ^{1}H NMR (500 MHz, *DMSO-d*$_6$) δ ppm 2.40 (m, 5H), 2.48 (overlapped with DMSO peak), 3.51–3.54 (m, overlapped with water peak), 4.48 (s, 2H), 6.07 (s, 1H), 7.37 (t, J = 7.75 Hz, 1H), 7.46 (d, J = 7.75 Hz, 1H), 7.52 (d, J = 7.75 Hz, 1H), 8.23 (s, 1H).

Results

Microbial Screening Plate. Based on their ability to catalyze oxidative transformations (references in Table 1 and unpublished data), twenty actinomycetes strains were used to construct the screening plate (Table 1). The strains were obtained from the American Type Culture Collection (Manassas, VA) (those strains with ATCC numbers) or from the Squibb Culture Collection (Bristol-Myers Squibb Company, Princeton, New Jersey) (those strains with SC numbers). A large number of 24-well deep-well screening plates was prepared and stored at −78°C until needed. A single medium (malt extract medium) was used for growing the cultures in the screening plate. Under these conditions, all the cultures achieved good growth in 2 days. The final concentrations of the compounds used in these studies ranged from 0.1 to 0.3 mM, and the incubations were typically run for 24 h. This system provided a simple way to screen microbial strains with a small amount (~4 mg) of parent compound. The entire screening process was completed in 4 days, including 1 day for analysis.

The ability of the microbial strains in the screening plate to selectively catalyze the formation of metabolites was first tested in incubations with diclofenac. After a 24-h incubation, diclofenac was depleted in most of the wells (Fig. 3). Among the active strains, those in wells C2, C5, D1, and D2 selectively catalyzed 4′-hydroxylation to produce 4′-hydroxydiclofenac in high yield. In contrast, the strains in wells A3, A4, and D5 selectively catalyzed the formation of di-

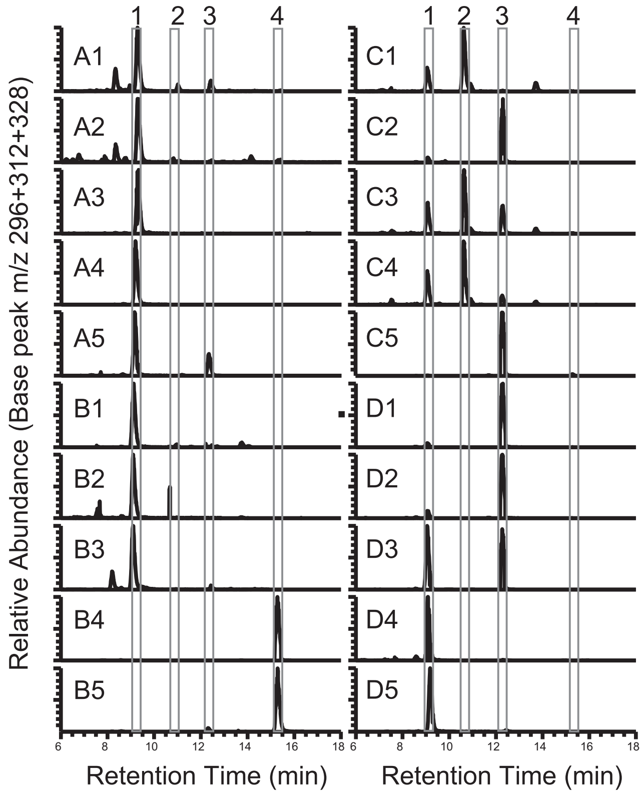


FIG. 3. HPLC/MS chromatograms of microbial reaction extracts with diclofenac in the screening plate. Microbial strains are identified with well positions. Di-hydroxydiclofenac, hydroxydiclofenac (unidentified isomer), 4′-hydroxydiclofenac, and diclofenac are marked with boxes 1, 2, 3, and 4, respectively.

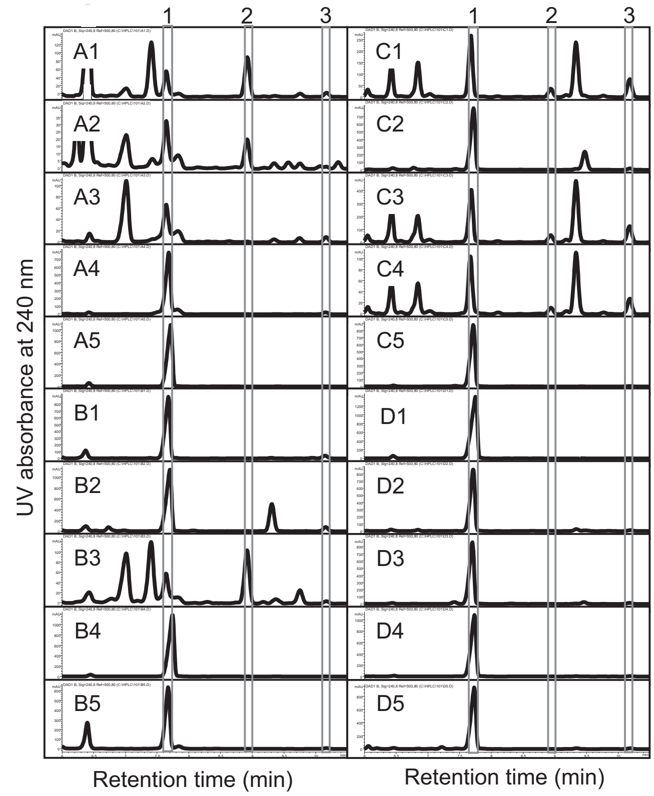


FIG. 5. HPLC/UV chromatograms of microbial reaction extracts with BMS-587101 in the screening plate. Microbial strains are identified with well positions. BMS-587101, the amide derivative of M6, and M6 eluted at 7.7 min (box 1), 8.9 min (box 2), and 10.2 min (box 3), respectively.

hydroxydiclofenac. Strains in other wells produced multiple metabolites in a nonselective manner.

Biosynthesis of BMS-587101 Metabolite M6. Screening was performed to identify strains that were capable of converting BMS-587101 to M6. Upon incubation with BMS-587101 in the screening plate, 16 strains produced an HPLC/UV peak that had an identical UV spectrum (Fig. 4) and HPLC retention time to M6 isolated from an RLM incubation (Fig. 5). The strain in well C1 (*Actinomycetes* sp. SC15850) produced the largest amount of M6 and was therefore used to scale-up the reaction in two shake flasks with a total of 200 ml of malt extract medium. At a loading concentration of 0.18 mM, a reasonable production of M6 was obtained at 9 h. Extending the reaction did not increase M6 concentration; rather, an additional peak with an identical UV spectrum to that of M6 began to become more prominent (Fig. 6). Therefore, the reaction was terminated at 12 h. M6 (2.5 mg) was isolated from the reaction and analyzed by NMR and LC/MS to confirm that it was identical to M6 isolated from the RLM reaction (Table 2).

The peak at 9 min (3.5 mg) with an identical UV spectrum to M6 was also isolated from the microbial reaction. Accurate mass analysis

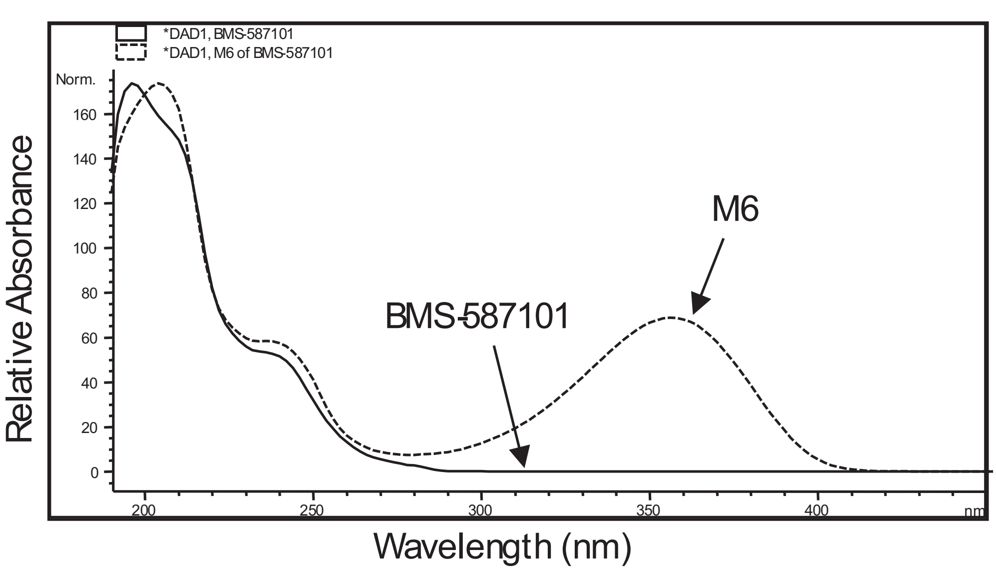


FIG. 4. UV absorbance spectra of BMS-587101 and metabolite M6.

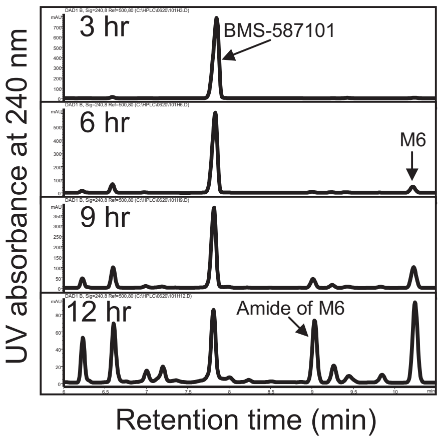


FIG. 6. HPLC/UV chromatograms of extracts from the reaction of BMS-587101 in *Actinomycetes* sp. SC15850 (well C1) at different reaction times.

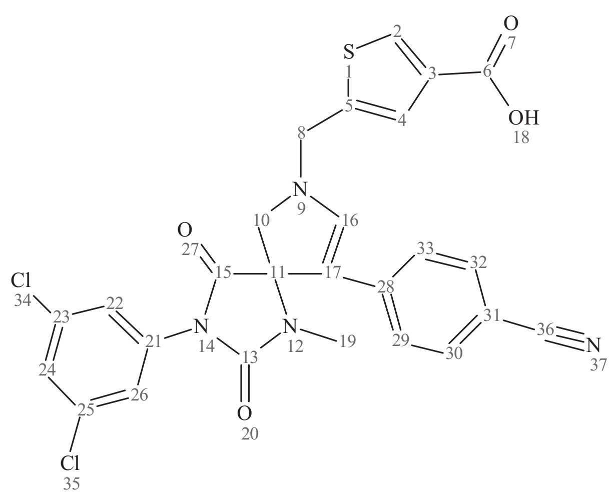
suggested that it was an amide of M6. Incubation of the metabolite with pig liver esterase demonstrated that this product could be quantitatively converted to M6.

Due to variations among cultures and because M6 was converted to the amide by the cultures, it would be hard to stop the reaction at the ideal time to obtain the maximum yield of M6 from each flask. Therefore, the reaction was conducted to obtain a high yield of the amide, which was then hydrolyzed by pig liver esterase to yield M6. Using this two-step process, more than 10 mg of M6 was generated, enabling the further evaluation of this metabolite.

Biosynthesis of Dasatinib Metabolites M20 and M24. Dasatinib metabolites M20 and M24 both produced the same protonated molecule at *m/z* 504, and they often coeluted in LC/MS analyses. Their retention times were sensitive to sample and column conditions and varied among analyses. Therefore, MS2 spectra of the *m/z* 504 ions were used to distinguish M20 and M24. M20 produced a product ion at *m/z* 417, whereas M24 yielded product ions at *m/z* 486 and *m/z* 347. Thus, both MS and UV absorbance at 320 nm were used to determine that strains in wells A3, A4, C5, D1, D2, D3, and D4 were able to catalyze conversion of dasatinib to M20 and M24 in useful amounts (Fig. 7). HPLC/UV analysis with dasatinib HPLC method 2 indicated that the strain in well A4 yielded the highest amounts of M20 and a high ratio (~4:1) of M20 to M24. The strain in this well, *Streptomyces* sp. SC15761, was therefore chosen for scaling up to a larger reaction. More than 20 milligrams of M20 was prepared from approximately 0.5-liter microbial incubation with *Streptomyces* sp. SC15761. NMR and LC/MS/MS analyses confirmed that it was identical to M20 isolated from an HLM reaction (Table 3). Furthermore, M20 isolated from both the microbial biotransformation and M20 isolated from an HLM incubation coeluted under various HPLC conditions.

TABLE 2

^{1}H and ^{13}C NMR chemical shift assignment of BMS-587101 metabolite M6 produced by RLM



Position	^{1}H ppm (*d4*-Methanol: d 3.30 ppm)	^{13}C ppm (*d4*-Methanol: d 49 ppm)	Key HMBC (^{1}H-^{13}C) Correlations
2	8.13 (s, 1H)		
3		134	
4	7.45 (s, 1H)	129	142 (C-5)
5		142	
8	a, 4.53 (d, *J* = 15.4 Hz, 1H); b, 4.68 (d, *J* = 15.4 Hz, 1H)	49	60 (C-10), 142 (C-5), 148 (C-16)
9 (N)			
10	a, 3.76 (d, *J* = 12.4 Hz, 1H); b, 3.81 (d, *J* = 12.4 Hz, 1H)	60	76 (C-11), 106 (C-17), 148 (C-16), 174 (C-15)
11		76	
13		154	
15		174	
16	7.62 (s, 1H)	148	60 (C-10), 76 (C-11), 106 (C-28)
17		106	
19	2.81 (s, 3H)		154 (C-13)
22 and 26	7.53 (d, *J* = 1.8 Hz, 2H)		
23 and 25			
24	7.50 (t, *J* = 1.8, 1H)		
29 and 33	7.55 (d, *J* = 8.6 Hz, 2H)	123	120 (C-30 and C-32)
30 and 32	7.17 (d, *J* = 8.8 Hz, 2H)	120	106 (C-17), 123 (C-29 and C-33)

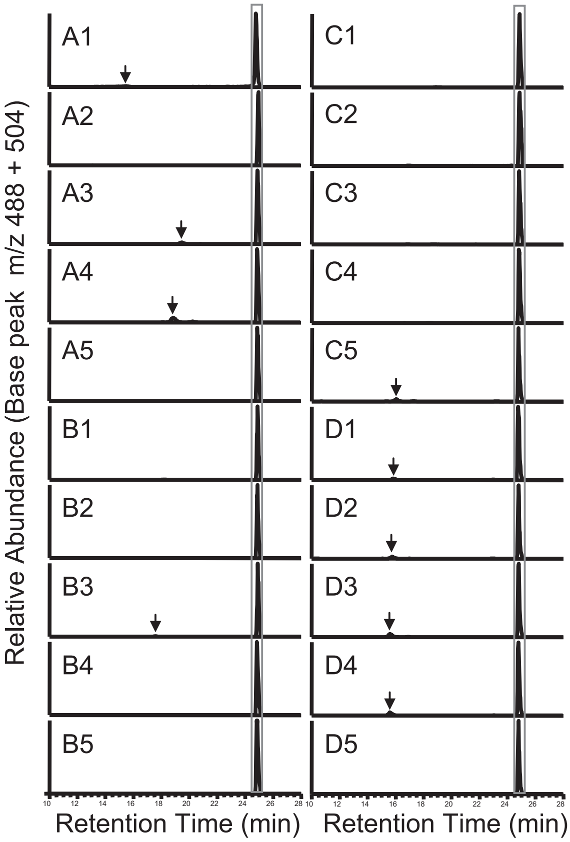


FIG. 7. HPLC/MS chromatograms of microbial reaction extracts with dasatinib in the screening plate (obtained with dasatinib HPLC method 1). Microbial strains are identified with well positions. Dasatinib eluted at 25 min (boxed). M20 and M24 coeluted, and their retention time varied among the chromatographs (marked with an arrow).

The amounts of M24 produced by the strains in wells A4 and D4 were similar; however, the amount of M20 produced by the strain in well D4 was much lower than the strain in well A4. Because M20 and M24 had very similar chromatographic properties, a high level of M20 would make purification of M24 very difficult. Therefore, the strain in well D4, *S. griseus* ATCC 10137, was chosen for production of M24. M24 (2.8 mg) was prepared from an approximately 0.3-liter microbial incubation of *S. griseus* ATCC 10137. The identity of the microbial synthesized M24 was confirmed by NMR and LC/MS/MS analyses (Table 4) and coelution with M24 isolated from an HLM incubation under various HPLC conditions.

Discussion

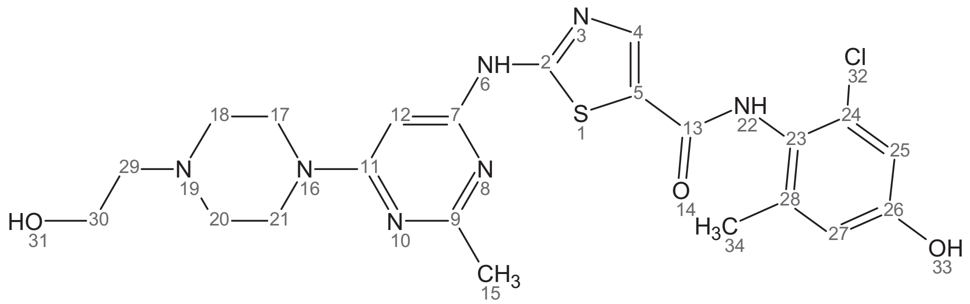
Many fungal and bacteria strains with the capacity for oxidative biotransformation are useful for the biosynthesis of otherwise difficult to prepare chemicals. Much of the knowledge about these microbial techniques comes from screening efforts to identify strains that metabolize steroid-like molecules. In fact, the microbial hydroxylation of steroids has been a synthetic route for the production of several steroids since the early 1950s (Peterson et al., 1952). Evidence indicates that many fungi possess numerous genes that encode enzymes capable of oxidative biotransformations and many of these enzymes are monooxygenases belonging to the cytochrome P450 superfamily (van den Brink et al., 1998). Although cultures of these fungi may provide a convenient and abundant capacity for enzymatic oxidations, fermentations in a microtiter plate or a shake flask can be difficult because the cultures tend to form large mycelium aggregates. Cross-contamination when using plate formats is also an issue because spores formed on the surface of mycelium aggregates or in the liquid can be transported from well to well. On the other hand, actinomycetes strains do not have such limitations, as they are often easy to grow and do not form large mycelium aggregates. P450 enzymes, as well as other monooxygenases and dioxygenases, have been identified in actinomycetes and shown to be capable of oxidative transformation of xenobiotics (Sariaslani and Omer, 1992; Trower et al., 1992; Lamb et al., 2002; Basch and Chiang, 2007). These attributes illustrate that actinomycetes strains offer a balance of ease of use and substantial biotransformation capability.

Much of the work in microbial models of mammalian metabolism has focused on finding one or a limited number of "super" strains capable of producing most of the mammalian metabolites of a given drug or drug candidate, so that the majority of the metabolites of interest can be obtained with a minimal number of incubations (Cannell, 1995; Joanna, 1999; Zhang et al., 2006; Zmijewski et al., 2006). Alternatively, for the purpose of preparing specific key mammalian metabolites, it may be advantageous to have an array of microbial strains with differing selectivities for the separate production of individual metabolites. If the interest is in preparing one or a very limited number of metabolites, a strain capable of producing many metabolites lowers the yield and increases the difficulty of purification. The advantages of selective metabolite formation were demonstrated with diclofenac, the metabolism of which has been well characterized in various biological systems including microbial systems. When diclofenac metabolism was screened in the microbial panel, the strains in wells A1-A5, B1-B3, C1, C3, C4, D4, and D5 were shown to be highly efficient in the turnover of diclofenac (Fig. 3), but these strains were not as useful as those in wells C2, C5, D1, and D2 for preparing 4′-hydroxydiclofenac. This illustrates the value of identifying those strains with a high degree of selectively toward the formation of a specific metabolite. Each actinomycetes strain bears oxygenases with different regio- and stereo-selectivity, and, accordingly, different substrate specificities. It is possible to take advantage of the variety in a limited number of highly active strains to produce a wide range of oxidative metabolites of interest without sacrificing yield and purity. The key to the successful use of this approach is the ability to rapidly screen microbial strains. The screening plate system introduced here provides an efficient way to access the power of a microbial strain array.

When selecting strains for a screening plate, it is critical to consider both the oxidative capabilities and the growth characteristics of the strain. There is a wealth of literature reports on highly active fungi and bacteria strains, and these various strains can be roughly divided into two groups: the first group is those known to produce natural products with oxidative steps in the biosynthetic pathways, and the second group is those known to perform oxidative transformations of xenobiotics. Many of these strains can be obtained from culture collections such as the American Type Culture Collection (strain identifier starting with ATCC) and the National Center for Agricultural Utilization Research (Peoria, IL) (strain identifier starting with NRRL). If the strain reported is not available from the culture collections, other strains in the same genus may be obtained from the culture collections and will likely have similar biotransformation properties. The growth characteristics of the strains should be chosen such that strains in the same plate have similar growth rates. To further simplify the screening procedures, the strains should be able to grow in the same medium. The malt extract medium described in this report is able to support the growth of various actinomycetes strains and supports good oxidative biotransformation activity. The oxidative capacity of several of the strains used in this study has been previously demonstrated

TABLE 3

[1]H, [13]C, and [15]N NMR chemical shift assignment of dasatinib metabolite M20 produced by microbial biotransformation



Position	^{1}H ppm (*d6*-DMSO, d 2.50 ppm)	^{13}C ppm (*d6*-DMSO, d 39.5 ppm)	^{15}N ppm (*d6*-DMSO, Referenced to $^{15}NH_4{}^{15}NO_3$ at δ20.7 and δ376.3 ppm)
2		162.2	
3 (N)			267.8
4	8.17 (s, 1H)	140.9	
5		126.3	
6 (N)	11.44 (b, s, 1H)		119.1
7		157.1	
8 (N)			226.7/237.5
9		165.6	
10 (N)			226.7/237.5
11		163.1	
12	6.05 (s, 1H)	82.9	
13		160.7	
15	2.40 (s, 1H)	26.0	
16 (N)			87.5
17 and 21	3.50 (b, t, 4H)	44.0	
18 and 20	2.48 (t, *J* = 5.05 Hz, 4H)	53.2	
19 (N)			43.7
22 (N)	9.61 (s, 1H)		115.5
23		125.2	
24		132.8	
25	6.76 (d, *J* = 2.65 Hz, 1H)	113.8	
26		156.9	
27	6.67 (d, *J* = 2.65 Hz, 1H)	116.3	
28		139.6	
29	2.42 (t, *J* = 6.19 Hz, 2H)	60.7	
30	3.53 (t, *J* = 6.19 Hz, 2H)	59.0	
34	2.13 (s, 1H)	18.9	

(Table 1, strains with references). The other strains have not been previously described but were found to possess useful oxidative activities (data not shown). The strains described here are one possible starting point for screening plates. There are likely to be other strains of actinomycetes capable of producing similar results.

The actinomycetes strains used in this study grew well in the 24-well deep-well plate. Plate formats with a larger number of wells would allow screening of more strains with each plate, but low aeration rates would negatively affect growth of cultures and efficiency of biotransformation unless modifications were made to the fermentation conditions (Duetz and Witholt, 2001). Additionally, the experimental design described in this study using 20 high-activity strains has been shown to be sufficient to find conditions under which most metabolites of interest can be produced. This study and a recent study showed that, collectively, the microbial enzymes encompassed by the 20 strains in Table 1 were able to mimic mammalian P450 enzymes and perform typical P450 probe reactions, such as the 4′-hydroxylation of diclofenac and the 1′- or 4-hydroxylation of midazolam (Li et al., 2005).

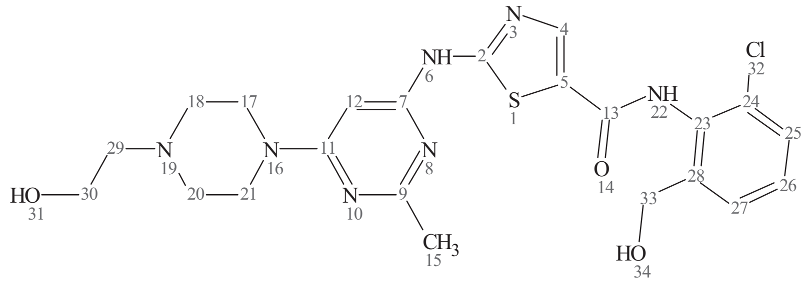
After analysis of the screening reactions, the selection of strains for scale-up reactions was based on the yield of desired metabolites as well as the number and relative proportions of undesired metabolites. Scaling up from microtiter plates to shake flasks (100 ml culture in 500-ml flask) was relatively straightforward, probably because the oxidative biotransformations proceeded faster in the flask due to higher aeration rates. Biotransformations in flasks can be used to make gram quantities of a desired product, as shown for the 21-hydroxylation of epothilone B (Li et al., 2004).

BMS-587101 is a small molecule antagonist of the leukocyte function-associated antigen-1 (Potin et al., 2006). During the development of BMS-587101 it was determined that M6 was a circulating metabolite in rat, dog, and human (data not shown). MS analysis indicated that it was two mass units less than BMS-587101. The possible sites for this net dehydrogenation were limited in the molecule and therefore the likely site was determined to be between C-16 and C-17. This was confirmed by NMR analysis of material purified from an RLM incubation. Milligram quantities of this metabolite were needed for further characterization, but the yield of M6 in RLM (0.2%) was extremely low, and it was not feasible to use any other LM incubation for a larger scale preparation. Chemical synthesis of M6 would have required considerable effort because it would require a completely revamped synthetic route to install a double bond between C-16 and C-17 (Potin et al., 2006). The microbial biosynthesized material allowed for the necessary initial characterization of the metabolite.

It is interesting to note the conversion of the carboxylic acid moiety of M6 and BMS-587101 to amides by the microbial cultures. Although amide formation from a carboxylic acid of an amino acid has been reported (Steffensky et al., 2000), there are only a few reports of this type of transformation in bacteria. In the case of amidation of polyaromatic carboxylic acids in *Bacillus cereus*, it was shown that

TABLE 4

[1]H and [13]C NMR chemical shift assignment of dasatinib metabolite M24 produced by microbial biotransformation



Position	^{1}H ppm (*d6*-DMSO, d 2.50 ppm)	^{13}C ppm (*d6*-DMSO, d 39.5 ppm)
2		160.5
3 (N)		
4	8.23 (s, 1H)	141.4
9		165.5
12	6.07 (s, 1H)	83.1
13		163.1
15	2.40[a]	26.2
17 and 21	3.50[a]	44.1
18 and 20	2.48[a]	53.1
23		131.4
24		132.6
25	7.46 (d, J = 7.75 Hz, 1H)	127.8
26	7.37 (t, J = 7.75 Hz, 1H)	128.3
27	7.53 (d, J = 7.75 Hz, 1H)	125.7
28		143.7
29	2.41[a]	60.7
30	3.53[a]	59.0
33	4.48 (s, 1H)	59.8

[a] No integration or coupling constant data are available because of peak overlap.

the nitrogen atom of the amides came from an amino group of an amino acid and not from ammonia or an alkylamine (Maruyama et al., 2001).

Dasatinib is a potent inhibitor of SRC and BCR-ABL kinases (Das et al., 2006). It has been approved in the United States and Europe for the treatment of chronic myelogenous leukemia in imatinib (Gleevec)-resistant and imatinib-sensitive patients. Hydroxylation on the 2-chloro-6-methylphenyl ring to form metabolites M20 and M24 was one of the major routes identified in the in vitro and in vivo biotransformation of dasatinib (L. Christopher, D. Cui, C. Wu, R. Luo, J. Manning, S. J. Bonacorsi, M. Lago, A. Allentoff, F. Y. F. Lee, B. McCann, D. Reitberg, S. Galbraith, K. He, A. Barros, A. Blackwood-Chirchir, W. G. Humphreys, and R. A. Iyer, submitted manuscript; D. Cui, W. Li, L. Chistopher, A. Barros, V. Arora, H. Zhang, L. Wang, D. Zhang, J. A. Manning, K. He, A. M. Fletcher, M. Ogan, M. Lago, S. J. Bonacorsi, W. G. Humphreys, and R. A. Iyer, submitted manuscript). M20 accounted for approximately 31% of the administered dose in humans (L. Christopher, D. Cui, C. Wu, R. Luo, J. Manning, S. J. Bonacorsi, M. Lago, A. Allentoff, F. Y. F. Lee, B. McCann, D. Reitberg, S. Galbraith, K. He, A. Barros, A. Blackwood-Chirchir, W. G. Humphreys, and R. A. Iyer, submitted manuscript). Quantities of M20 and M24 generated in HLM incubations were sufficient for structural elucidation by NMR analysis (D. Cui, W. Li, L. Chistopher, A. Barros, V. Arora, H. Zhang, L. Wang, D. Zhang, J. A. Manning, K. He, A. M. Fletcher, M. Ogan, M. Lago, S. J. Bonacorsi, W. G. Humphreys, and R. A. Iyer, submitted manuscript). However, a larger quantity of M20 and M24 was needed for activity testing and to serve as an analytical standard in the analysis of animal and human samples. Although efforts toward chemical synthesis of these metabolites met with great difficulty, the microbial technique provided a relatively facile means for rapid generation of M20 and M24.

It is not clear whether a single microbial enzyme in each strain was responsible for formation of both M20 and M24 or the two metabolites were products of multiple enzymes. Each microbial strain that produced M20 also generated M24, which may imply that they are formed by a single enzyme. Obviously, variations existed among the strains, as each strain produced a different ratio of M20 to M24.

In summary, the 24-well plate system described in this study is an extremely useful and efficient method for rapidly screening actinomycetes strains for biotransformation activity. The utility of this method was demonstrated with two examples in which mammalian oxidative metabolites of two development-stage compounds were produced by microbial routes. In both of these examples, the metabolites were not readily accessible by chemical synthesis, and scale-up of mammalian systems would have required significant resources. A simple shake-flask microbial biotransformation method subsequent to the initial screening was shown to be readily capable of producing up to 20 mg of metabolite with a half-liter fermentation and was used for obtaining material for initial metabolite characterization work such as testing for pharmacological activity or as a standard for the development of quantitative LC/MS assays.

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