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(54) **EFFICIENT MICROBIAL PREPARATION OF CAPRAVIRINE METABOLITES M4 AND M5**

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(57) **ABSTRACT**

The present invention provides a method for producing metabolites of capravirine (2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole) via whole cell biotransformation using fungi and bacterial cells as oxygenation catalysts.

EFFICIENT MICROBIAL PREPARATION OF CAPRAVIRINE METABOLITES M4 AND M5

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Ser. No. 60/496,635, filed Aug. 19, 2003, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to the production of capravirine metabolites M4 and M5 by using microbial cell strains as oxygen transfer catalysts. The method can be used to selectively prepare sufficient quantities of M4 and M5 for use in various drug activity studies. These two metabolites have potent antiviral activity, while exhibiting less toxicity than capravirine itself.

[0003] Capravirine (CPV, also known as S-1153), which is also known as 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole, is classified as a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is a potent anti-HIV agent. Capravirine has demonstrated activity against HIV strains that are resistant to other antiviral agents. U.S. Pat. No. 5,910,506 describes capravirine and other imidazole derivatives that are useful as anti-HIV agents, while U.S. Pat. No. 6,083,958 describes, in part, anti-HIV compositions that contain such imidazole derivatives.

[0004] Two proposed metabolites of capravirine, M4 and M5, were structurally postulated as being hydroxylated metabolites of the capravirine isopropyl group (see Ohkawa, T. et al. *Xenobiotica*, 1998, 28, 877). The antiviral activity and relative toxicity of these metabolites has not previously been determined. Also, the two metabolites have to date not been prepared or characterized, due to difficulties in their synthesis. In particular, it is difficult to use human-liver derived in vitro systems (e.g., human liver homogenates also known as microsomes) (Pelkonen O, Maenpaa J, Taavitsainen P, Rautio A, Raunio H. Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* 28: 1203-1253, 1998) to prepare a sufficient quantity of metabolites for structural characterization, since in general they can only be used to generate nanogram (ng) to microgram (μ g) amounts of materials. Human liver samples used for these studies are usually obtained from human donors, and not only the ethical implications of such methodology, but also the limited amounts in which microsomes are offered from commercial sources, pose a great limitation for their use on an industrial scale. Microbial models of mammalian metabolism have been reported in the literature as an inexpensive, renewable and simple alternative for the preparation of drug metabolites (R. V. Smith and J. P. Rosazza: Microbial models of mammalian metabolism. *J. Pharm. Sci.* 11,1737-1759).

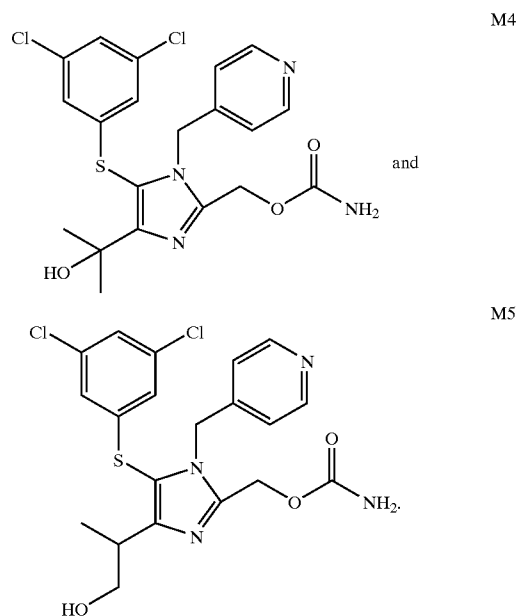
[0005] Accordingly, a need exists for preparing capravirine metabolites M4 and M5 in sufficient quantities of scale in order to characterize their relative antiviral activity,

associated toxicity and to elucidate their structure. The present invention describes the use of microbial cells to obtain sufficient amounts of metabolites M4 and M5 for such activity studies and structural characterization.

SUMMARY OF THE INVENTION

[0006] The present invention is directed to a method for preparing a metabolite of 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole from a cell strain, comprising reacting the cell strain with 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole, and collecting the metabolite. The invention is further directed to the preparation of CPV metabolites from dioxygenated precursors.

[0007] Preferred metabolites produced via the invention include:



[0008] Preferred cell strains for use in the method include *Streptomyces griseus* ATCC 13273, *Streptomyces griseolus* ATCC 11796, *Syncephalastrum racemosum* ATCC 18192, *Actinoplanes* sp. ATCC 53771, *Streptomyces rimosus* ATCC 10970, *Absidia pseudocylindrospora* ATCC 24169, *Mortierella isabellina* ATCC 42613 and *Verticillium theobromae* ATCC 12474.

DETAILED DESCRIPTION OF THE INVENTION

[0009] The preparation of metabolites M4 and M5 was achieved using microbial cell strains as oxygen transfer catalysts. Using this method, M4 and M5 can be produced

at milligram to grams scale, and they can also be generated in a selective fashion. The methods described herein include a screening procedure, followed by a process optimization where fermentation parameters were optimized. In addition, a chemical method to convert undesired metabolites into M4 and M5 is also presented. Suitable bacterial and fungal strains were identified (see Table I and procedure below) from performing a microbial screening. Two particular bacterial strains, *Streptomyces griseus* ATCC 13273 and *Streptomyces griseolus* ATCC 11796, were found to be efficient in producing a mixture of M4 and M5 precursors which were chemically converted into M4 and M5. One fungal strain, *Syncephalastrum racemosum* ATCC 18192, selectively produced M4, which greatly facilitated the structural studies on this metabolite.

DEFINITIONS

[0010] The term “ACN”, as used herein, refers to acetonitrile.

[0011] The term “ CC_{50} ”, as used herein, means the 50% cytotoxicity concentration, which is calculated as the concentration of compound that decreases the viability of uninfected, compound-treated cells to 50% of that of uninfected, compound-free cells.

[0012] The term “ EC_{50} ”, as used herein, means the statistically derived concentration of a toxicant that can be expected to cause a defined non-lethal effect in 50% of a given population of organisms under defined conditions.

[0013] The term “ EC_{90} ”, as used herein, means the statistically derived concentration of a toxicant that can be expected to cause a defined non-lethal effect in 90% of a given population of organisms under defined conditions.

[0014] The term “HPLC”, as used herein, refers to High Performance Liquid Chromatography, which is also often referred to as High Pressure Liquid Chromatography.

[0015] The term “MeOH”, as used herein, refers to methanol.

[0016] The term “min.”, as used herein, refers to minutes.

[0017] The term “NMR”, as used herein, refers to Nuclear Magnetic Resonance spectroscopy.

[0018] The term “RT”, as used herein, refers to room temperature.

[0019] The term “TFA”, as used herein, refers to trifluoroacetic acid.

[0020] The term “TLC”, as used herein, refers to Thin Layer Chromatography.

[0021] Experiments for Biosynthesis of CPV Metabolites M4 and M5

[0022] 1. Microbial screening of CPV hydroxylators

[0023] Most of the microorganisms that were found to perform the desired reaction could also produce other dioxygenated metabolites such as M2 and M3, and in some cases dioxygenated compounds C12, C14 and M6 (SCHEME 1).

SCHEME 1

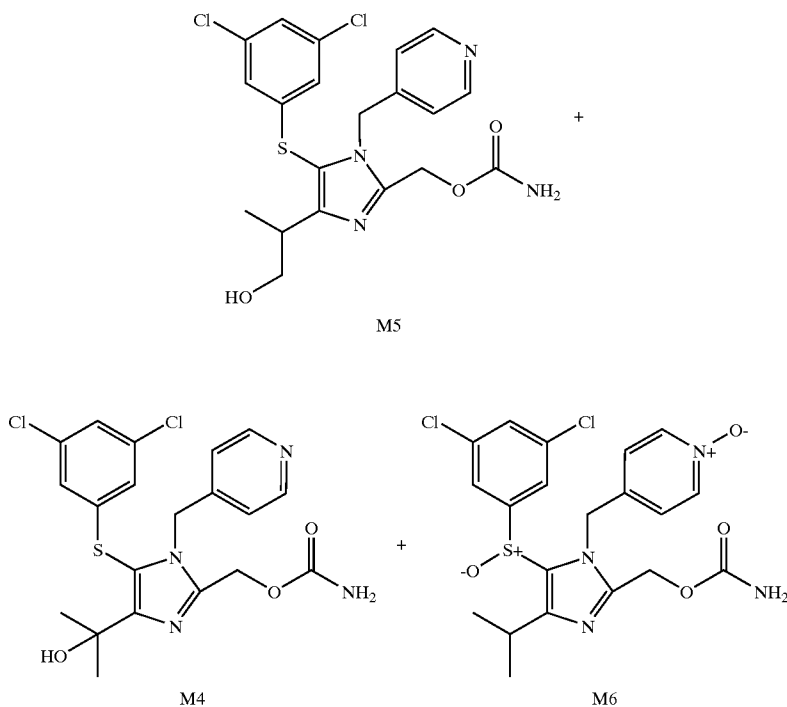


TABLE I-continued

ATCC Number	Fungi
10137	<i>Streptomyces griseus</i> sp. griseus
13273	<i>Streptomyces griseus</i>
10970	<i>Streptomyces rimosus</i>
51531	<i>Chelatococcus asaccharovorans</i>
31338	<i>Nocardia corallina</i>
15592	<i>Rhodococcus</i> sp.
29347	<i>Pseudomonas oleovorans</i>
12633	<i>Pseudomonas putida</i>
17699	<i>Ralsonia eutropha</i>
21457	<i>Achromobacter lyticus</i> Isono
19795	<i>mycolatopsis orientalis</i>
35203	<i>Pseudocardia autotrophica</i>
34541	<i>Phanerochaete chiysosporium</i>
10404	<i>Rhizopus oryzae</i>
1008	<i>Aspergillus ochraceus</i>
18192	<i>Syncephalastrum racemosum</i>
9245	<i>Cunninghamella echinulata</i> var. Elegans
22751	<i>Absidia cylindrospora</i>
42613	<i>Mortierella isabellina</i>
18191	<i>Thamnidium elegans</i>
24169	<i>Absidia pseudocylindrospora</i>
10864	<i>Aspergillus niger</i>
9142	<i>Aspergillus niger</i>
15517	<i>Aspergillus parasiticus</i>
10029	<i>Aspergillus terreus</i>
7158	<i>Beauveria bassiana</i>
13144	<i>Beauveria bassiana</i>
10571	<i>Candida rugosa</i>
36190	<i>Cunninghamella echinulata</i>
13633	<i>Cutvularia lunata</i>
4740	<i>Mucor plumbeus</i>
36060	<i>Rhizopogon</i> sp
34541	<i>Cunninghamella echinulata</i> var. Elegans
12724	<i>Epicoccum oryzae</i>
12726	<i>Epicoccum</i> sp.
12725	<i>Epicoccum yuccae</i>
12722	<i>Epicoccum humicola</i>
16373	<i>Caldariomyces fumago</i>
28300	<i>Verticillium lecanii</i>
12474	<i>Verticillium theobromae</i>

[0025] Four bacterial strains were found to efficiently metabolize CPV under screening conditions: *Actinoplanes* sp. ATCC 53771, *Streptomyces griseolus* ATCC 11796,

Streptomyces griseus ATCC 13273, and *Streptomyces rimosus* ATCC 10970. Of those, *Streptomyces griseolus* ATCC 11796 and *Streptomyces griseus* ATCC 13273 showed greater amounts of metabolites with almost complete consumption of starting material. M4 and M5, as well as the other metabolites shown in SCHEME 1, were observed during the screening process using these strains.

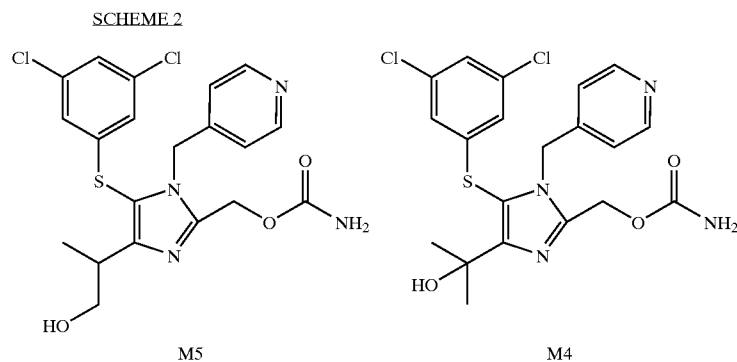
[0026] Four fungal strains were found to metabolize CPV under screening conditions: *Absidia pseudocylindrospora* ATCC 24169, *Mortierella isabellina* ATCC 42613, *Verticillium theobromae* ATCC 12474 and *Syncephalastrum racemosum* ATCC 18192. The fungus *Syncephalastrum racemosum* ATCC 18192 is preferred for selective conversion to the hydroxylated product M4. Other compounds present after the whole cell reaction included metabolites M2, M3 and unreacted CPV. Representative methods and reaction scale-up are shown for *Syncephalastrum racemosum* ATCC 18192 using conditions similar to the ones used in the screen.

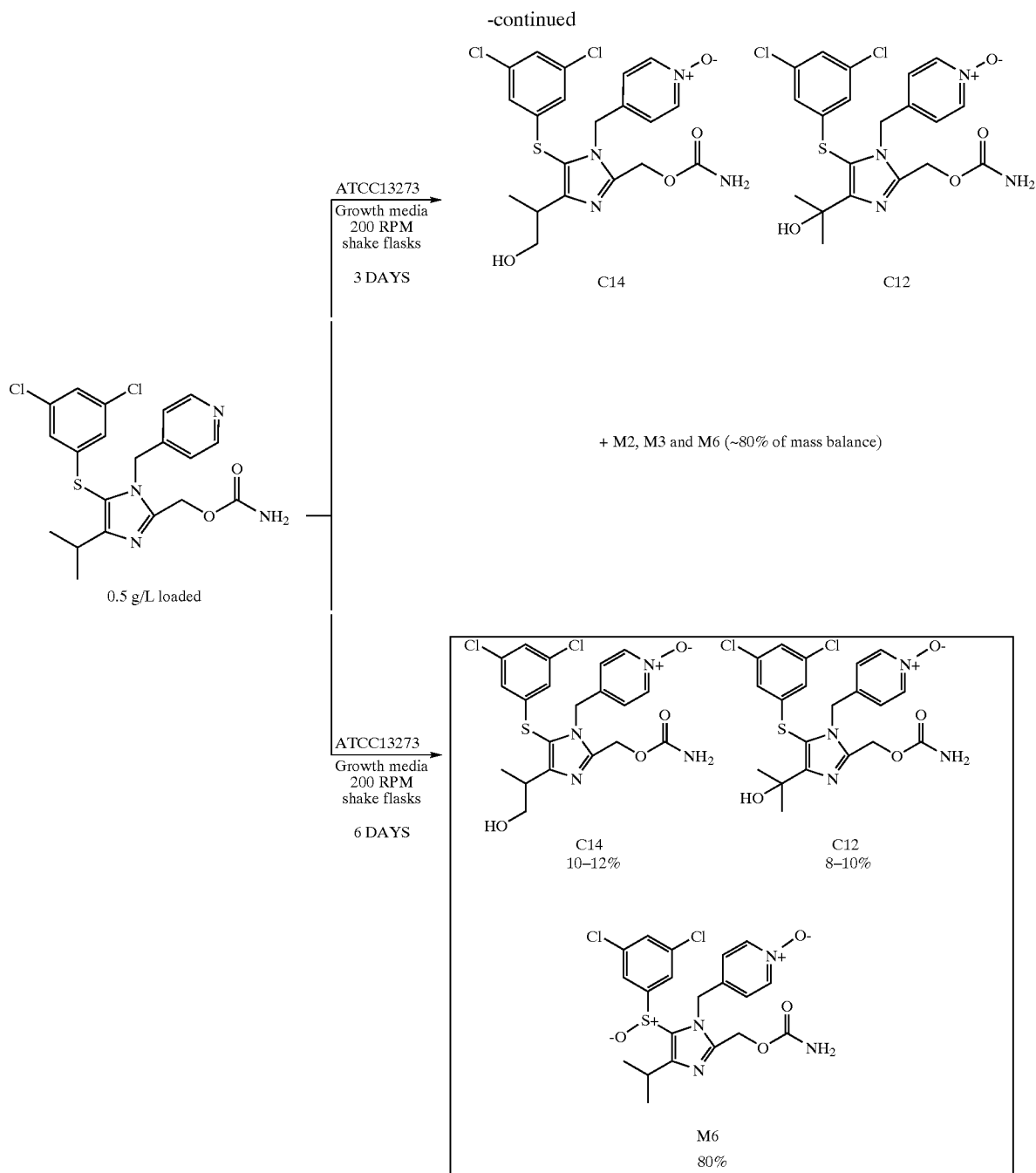
[0027] 2. Optimization studies using *Streptomyces griseus* ATCC 13273

[0028] Several experiments were conducted on bacterial strain *Streptomyces griseus* ATCC 13273 in order to optimize the entire cell reaction.

[0029] (a) Growth conditions

[0030] Glycerol-based media resulted in stable and high growth culture that ensured reproducibility of the procedure. A two-stage fermentation procedure was set up where preculture (first stage) was grown from fresh inoculum (colonies washed from agar plate) in shake flasks for 2 days. The second stage culture was started by adding preculture to fresh media ($1/50$ - $1/100$ dilution) and the resulting culture was grown for 1 day before substrate was added from a 10% ethanol solution. SCHEME 2 below illustrates the conversion of CPV into metabolites C12, C14, M4, M5, and M6.





[0031] Close monitoring of reaction outputs indicated that the conversion of CPV into metabolites M4 and M5 peaked at about 3 days, followed by dioxygenation of those into C12 and C14, which continued until about 6 days (see SCHEME 2). The final crude materials after 6 days contained only three components: C12, C14 and M6.

[0032] (b) Development of HPLC method for the purification of C12 and C14

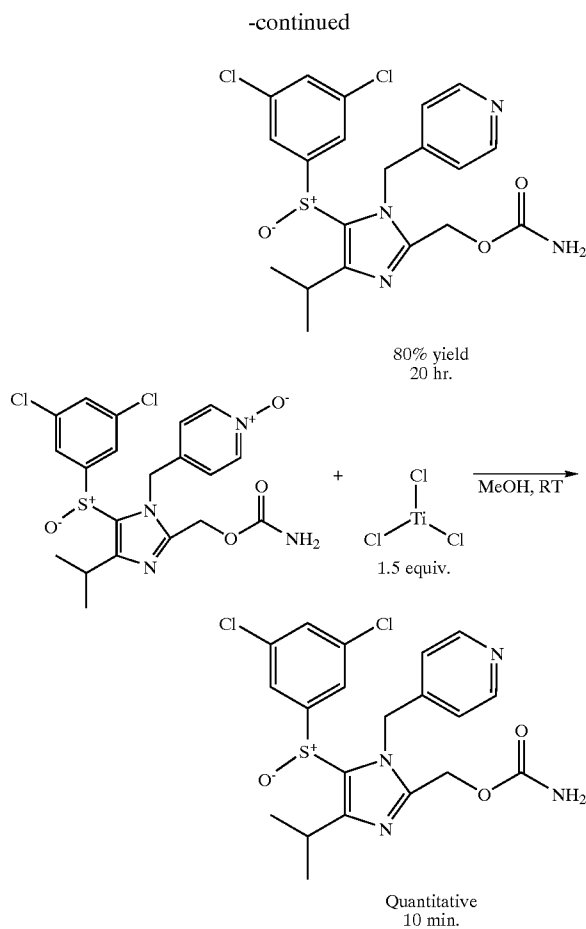
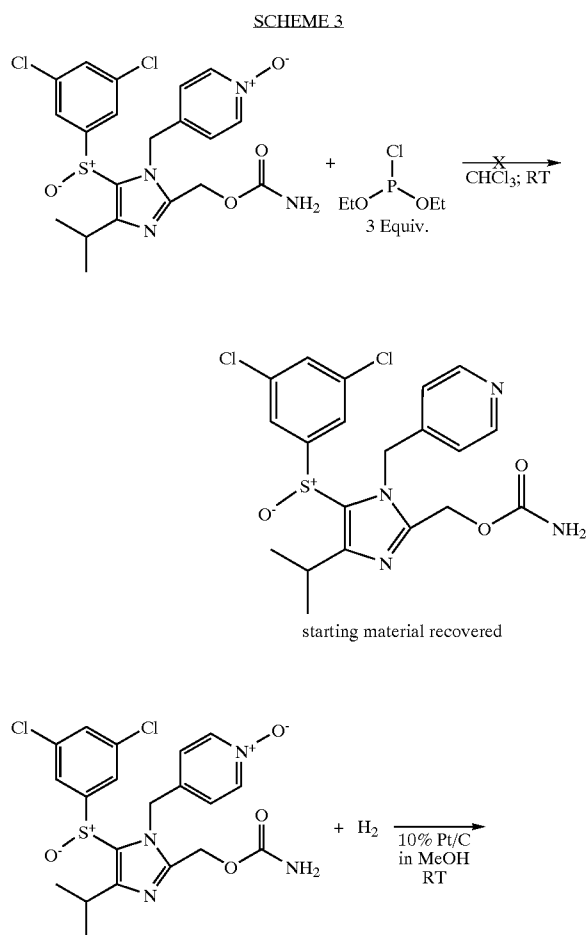
[0033] The crude extracts from the biotransformation of CPV were fractionated by semi-preparative chromatography

on an Agilent HPLC preparative system. Multiple injections (extract dissolved in MeOH) loaded onto a 21.2x150 mm Phenomenex Max RP column (80 521, 4 μ m) were performed with UV detection at 254 nm and peak-level detection for fractionation adjusted to the injection volume. Gradient elution with a flow rate of 20 ml/min was used: 5% ACN/(0.1% TFA in water) for 2.9 min.; 5% to 15% in 0.1 min.; 15% to 45% in 12 min.; 45% to 98% in 1 min.; 98% for 4 min.; then reequilibration. Desired fractions were isolated and subsequent analysis by LC/MS and NMR

showed that desired M4 and M5 fractions contained the corresponding N-oxide (C12 and C14 respectively). NMR and LC analysis further showed that the major component of each mixture was a higher oxidation product, C12 and C14. Therefore, these fractions were subjected to reduction conditions discussed below and were later repurified using the identical HPLC method to ensure >98% purity of product for clinical studies.

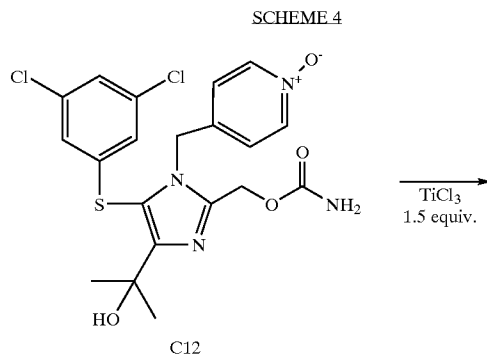
[0034] (c) Studies toward the N—O reduction of dioxygenated precursors C6:

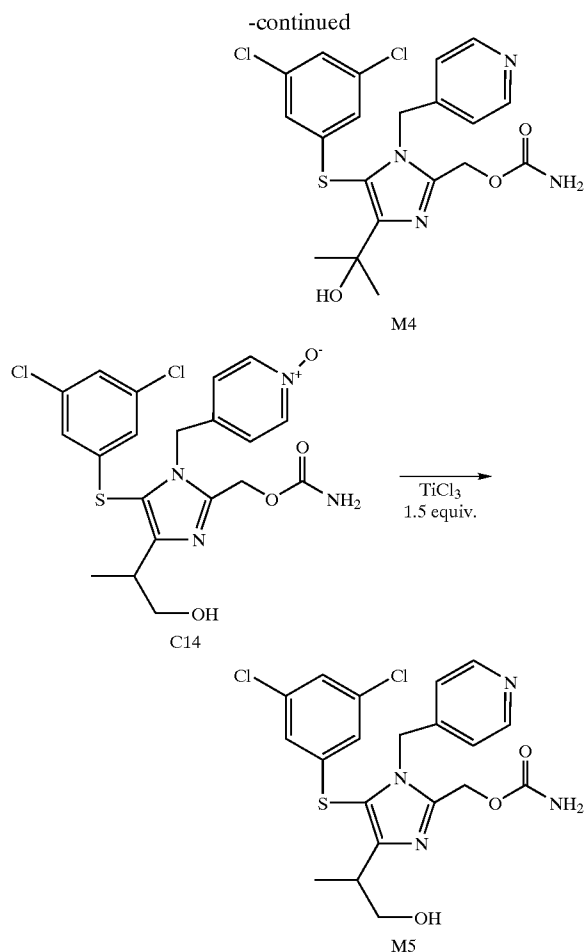
[0035] Due to the small amount of pure M4 and M5 products and the presence of dioxygenated species C12 and C14, a model reaction for the reduction of M4 and M5 byproducts was devised (see SCHEME 3). Compound M6 was used as the test compound to study the reduction of the N-oxide moiety in these metabolites. The first method tested involved the use of diethylchlorophosphite (DECP). The reaction was unsuccessful and no further conditions were tested.



[0036] The use of platinum on carbon was effective for the hydrogenolysis reaction. However, titanium trichloride performed the reaction much faster, and it was selected as the preferable reagent to test for the reduction of C12 and C14.

[0037] (d) Preparation of M4 and M5 from dioxygenated precursors C12 and C14 (SCHEME 4): Reduction of pure compounds C12 and C14 in the presence of TiCl_3 solution (1.5 eq from a 15% TiCl_3 stock solution in aqueous HCl) in MeOH was completed in 10 min., according to general SCHEME 4, below.





[0038] The reaction was quenched with 1 volume of 100 mM Phosphate buffer pH 8.0, stirred at room temperature for 10 min. and centrifuged at 5,000 RPM for 10 min. The supernatant was concentrated to remove methanol and then extracted (5 times) with 1 volume of chloroform to afford pure M4 and M5 after evaporation of the organic solvent. Almost quantitative recovery was observed in most runs (see procedure below for the production of M5).

[0039] 3. Whole cell biotransformations for the Preparation of M4 and M5

[0040] Once reproducible and efficient cell strains were identified, 1 L reactions were run using shake flasks as culture vessels. The procedures presented below were validated at the 10 L scale.

[0041] (a) Whole cell biotransformation using *Streptomyces griseus* ATCC 13273

[0042] *Streptomyces griseus* was grown from an agar plate into a 100 ml preculture using the screening medium containing glycerol as carbon source. After 2 days culture, 10 ml of the preculture was inoculated into a 1 L culture containing fresh culture media (2% glucose as carbon source) on a 4 L shake flasks. The culture was grown for 24 hr. and substrate was added in two portions (0.2 g after 24 hr. and 0.3 g after

48 hr.). Oxidation was followed by HPLC, monitoring the amount of metabolite C12 and C14 (until approximately 10% conversion each). The cells were removed from the culture by centrifugation at 10,000 RPM and the oxidation products extracted 3 times with one volume of chloroform each. After removal of CHCl_3 in vacuo, crude product (550 mg) was obtained. The crude product was purified by preparative HPLC chromatography using the same conditions described in the analytical method (see section 2(b) above). Fifty-four mg of C12 and 60 mg of C14 were recovered. The pure deoxygenated products were then treated with TiCl_3 to afford pure M4 (35 mg) and M5 (40 mg), respectively.

[0043] (b) Whole cell biotransformation using *Syncephalastrum racemosum* ATCC 18192

[0044] *Syncephalastrum racemosum* was grown from an agar plate into a 100 ml preculture using the screening medium and conditions. After 2 days, 10 ml of the preculture was inoculated into 1 L culture on a 4 L shake flask. The culture was grown for 24 hr. and substrate was added (0.2 g/L substrate load). Oxidation was followed by reverse phase HPLC and the reaction stopped after the concentration of metabolite M4 has reached approximately 20% conversion. The mycelium was removed from the culture by filtration and the oxidation products extracted 3 times with one volume of chloroform each. After removal of CHCl_3 in vacuo, crude product (150 mg) was obtained. The crude product was purified by silica gel flash chromatography, using $\text{CH}_2\text{Cl}_2/\text{Acetone}/\text{MeOH}$ (40:1:1 and 10:1:1) as eluent, to afford 25 mg of pure M4 as the only hydroxylated product based on TLC, HPLC/MS and NMR analysis.

[0045] (c) Structural characterization of M4 and M5

[0046] $^1\text{H-NMR}$ spectra were recorded on a Bruker DPX-300 using a QNP probe operating at 300 MHz and $^{13}\text{C-NMR}$ spectra were recorded operating at 75 MHz. Spectra were obtained as CDCl_3 solutions (reported in ppm), using chloroform as the reference standard (7.27 ppm and 77.00 ppm) unless otherwise noted. Where peak multiplicities are reported, the following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broadened multiplet), bs (broadened singlet), dd (doublet of doublets), dt (doublet of triplets). Coupling constants, when given, are reported in Hertz (Hz).

[0047] M4: ESI: $[\text{M}+1]^+467.0726$; calc. for $\text{C}_{20}\text{H}_{21}\text{Cl}_2\text{N}_4\text{O}_3\text{S}$ 467.0711, $^1\text{H NMR}$ (CDCl_3) δ 8.24 (br.d, 2H), 7.07 (br.t, 1H), 6.80 (d, 2H), 6.72 (d, 2H), 5.25 (s, 1H), 5.18 (s, 1H), 1.62 (s, 6H); $^{13}\text{C NMR}$ (CDCl_3) δ 156.12, 154.72, 148.99, 144.14, 138.45, 134.87, 127.63, 125.64, 122.85, 120.10, 110.97, 69.43, 57.44, 45.72, 29.55.

[0048] M5: ESI: $[\text{M}+1]^+467.0726$; calc. for $\text{C}_{20}\text{H}_{21}\text{Cl}_2\text{N}_4\text{O}_3\text{S}$ 467.0711; $^1\text{H NMR}$ (CDCl_3) δ 8.49 (br.d, 2H), 7.40 (br.d, 2H), 7.09 (t, 1H), 6.90 (t, 2H), 5.59 (s, 2H), 5.15 (s, 2H), 1.70 (m, 2H), 3.40 (m, 1H), 1.15 (d, 3H). $^{13}\text{C NMR}$ (CDCl_3) δ 156.12, δ 148.89, 139.59, 136.03, 127.78, 126.45, 122.85, 120.10, 115.05, 67.52, 59.14, 48.48, 36.72, 17.56.

[0049] TABLE 2 provides a comparison of the antiviral activity and cytotoxicity data for CPV and the M4 and M5 metabolites.

TABLE 2

Antiviral activity and cytotoxicity of CPV and CPV metabolites ^a					
Compound	EC ₅₀ (uM)	EC ₉₀ (uM)	CC ₅₀ (uM)	TI ^b	Activity
CPV	0.0015	0.0032	69	45,667	+
M4	0.048	0.11	>320	>6,737	+
M5	0.047	0.11	>320	>6,882	+

^aAntiviral activity and cytotoxicity were determined measuring XTT dye reduction. Results for M4 and M5 represent the mean of two to four experiments. Results for CPV represent the mean of 9 experiments.

^bTherapeutic index = CC₅₀/EC₅₀.

[0050] While the invention has been illustrated by reference to specific and preferred embodiments, those skilled in the art will recognize that variations and modifications may be made through routine experimentation and practice of the invention. Thus, the invention is intended not to be limited by the foregoing description, but to be defined by the appended claims and their equivalents.

We claim:

1. A method for preparing a metabolite of 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole comprising:

(a) providing a cell strain selected from the group consisting of:

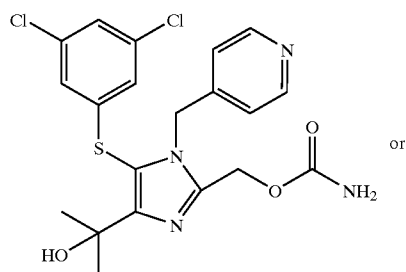
Streptomyces griseus, *Streptomyces griseolus*, *Syncephalastrum racemosum*, *Actinoplanes* sp., *Streptomyces rimosus*, *Absidia pseudocylindrospora*, *Mortierella isabellina* and *Verticillium theobromae*;

(b) reacting said cell strain with 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole; and

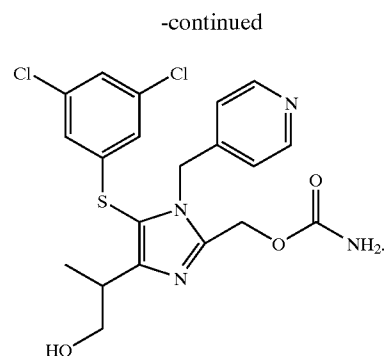
(c) collecting said metabolite.

2. The method of claim 1 wherein said metabolite is an oxidative metabolite.

3. The method of claim 1 wherein said metabolite is



M4



M5

4. The method of claim 1 wherein said cell strain is *Streptomyces griseus*, *Streptomyces griseolus* or *Syncephalastrum racemosum*.

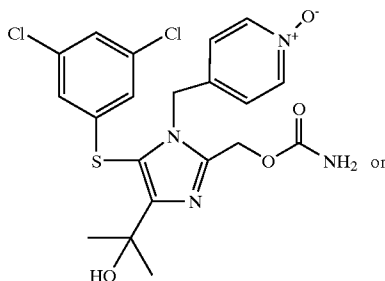
5. A method for preparing a metabolite of 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole comprising:

(a) providing a bacteria cell strain selected from the group consisting of:

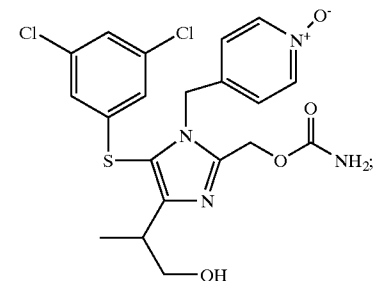
Actinoplanes sp., *Streptomyces griseolus*, *Streptomyces griseus*, and *Streptomyces rimosus*;

(b) reacting said bacteria cell strain with 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole;

(c) producing a compound of formula



C12

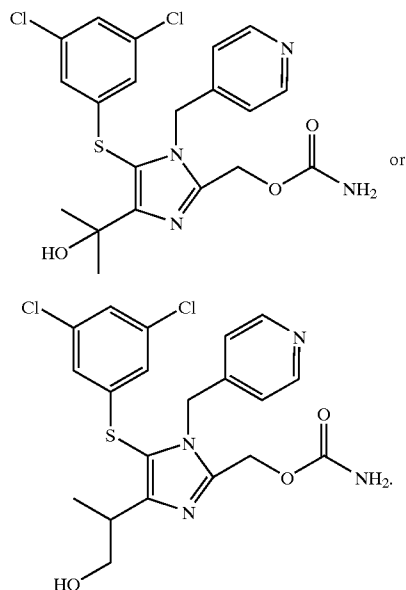


C14

(d) reacting said compound from step (c) with TiCl₃; and

(e) collecting said metabolite.

6. The method of claim 5 wherein said metabolite is



7. The method of claim 5 wherein said bacteria cell strain is *Streptomyces griseus* or *Streptomyces griseolus*.

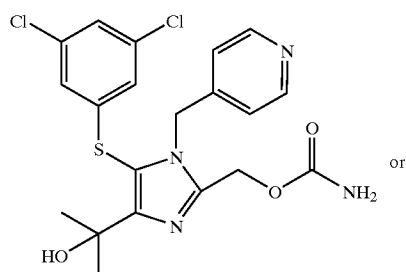
8. A method for preparing a metabolite of 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole comprising:

(a) providing a fungus cell strain selected from the group consisting of:

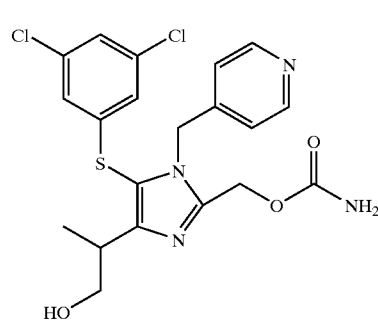
Syncephalastrum racemosum, *Absidia pseudocylindrospora*, *Mortierella isabellina* and *Verticillium theobromae*;

(b) reacting said cell strain with 2-carbamoyloxymethyl-5-(3,5-dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazole;

(c) producing a compound of formula



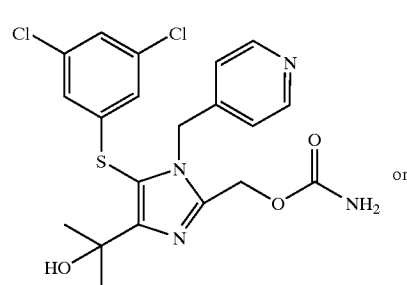
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(d) reacting said compound from step (c) with TiCl_3 ; and

(e) collecting said metabolite.

9. The method of claim 8 wherein said metabolite is



10. The method of claim 8 wherein said fungus cell strain is *Syncephalastrum racemosum*.

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