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SPECIFICATION

PROCESS FOR PRODUCING OPTICALLY ACTIVE PYRIDINEETHANOL
DERIVATIVES

5

TECHNICAL FIELD

The present invention relates to a method of producing an optically active pyridineethanol derivative. More particularly, it relates to a method of producing an optically active polycyclic pyridineethanol derivative by causing an enzyme or enzyme source to act on polycyclic acetylpyridine derivatives.

The present invention also relates to a novel enzyme which can be used in the production method mentioned above, a DNA coding for said enzyme, a recombinant vector having said DNA, and a transformant having said recombinant DNA.

The invention further relates to a method of producing an optically active polycyclic pyridineethanol derivative by causing the above novel enzyme or the above transformant to act on an optically inactive polycyclic pyridineethanol derivative.

BACKGROUND ART

Optically active pyridineethanol derivatives are compounds useful as starting materials and intermediates of the synthesis of medicinals, agrochemicals and the like, which are required to be optically active.

As for the production of an optically active monocyclic pyridineethanol derivative, a method is known which comprises converting an acetylpyridine to the simplest optically active pyridineethanol derivative, namely hydroxyethylpyridine, using baker's yeast or like microorganisms (Japanese Kokai Publication Sho-61-22791), for instance.

As for the production of an optically active polycyclic pyridineethanol derivative, some methods are known: the method

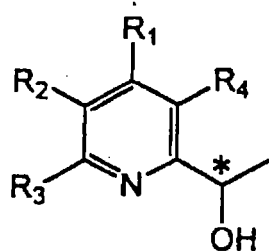
comprising effecting optical resolution of racemic 5-(1-hydroxyethyl)furo[2,3-c]pyridine or 5-(1-hydroxyethyl)-3-methylfuro[2,3-c]pyridine through asymmetric esterification using lipase type 2 from swine pancreatic (WO 9635678), and the
5 method comprising effecting optical resolution of racemic 7-chloro-5-(1-hydroxyethyl)furo[2,3-c]pyridine through asymmetric esterification using Candida antarctica lipase (Synlett, 41, (1999)), for instance. However, these methods are based on optical resolution, hence the yield of one
10 enantiomer is at most 50%, which is low and unsatisfactory.

Further, a method is known which comprises chemically reducing 5-(1-acetyl)-7-chloro-3-methylfuro[2,3-c]pyridine with (-)-chlorodiisopinocampheylborane in tetrahydrofuran to thereby obtain (S)-7-chloro-5-(1-hydroxyethyl)-3-
15 methylfuro[2,3-c]pyridine (Journal of Organic Chemistry, 63, 7851 (1998)). Since, however, the expensive reducing agent is used in large amounts, it is difficult to put the method into practical use.

20 DISCLOSURE OF THE INVENTION

As a result of intensive investigations made by the present inventors to develop an efficient method of producing an optically active polycyclic pyridineethanol derivative, they discovered an enzyme source which has not been reported
25 as yet but which is capable of stereoselectively reducing an acetylpyridine derivative and thus converting it to an optically active pyridineethanol derivative and this finding has now led to completion of the present invention.

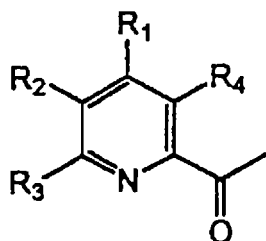
Thus, the present invention is concerned with a method
30 of producing an optically active pyridineethanol derivative represented by the general formula [2]:



[2]

- wherein R_1 and R_2 are bound to each other to form a 5- to 8-membered monocyclic heterocycle containing at least one hetero atom selected from the group consisting of oxygen, sulfur and nitrogen atoms, which heterocycle may optionally have a substituent(s), or a polycyclic heterocycle resulting from the condensation of such monocyclic heterocycle with another ring, which polycyclic heterocycle may optionally have a substituent(s),
- R_3 and R_4 are the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms, which may optionally have a substituent(s), or an alkoxy group containing 1 to 12 carbon atoms, which may optionally have a substituent(s),
- and * indicates that the asterisked carbon atom is an asymmetric one,

which method comprises stereoselectively reducing an acetylpyridine derivative represented by the general formula [1]:



[1]

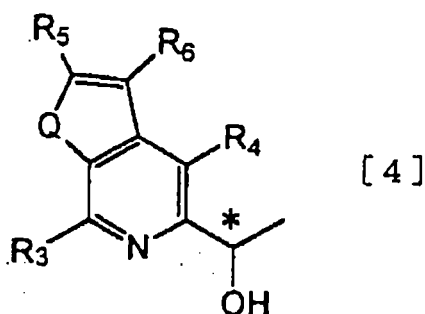
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wherein R_1 , R_2 , R_3 and R_4 are as defined above, by causing an enzyme or enzyme source capable of asymmetrically reducing the same to act thereon.

In a preferred mode of embodiment, the invention relates to a method of producing an optically active pyridineethanol

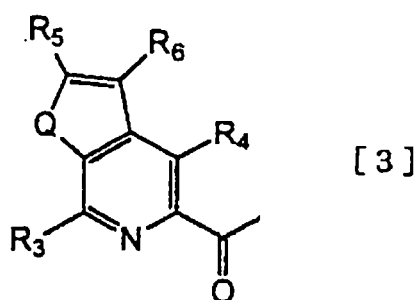
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derivative represented by the general formula [4]:



wherein Q represents an oxygen or sulfur atom or a group of the
 5 general formula -N(D)-, in which N is a nitrogen atom and D
 represents a hydrogen atom or a monovalent protective group,
 R₃, R₄, R₅ and R₆ are the same or different and each represents
 a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group
 10 containing 1 to 12 carbon atoms, which may optionally have a
 substituent(s), or an alkoxy group containing 1 to 12 carbon
 atoms, which may optionally have a substituent(s),
 and * indicates that the asterisked carbon atom is an asymmetric
 one,

which method comprises stereoselectively reducing an
 15 acetylpyridine derivative represented by the general formula
 [3]:



wherein Q, R³, R⁴, R⁵ and R⁶ are as defined above,
 by causing an enzyme or enzyme source capable of asymmetrically
 20 reducing the same to act thereon.

The invention further relates to an enzyme having the
 following physical and chemical properties (1) to (3):

(1) Activity: It stereoselectively reduces 5-acetylfuro[2,3-c]pyridine, in the presence of reduced form nicotinamide adenine dinucleotide as a coenzyme, to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine;

5 (2) Specificity: It has reducing ability against ketones and aldehydes but is very low in reducing activity against carbocyclic ketones and the α -position keto group of α -keto acids;

(3) Molecular weight: It shows a molecular weight of about
10 60,000 in gel filtration analysis and a molecular weight of about 29,000 in SDS polyacrylamide electrophoresis.

The invention also relates to an enzyme specified below under (a) or (b):

(a) An enzyme comprising an amino acid sequence shown under
15 SEQ ID NO:1 in the sequence listing;

(b) An enzyme comprising an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:1 in the sequence listing by deletion, substitution and/or addition of one or several amino acids and having an activity by which 5-
20 acetylfuro[2,3-c]pyridine is stereoselectively reduced to 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine.

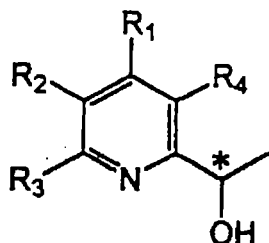
When these enzymes are used as the enzyme in the production method mentioned above, an optically active pyridineethanol derivative having the R absolute configuration
25 is obtained.

Furthermore, the invention relates to a DNA coding for such enzyme or a DNA comprising a base sequence shown under SEQ ID NO:2 in the sequence listing. It further relates to a recombinant vector containing such DNA and to a transformant
30 having such recombinant vector.

When this transformant is used as a source of said enzyme in the above production method, an optically active pyridineethanol derivative having the R absolute configuration is obtained.

35 Still further, the invention relates to a method of

producing an optically active pyridineethanol derivative having the S absolute configuration and represented by the general formula [6]:



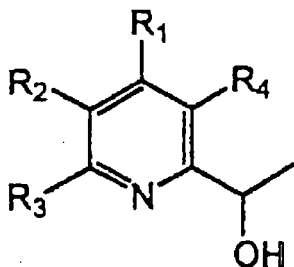
[6]

5

wherein R_1 and R_2 are bound to each other to form a 5- to 8-membered monocyclic heterocycle containing at least one hetero atom selected from the group consisting of oxygen, sulfur and nitrogen atoms, which heterocycle may optionally have a substituent(s), or a polycyclic heterocycle resulting from the condensation of such monocyclic heterocycle with another ring, which polycyclic heterocycle may optionally have a substituent(s),

R_3 and R_4 are the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms, which may optionally have a substituent(s), or an alkoxy group containing 1 to 12 carbon atoms, which may optionally have a substituent(s), and * indicates that the asterisked carbon atom is an asymmetric one,

which method comprises causing the enzyme and/or transformant mentioned above to act on a pyridineethanol derivative represented by the general formula [5]:



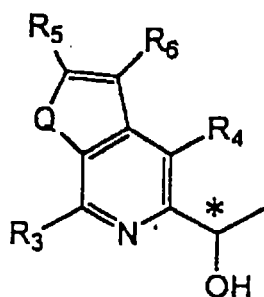
[5]

wherein R_1 , R_2 , R_3 and R_4 are as defined above,
to thereby preferentially oxidize the R form of the
pyridineethanol derivative

and recovering the remaining S form of the pyridineethanol
5 derivative.

In a preferred mode of embodiment, the invention relates
to a method of producing an optically active pyridineethanol
derivative having the S absolute configuration and represented
by the general formula [8]:

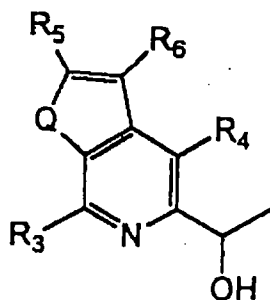
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[8]

wherein Q represents an oxygen or sulfur atom or a group of the
general formula $-N(D)-$, in which N is a nitrogen atom and D
represents a hydrogen atom or a monovalent protective group,
15 R_3 , R_4 , R_5 and R_6 are the same or different and each represents
a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group
containing 1 to 12 carbon atoms, which may optionally have a
substituent(s), or an alkoxy group containing 1 to 12 carbon
atoms, which may optionally have a substituent(s),
20 and * indicates that the asterisked carbon atom is an asymmetric
one,

which method comprises causing the enzyme and/or
transformant mentioned above to act on a pyridineethanol
derivative represented by the general formula [7]:



[7]

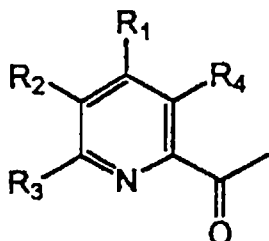
wherein Q, R₃, R₄, R₅ and R₆ are as defined above,
to thereby preferentially oxidize the R form of the
pyridineethanol derivative

- 5 and recovering the remaining S form of the
pyridineethanol derivative.

In the following, the present invention is described in
detail.

- 10 First, the method of producing an optically active
pyridineethanol derivative [2] by causing an enzyme or enzyme
source having asymmetrically reducing activity to act on an
acetylpyridine derivative [1] to thereby stereoselectively
reduce the same is described in detail.

- 15 The acetylpyridine derivative to be used as the substrate
in the production method of the invention is represented by the
following general formula [1]:



[1]

- 20 In the above general formula [1], R₁ and R₂ are bound to
each other to form a 5- to 8-membered monocyclic heterocycle
containing at least one hetero atom selected from the group
consisting of oxygen, sulfur and nitrogen atoms, which
heterocycle may optionally have a substituent(s), or a
polycyclic heterocycle resulting from the condensation of such

monocyclic heterocycle with another ring, which polycyclic heterocycle may optionally have a substituent(s).

Specifically, the 5-membered monocyclic heterocycle includes, among others, the furan, dihydrofuran, pyrrole, pyrroline, dehydrodioxolane, pyrazole, pyrazoline, imidazole, oxazole, isoxazole, oxadiazole, triazole, thiazole, thiophene, dihydrothiophene and like rings. The 6-membered monocyclic heterocycle includes, among others, the pyran, dihydropyran, pyridine, dihydropyridine, tetrahydropyridine, dehydrodioxane, dehydromorpholine, pyridazine, dihydropyridazine, pyrimidine, dihydropyrimidine, tetrahydropyrimidine, pyrazine, dihydropyrazine and like rings. The 7-membered monocyclic heterocycle includes, among others, the cycloheptane, cycloheptadiene and cycloheptatriene rings each substituted by a nitrogen, oxygen or sulfur atom or atoms, and the thiazepine and like rings. The 8-membered monocyclic heterocycle includes, among others, the cyclooctene, cyclooctadiene and cyclooctatetraene rings each substituted by a nitrogen, oxygen or sulfur atom or atoms and the like rings. The polycyclic heterocycle includes, among others, the benzofuran, isobenzofuran, chromene, indolidine, indole, isoindole, isoquinoline, phthalazine, naphthyridine, quinoxaline and benzothiophene rings, hydrogenated versions of these rings, and so forth.

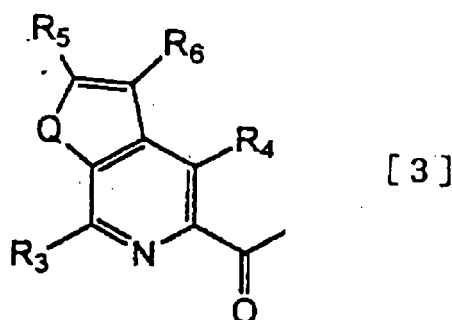
These heterocycles may each have a substituent(s). As such substituent, there may be mentioned, for example, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms and an alkoxy group containing 1 to 12 carbon atom.

Among the heterocycles specifically mentioned above, 5-membered monocyclic heterocycles are preferred. The furan ring, either substituted or unsubstituted, is more preferred and the furan ring is most preferred.

In the above general formula [1], R_3 and R_4 are the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms,

which may optionally have a substituent(s), or an alkoxy group containing 1 to 12 carbon atoms, which may optionally have a substituent(s). Specifically, there may be mentioned a hydrogen atom, chlorine atom, bromine atom, fluorine atom, hydroxyl group, methyl group, ethyl group, methoxy group and ethoxy group, etc. As the substituent(s) which the above alkyl and alkoxy groups may have, there may be mentioned a hydroxyl group, a halogen atom and an alkoxy group containing 1 to 12 carbon atoms, among others. R_3 and R_4 each is preferably a hydrogen or chlorine atom and more preferably a hydrogen atom.

Among the above-mentioned acetylpyridine derivatives of the general formula [1], those represented by the following general formula [3] are particularly preferred:



15

In the above general formula [3], Q represents an oxygen, sulfur atom or a group of the general formula $-N(D)-$ (in which N is a nitrogen atom and D represents a hydrogen atom or a monovalent protective group). The monovalent protective group is generally well known for protecting the amino group, and includes for example, acetyl, methoxycarbonyl or benzyl. Preferred as Q is an oxygen atom.

R_3 , R_4 , R_5 and R_6 are the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms, which may optionally have a substituent(s), or an alkoxy group containing 1 to 12 carbon atoms, which may optionally have a substituent(s). More specifically, there may be mentioned the same species as

those mentioned hereinabove referring to R_3 and R_4 occurring in the general formula [1].

In a preferred mode of embodiment, the substrate to be used in the production method of the invention is a compound of the general formula [3] in which Q is an oxygen atom, R_3 is a hydrogen or chlorine atom, R_4 is a hydrogen atom, R_5 is a hydrogen atom and R_6 is a hydrogen atom or a methyl group.

In a particularly preferred mode of embodiment, the substrate to be used in the production method of the invention is that compound of the general formula [3] in which Q is an oxygen atom and each of R_3 , R_4 , R_5 and R_6 is a hydrogen atom, namely 5-acetylfuro[2,3-c]pyridine.

The above-mentioned acetylpyridine derivatives of the general formula [1] can be readily obtained by production methods known in the art. For example, 5-acetylfuro[2,3-c]pyridine can be synthesized by the method described in EP 911335. 5-Acetyl-7-chlorofuro[2,3-c]pyridine can be prepared by synthesizing 7-chloro-5-(1-hydroxyethyl)furo[2,3-c]pyridine by the method described in J. Org. Chem., 63, 7851(1998) and oxidizing the hydroxyl groups thereof.

The enzyme or enzyme source which can be used in the production method of the invention is derived from a microorganism capable of converting an acetylpyridine derivative [1] to the corresponding optically active pyridineethanol derivative [2]. For instance, cells or a culture of such microorganism, or a material derived therefrom, or the enzyme obtained from such microorganism can be used. These may be used alone or in combination of two or more.

The microorganism capable of converting an acetylpyridine derivative [1] to the corresponding optically active pyridineethanol derivative [2] can be screened out by the method described below. When the acetylpyridine derivative [1] is 5-acetylfuro[2,3-c]pyridine, the screening is carried out as followed. A liquid broth (pH 7; 5 ml; composition, per liter: 40 g of glucose, 3 g of yeast extract,

6.5 g of diammonium hydrogen phosphate, 1 g potassium dihydrogenphosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of manganese sulfate tetrahydrate, 100 mg of sodium chloride) is placed in each test tube and, after sterilization, aseptically inoculated with the test microorganism, and shake culture is carried out at 30 °C for 2 to 3 days. Thereafter, cells are collected by centrifugation. They are suspended in 1 to 5 ml of a phosphate buffer solution containing 2 to 10% of glucose. The suspension is added to a test tube containing 2.5 to 25 mg of 5-acetylfuro[2,3-c]pyridine placed therein beforehand and the whole is shaken at 30 °C for 2 to 3 days. In this step, the cells collected by centrifugation may also be used in a form dried in a desiccator or with acetone. Further, in reacting such microorganism or a material derived therefrom with 5-acetylfuro[2,3-c]pyridine, oxidized form nicotinamide adenine dinucleotide (hereinafter referred to as "NAD⁺" for short), reduced form nicotinamide adenine dinucleotide (hereinafter referred to as "NADH" for short), oxidized form nicotinamide adenine dinucleotide phosphate (hereinafter referred to as "NADP⁺" for short), reduced form nicotinamide adenine dinucleotide phosphate (hereinafter referred to as "NADPH" for short) or the like and a glucose dehydrogenase or formate dehydrogenase may be added. After the conversion reaction, 5 volumes of ethyl acetate is added to the reaction mixture for product extraction. The extract is analyzed by high performance liquid chromatography (column: Chiralpak AS, product of Daicel Chemical Industries; eluate: hexane/ethanol/diethylamine = 92/8/0.1; flow rate: 1 ml/min; detection: 254 nm, column temperature: room temperature; elution time: 5-acetylfuro[2,3-c]pyridine 8.8 min, 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine 11.7 min, 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine 17.5 min).

The microorganism to be used in the practice of the

invention may be any of those microorganisms capable of converting an acetylpyridine derivative [I] to the corresponding optically active pyridineethanol derivative [III]. For example, there may be mentioned microorganisms

5 belonging to the genera Ashbya, Candida, Cryptococcus, Clavispora, Debaryomyces, Dipodascus, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hyphopichia, Issatchenkia, Kluyveromyces, Kuraishia, Lodderomyces, Metschnikowia, Ogataea, Pachysolen, Pichia,

10 Rhodospiridium, Rhodotorula, Saccharomycopsis, Schwanniomyces, Sporidiobolus, Sporobolomyces, Schizoblastosporion, Stephanoascus, Torulaspora, Trigonopsis, Trichosporon, Willopsis, Yamadazyma, Zygosaccharomyces, Alcaligenes, Bacillus, Brevibacterium, Cellulomonas,

15 Corynebacterium, Jensenia, Ochrobactrum, Pseudomonas, Rhodococcus and Tsukamurella.

In particular when the conversion to a pyridineethanol derivative having the S absolute configuration is intended, microorganisms belonging to the following genera are preferred:

20 Ashbya, Candida, Cryptococcus, Clavispora, Debaryomyces, Dipodascus, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hyphopichia, Issatchenkia, Kluyveromyces, Kuraishia, Lodderomyces, Metschnikowia, Ogataea, Pachysolen, Pichia, Rhodospiridium, Rhodotorula,

25 Saccharomycopsis, Schwanniomyces, Sporidiobolus, Sporobolomyces, Schizoblastosporion, Stephanoascus, Torulaspora, Trigonopsis, Trichosporon, Willopsis, Yamadazyma, Zygosaccharomyces, Alcaligenes, Bacillus, Brevibacterium, Cellulomonas, Corynebacterium, Jensenia, Ochrobactrum,

30 Pseudomonas, Rhodococcus and Tsukamurella.

When the conversion to a pyridineethanol derivative having the R absolute configuration is intended, microorganisms belonging to the following genera are preferred: Candida, Ogataea, Pichia, Yamadazyma, Brevibacterium and

35 Corynebacterium.

As specific examples to be used for obtaining a pyridineethanol derivative having the S absolute configuration, there may be mentioned, among others, Ashbya gossypii IFO 0560, Candida fennica CBS 6087, Candida guilliermondii IFO 0454, 5 Candida intermedia IFO 0761, Candida krusei IFO 0011, Candida magnoliae IFO 0705, Candida maltosa CBS 5612, Candida versatilis IFO 1908, Candida mogii IFO 0436, Candida norvegensis IFO 1020, Candida parapsilosis IFO 0585, Candida pseudotropicalis IAM 4840, Candida rugosa IFO 0750, Candida 10 oleophila CBS 2219, Candida stellata IFO 0701, Candida tropicalis IFO 0006, Candida bodinii IFO 10574, Candida saitoana IFO 0380, Candida albicans IFO 0759, Candida cariosilignicola IFO 1910, Candida solani IFO 0762, Candida tenuis IFO 0716, Cryptococcus albidus var. albidus IFO 0378, 15 Cryptococcus humicola CBS 1896, Cryptococcus terreus IFO 0727, Clavispora lusitaniae IFO 1019, Debaryomyces hansenii IFO 0082, Debaryomyces marama IFO 0668, Debaryomyces carsonii IFO 0946, Debaryomyces castellii IFO 1359, Dipodascus ovetensis IFO 1201, Dipodascus tetrasperma CBS 765.70, Galactomyces reessii CBS 20 179.60, Geotrichum candidum CBS 178.71, Geotrichum fragrans CBS 164.32, Geotrichum candidum CBS 187.67, Guilliermondella selenospora IFO 1850, Hanseniaspora valbyensis IFO 0115, Hansenula polymorpha DL1 AKU 4752, Hyphopichia burtonii IFO 0844, Issatchenkia orientalis IFO 1279, Kluyveromyces lactis 25 IFO 1090, Kluyveromyces thermotolerans IFO 0662, Kuraishia capsulata IFO 0721, Lodderomyces elongisporus IFO 1676, Metschnikowia bicuspidata IFO 1408, Metschnikowia gruessii IFO 0749, Ogataea minuta var. minuta IFO 0975, Ogataea minuta var. nonfermentans IFO 1473, Ogataea polymorpha IFO 0799, Pachysolen 30 tannophilus IFO 1007, Pichia rhodanensis IFO 1272, Pichia trehalophila IFO 1282, Pichia wickerhamii IFO 1278, Rhodosporidium diobovatum IFO 0688, Rhodosporidium sphaerocarpum IFO 1438, Rhodosporidium toruloides IFO 0559, Rhodotorula araucariae IFO 10053, Rhodotorula glutinis IFO 1099, 35 Rhodotorula glutinis var. dairenensis IFO 0415, Rhodotorula

- graminis IFO 0190, Saccharomycopsis fibuligera IFO 0104, Saccharomycopsis malanga IFO 1710, Schwanniomyces occidentalis var. occidentalis IFO 0371, Sporidiobolus johnsonii IFO 6903, Sporobolomyces salmonicolor IAM 12249, 5 Sporobolomyces roseus IFO 1106, Schizoblastosporion kobayashii IFO 1644, Stephanoascus ciferrii IFO 1854, Torulaspora globosa IFO 0016, Trigonopsis variabilis IFO 0671, Trichosporon aquatile ATCC 22310, Trichosporon cutaneum IFO 1198, Trichosporon fermentans ATCC 10675, Willopsis saturnus var. 10 suaveolens IFO 0809, Willopsis saturnus var. mrakii IFO 0895, Yamadazyma haplophila IFO 0947, Zygosaccharomyces bailii IFO 0488, Zygosaccharomyces rouxii IFO 0493, Alcaligenes xylosoxidans IFO 13495, Alcaligenes xylosoxidans subsp. dentrificans IFO 12669, Bacillus megaterium, Bacillus amyloliquefaciens IFO 3022, Brevibacterium incertum IFO 12145, 15 Cellulomonas fimi IAM 12107, Corynebacterium sp. ATCC 21245, Jensenia canicruria IFO 13914, Ochrobactrum sp. IFO 12950, Pseudomonas stutzeri IFO 13596, Pseudomonas chlororaphis IFO 3904, Pseudomonas mendocina IFO 14162, Rhodococcus 20 erythropolis IFO 12320, Rhodococcus rhodochrous IFO 3338, and Tsukamurella paurometabola IFO 12160.

For obtaining a pyridineethanol derivative having the R absolute configuration, there can be mentioned Candida etchellsii IFO 1942, Candida lactiscondensi IFO 1286, Candida 25 maris IFO 10003, Ogataea wickerhamii IFO 1706, Pichia farinosa IFO 0602, Pichia membranaefaciens IFO 0460, Pichia naganishii IFO 1670, Yamadazyma farinosa IFO 0534, Brevibacterium iodinam IFO 3558 and Corynebacterium acetoacidophilum ATCC 21476, among others.

30 These microorganisms can be obtained from stock cultures readily available or purchasable or can be isolated from the natural world. It is also possible to obtain strains having favorable properties for this reaction by causing mutation of these microorganisms.

35 In culturing these microorganisms, any of the media

containing nutrient sources assimilable by these microorganisms can generally be used. For example, use may be made of ordinary media prepared by mixing and incorporating appropriate amounts of carbon sources, for example saccharides such as glucose, sucrose, maltose, etc., organic acids such as lactic acid, acetic acid, citric acid, propionic acid, etc., alcohols such as ethanol, glycerol, etc., hydrocarbons such as paraffins, fats and oils such as soybean oil, rapeseed oil, etc., or mixtures of these; nitrogen sources such as ammonium sulfate, ammonium phosphate, urea, yeast extract, meat extract, peptone, corn steep liquor, etc.; and, further, other nutrients sources, for example other inorganic salts, vitamins, etc. The medium to be used may be selected from among these media according to the microorganism to be used.

The culture of the microorganism can be generally carried out under ordinary conditions, preferably at a pH of 4.0 to 9.5 and a temperature within the range of 20 °C to 45 °C under aerobic conditions for 10 to 96 hours, for instance. In reacting the microorganism with an acetylpyridine derivative [1], the culture broth containing cells of the microorganism can generally be used as it is. The culture broth may also be used in a concentrated form. In cases a certain component in the culture broth adversely affects the reaction, cells or a material derived therefrom as obtained by centrifugation and/or other treatments of the culture broth may also be used.

The material derived from cells of the microorganism is not particularly restricted but includes, among others, dried cells obtained by dehydration treatment with acetone or diphosphorus pentoxide or by drying utilizing a desiccator or electric fan, materials derived by surfactant treatment, materials derived by lysozyme treatment, immobilized cells and cell-free extract preparations derived from disruption of cells. It is also possible to purify an enzyme catalyzing the asymmetric reduction reaction from the culture and use the same.

In carrying out the reduction reaction in accordance with

the invention, the substrate acetylpyridine derivative [1] may be added all at once at the beginning of the reaction or in divided portions with the progress of the reaction. The temperature during the reaction is generally 10 to 60 °C, preferably 20 to 40 °C, and the pH during the reaction is within the range of 2.5 to 9, preferably 5 to 9. The amount of the enzyme or enzyme source in the reactant mixture may be adequately selected according to the ability thereof to reduce the substrate. The substrate concentration in the reactant mixture is preferably 0.01 to 50% (w/v), more preferably 0.1 to 30% (w/v). The reaction is generally carried out with shaking or with aeration and stirring. The reaction time is adequately selected according to the substrate concentration, enzyme or enzyme source amount and other reaction conditions. Generally, it is preferred to select such reaction conditions so that the reaction may be complete in 2 to 168 hours.

For promoting the reduction reaction according to the invention, the addition, in an amount of 0.5 to 30%, of such an energy source as glucose, ethanol or isopropanol to the reactant mixture is preferred since better results are obtained.

The reaction can also be promoted by adding a coenzyme generally required for biochemical reduction reactions, such as NADH and NADPH. In this case, specifically, such coenzyme is directly added to the reactant mixture.

Further, for promoting the reduction reaction, it is preferred to carry out the reaction in the presence of an enzyme reducing NAD^+ and/or NADP^+ to the respective reduced forms and a substrate for the reduction, since better results are obtained. For example, a glucose dehydrogenase can be caused to exist as the enzyme for the reduction to the reduced form and glucose can be caused to exist as the substrate for reduction, or a formate dehydrogenase can be caused to exist as the enzyme for the reduction to the reduced form and formic acid can be caused to exist as the substrate for reduction.

It is also effective to add a surfactant such as Triton (product of Nakalai Tesque), Span (product of Kanto Chemical) or Tween (product of Nakalai Tesque) to the reactant mixture. A water-insoluble organic solvent such as ethyl acetate, butyl acetate, isopropyl ether, toluene and hexane may be added to the reactant mixture for the purpose of avoiding the inhibition of the reaction by the acetylpyridine derivative [1] and/or optically active pyridineethanol derivative [2]. For increasing the solubility of the acetylpyridine derivative [1], a water-soluble organic solvent, such as methanol, ethanol, acetone, tetrahydrofuran and dimethyl sulfoxide may also be added.

In the following, the enzyme, the DNA coding for said enzyme, the recombinant vector containing said DNA and the transformant having said recombinant vector according to the invention are described in detail.

The enzyme of the invention has the following physical and chemical properties (1) to (3):

- (1) Activity: It stereoselectively reduces 5-acetylfuro[2,3-c]pyridine, in the presence of NADH as a coenzyme, to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine;
- (2) Specificity: It has reducing ability against ketones and aldehydes but is very low in reducing activity against carbocyclic ketones and the α -position keto group of α -keto acids;
- (3) Molecular weight: It shows a molecular weight of about 60,000 in gel filtration analysis and a molecular weight of about 29,000 in SDS polyacrylamide electrophoresis.

Preferably, the enzyme has the following physical and chemical properties (4) to (6) in addition to the physical and chemical properties (1) to (3):

- (4) Optimal temperature: 50 °C to 55 °C;
- (5) Optimal pH: 5.0 to 6.0;
- (6) Inhibitor: It is inhibited by the mercury ion.

In the practice of the invention, the reducing activity

of the enzyme is determined by carrying out the reaction in 3.0 ml of a reactant mixture containing 1 mM substrate, 0.25 mM coenzyme NADH, 0.3% (vol/vol) dimethyl sulfoxide and an enzyme solution in 100 mM phosphate buffer (pH 6.5) at 30 °C for 3 minutes and then measuring the reduction in absorbance at 340 nm.

The term "very low in reducing activity" referring to the specificity means that when the reducing activity against 5-acetylfuro[2,3-c]pyridine is taken as 100%, the reducing activity against the substrate in question is not more than 10%. The term "carbocyclic ketones" means ketones, such as cyclohexanone and cyclopentanone, derived from alicyclic compounds by substituting -C(=O)- for a ring constituent -CH₂- thereof.

The molecular weight is determined by gel filtration analysis using a TSK-G 3000 SW column (7.8 mm I.D. × 30 cm) (product of Tosoh Corp.). It is calculated based on the relative elution times of standard proteins. The subunit molecular weight is calculated based on the relative mobilities of standard proteins as determined by 20% SDS-polyacrylamide gel electrophoresis.

The optimal pH and optimal temperature for the enzyme are determined, for example, by measuring the reducing activity while varying the reaction pH and reaction temperature of the reducing activity measuring system.

The inhibitor is found out, for example, by measuring the reducing activities of various compounds added to the reducing activity measuring system.

The microorganism to be used as a source of the enzyme of the invention may be any of the microorganisms having an enzyme capable of stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine. It may be a wild species or a mutant and, further, a recombinant microorganism derived by a genetic engineering technique such as cell fusion or gene

manipulation. Preferably, a microorganism belonging to the genus Candida is used. The species Candida maris is more preferred, and the strain Candida maris IFO 10003 is particularly preferred.

5 In the following, an example of the method of preparing an enzyme of the invention from a microorganism having the enzyme capable of stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine is described. It is to be
10 noted, however, that this example is by no means limitative of the scope of the invention. A crude enzyme solution can be obtained by cultivating, in an appropriate medium, a microorganism having an enzyme capable of stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-
15 hydroxyethyl)furo[2,3-c]pyridine, collecting cells from the culture broth by centrifugation, suspending the cells in an appropriate buffer solution, disrupting or lysing the cells by physical means such as glass beads or by biochemical means such as an enzyme and, further, removing the solid matter from the
20 solution by centrifugation. Alternately, a crude enzyme solution can also be obtained from the culture broth by the same purification procedure as mentioned above. Further, this crude enzyme solution can be purified by techniques generally used by those skilled in the art, for example precipitation with
25 ammonium sulfate, dialysis and chromatography, used either singly or in combination. The chromatography includes hydrophobic chromatography, ion exchange chromatography and gel filtration chromatography and these techniques may be used singly or in combination.

30 The enzyme of the invention may be a natural enzyme obtained from a microorganism as mentioned above, or a recombinant enzyme. As a natural enzyme, there may be mentioned an enzyme comprising the amino acid sequence shown under SEQ ID NO:1 in the sequence listing.

35 The enzyme of the invention may also be an enzyme

comprising an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:1 in the sequence listing by deletion, substitution and/or addition of one or several amino acids and capable of stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine. The terms "deletion, substitution and/or addition of one or several amino acids" mean that such a number of amino acids as capable of being deleted, substituted and/or added by a method well known in the art, for example site-specific mutagenesis, are deleted, substituted and/or added. The terms "capable of stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine" mean that when the enzyme is reacted with 5-acetylfuro[2,3-c]pyridine, 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine is formed in a yield of not less than 10%, preferably not less than 40%, particularly preferably not less than 60%, of the yield obtained with the enzyme comprising the amino acid sequence shown under SEQ ID NO:1 in the sequence listing. For determining such yield, the above-mentioned high performance liquid chromatography is used.

Once an enzyme has been obtained in a purified form, a DNA coding for the enzyme can be obtained by a method well known in the art. By introducing this DNA into another microorganism and cultivating the resulting recombinant microorganism, it is possible to produce the relevant enzyme source usable in the production method of the invention in large amounts.

In the following, an example of the process for obtaining a DNA coding for an enzyme of the invention is described. Of course, the present invention is not restricted to this process. First, the enzyme purified is digested with an appropriate endopeptidase, digested fragments are purified by reversed phase HPLC and partial amino acid sequences thereof are determined by means of a protein sequencer. Based on the partial amino acid sequences, PCR (polymerase chain reaction)

primers are synthesized. Then, chromosomal DNA is prepared from the microorganism, which is the source of the enzyme-encoding DNA, by a conventional method of DNA isolation, for example the Hereford method (Cell, 18, 1261 (1979)). Part of the enzyme-encoding DNA (core sequence) is amplified by performing PCR using the above PCR primers with the above chromosomal DNA as a template, and that part is sequenced. The sequencing can be carried out by the dideoxy chain termination method or like method using the ABI 373A DNA sequencer (Applied Biosystems), for instance. For revealing the base sequence in regions adjacent to the core sequence, the microbial chromosomal DNA is digested with a restriction enzyme having no recognition sequence in the core sequence and the resulting DNA fragment is allowed to self-circulize using T4 ligase to give a template DNA for inverse PCR (Nucleic Acids Res. 16, 8186 (1988)). Then, based on the core sequence, primers to serve as the initiation points for DNA synthesis toward the outsides of the core sequence are synthesized, and the regions neighboring the core sequence are amplified by inverse PCR. By revealing the base sequences of the thus-obtained DNAs, it is possible to reveal the DNA sequence of the whole coding region for the desired enzyme. Once the DNA sequence in question has been revealed, the DNA coding for the enzyme of the invention can be obtained from that microbial chromosomal DNA by PCR, for instance.

The DNA encoding the enzyme of the invention is inserted into a vector and the resulting recombinant vector is introduced into a host, whereupon the enzyme gene can be expressed in the resulting transformant. The vector to be used for this purpose may be any of those allowing the expression of the enzyme gene in an appropriate host. As such vector, there may be mentioned plasmid vectors, phage vectors and cosmid vectors, among others. Shuttle vectors capable of gene exchange with some other host may also be used. Such vectors each contain operatively joined regulator elements such as a promoter (lac UV5 promoter, trp

promoter, trc promoter, tac promoter, lpp promoter, tufB promoter, recA promoter, pL promoter) and can be used as an expression vector containing an expression unit operatively linked to the DNA of the invention. Thus, for example, pUCNT
5 (WO 94/03613) and the like can suitably be used.

The term "regulatory factors" as used herein means base sequences including a functional promoter, and if necessary related transcription elements (e.g. enhancer, CCAAT box, TATA box, SPI site, etc.).

10 The term "operatively joined" means that the DNA and various regulator elements, such as a promoter, enhancer, etc., are joined together so that they can operate in a host cell to cause gene expression. It is well known in the art that the regulator elements may vary in type and species according to
15 the host.

The host into which the recombinant vector containing the DNA of the invention includes bacteria, yeasts, filamentous fungi, plant cells, animal cells, etc. Escherichia coli is most preferred, however. The DNA of the invention can be introduced
20 into the host in the conventional manner. When Escherichia coli is used as the host cells, the DNA of the invention can be introduced therein by the calcium chloride method, for instance.

For stereoselectively reducing an acetylpyridine
25 derivative [1] using the enzyme or transformant of the invention to give the corresponding pyridineethanol derivative having the R absolute configuration, particularly preferably for stereoselectively reducing 5-acetylfuro[2,3-c]pyridine to give 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine, NADH is
30 required as a coenzyme. While the reduction can be carried out by adding a required amount of NADH to the reaction system, the amount of the expensive coenzyme can be markedly reduced by carrying out the reaction using the enzyme of the invention in combination with a coenzyme regeneration system, namely using
35 an enzyme capable of converting the oxidized form of that enzyme

(NAD⁺) to the reduced form (NADH) (hereinafter referred to as coenzyme regeneration ability) together with the substrate thereof. As the enzyme having coenzyme regeneration ability, use may be made of hydrogenase, formate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase and glucose dehydrogenase, among others. Glucose dehydrogenase and formate dehydrogenase are judiciously used, however.

Such reaction can be carried out by adding the coenzyme regeneration system to the asymmetric reduction reaction system. When a transformant resulting from the transformation with both the DNA coding for the enzyme of the invention and a DNA coding for glucose dehydrogenase is used, the reaction can efficiently be carried out without adding any enzyme having coenzyme regeneration ability as separately prepared. Such transformant can be produced by inserting a DNA coding for the enzyme of the invention and a DNA coding for glucose dehydrogenase into one and the same vector and introducing the recombinant vector into a host, or by inserting each of two DNAs into two vectors differing in incompatibility group separately and introducing these into one and the same host. Thus, a transformant having a recombinant vector containing a DNA coding for the enzyme of the invention and a DNA coding for glucose dehydrogenase, or a transformant having a first recombinant vector containing a DNA coding for the enzyme of the invention and a second recombinant vector containing a DNA coding for glucose dehydrogenase can be used.

In cases where the enzyme or transformant of the invention has coenzyme regeneration ability, the reaction for regeneration of NADH can be conducted simultaneously by adding a substrate for that regeneration to the reaction system and, thus, the amount of the expensive coenzyme to be used can be markedly reduced without supplementary adding another enzyme having coenzyme regeneration ability. For example, when the enzyme or transformant of the invention has isopropanol oxidizing activity, it becomes possible to carry out the

regeneration of NADH by adding isopropanol to the reduction system.

The production of an optically active pyridineethanol derivative [2] from an acetylpyridine derivative [1] using the transformant of the invention, in particular the production of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine, can be performed in the following manner. Such production, however, is not limited to the following process. First, the substrate 5-acetylfuro[2,3-c]pyridine [1], a coenzyme such as NAD⁺ and a culture of the transformant or a material derived therefrom or the like are added to an appropriate solvent, and the reaction is allowed to proceed at an adjusted pH with stirring. This reaction is carried out at a temperature of 10 to 70 °C and the pH of the reaction mixture is maintained at 4 to 10 during the reaction. The reaction can be conducted either batchwise or continuously. In the case of batchwise reaction, the reaction substrate can be added in a charge concentration of 0.1% to 70% (w/v). The material derived from the transformant, so referred to herein, includes, among others, crude enzyme solutions, cultured microbial cells, lyophilized microbial cells, acetone-dried microbial cells, triturated modifications thereof, and mixtures of these. Furthermore, the enzyme itself or microbial cells themselves may be used in a form immobilized by conventional means. Further, when, in carrying out the reaction, the transformant employed can produce both the enzyme of the invention and glucose dehydrogenase, the addition amount of coenzyme can be markedly reduced by further adding glucose to the reaction system.

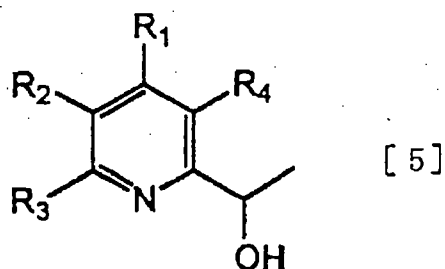
30

Now, the method of producing a pyridineethanol derivative having the S absolute configuration by reacting the above enzyme and/or transformant with a pyridineethanol derivative to thereby preferentially oxidizing the pyridineethanol derivative having the R absolute configuration and recovering

35

the remaining pyridineethanol derivative having the S absolute configuration is described in detail.

The pyridineethanol derivative to be used as the substrate in the production method according to the invention is represented by the general formula [5]:

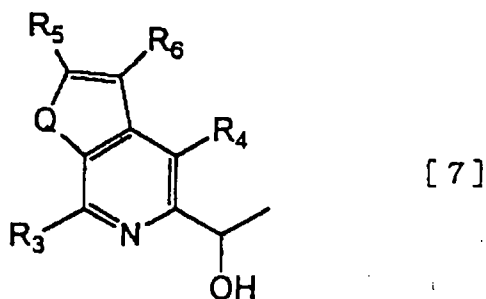


In the above general formula [5], R_1 and R_2 are as defined above in relation to the general formula [1]. Five-membered monocyclic heterocycles are preferred among others, a substituted or unsubstituted furan ring is more preferred and a furan ring is particularly preferred.

R_3 and R_4 are also as defined as above referring to the general formula [1]. A hydrogen atom or chlorine atom is preferred and a hydrogen atom is more preferred.

The pyridineethanol derivative [5] is not particularly restricted but has an optical purity of less than 100%. It may be entirely a racemate or have a certain degree of optical purity.

Among the pyridineethanol derivatives [5], particularly preferred species are represented by the general formula [7]:



In the above general formula [7], Q is as defined above

in reference to the general formula [3]. An oxygen atom is preferred as Q. R₃, R₄, R₅ and R₆ are also as defined above referring to the general formula [3].

5 In a preferred embodiment, the substrate to be used in the production method of the invention is a compound represented by the general formula [7] in which Q is an oxygen atom, R₃ is a hydrogen atom or chlorine atom, R₄ is a hydrogen atom, R₅ is a hydrogen atom and R₆ is a hydrogen atom or a methyl group.

10 In a particularly preferred embodiment, the substrate to be used in the production method of the invention is a compound represented by the general formula [7] in which Q is an oxygen atom, R₃, R₄, R₅ and R₆ each is a hydrogen atom, namely 5-(1-hydroxyethyl)furo[2,3-c]pyridine.

15 The pyridineethanol derivatives represented by the general formula [5] can be readily obtained by production methods known in the art. For example, 5-(1-hydroxyethyl)furo[2,3-c]pyridine can be synthesized by the method described in EP 911335.

For preferentially oxidizing an R-configuration
20 pyridineethanol derivative by reacting the pyridineethanol derivative [5] with the enzyme or transformant of the invention, NAD⁺ is required as a coenzyme. While the reaction can be carried out by adding a required amount of NAD⁺ to the reaction system, the required amount of the expensive coenzyme can be
25 markedly reduced by carrying out the reaction by using an enzyme capable of converting the reduced form of the coenzyme to the oxidized form and a substrate for that enzyme in combination with the enzyme of the invention. A microorganism or a material derived therefrom, which contains an enzyme capable of
30 converting the reduced form of the coenzyme to the oxidized form may also be used. Useful as the enzyme capable of converting the reduced form of the coenzyme to the oxidized form are, for example, NADH oxidase and NADH dehydrogenase.

In cases where the enzyme or transformant of the invention
35 has NAD⁺ regeneration ability, the NAD⁺ regeneration reaction

can be conducted simultaneously by adding a substrate for that regeneration to the reaction system. In that case, the amount of the expensive coenzyme can be markedly reduced without particularly adding another enzyme having NAD^+ regeneration ability. For example, when the enzyme or transformant of the invention has acetone reducing activity, the addition of acetone to the reaction system enables the regeneration of NAD^+ .

Further, when the transformant of the invention is used, the reaction can proceed owing to the NAD^+ occurring within the microbial cells and the NADH produced upon the reduction of NAD^+ is reoxidized within the microbial cells, hence the reaction can be conducted without particularly adding the coenzyme and an enzyme having NAD^+ regeneration ability.

The method of recovering the optically active pyridineethanol derivative obtained by any of the above-mentioned methods is not particularly restricted but the optically active pyridineethanol derivative can be readily obtained, in a highly pure form, by extracting the product from the reaction mixture, directly or after separation of microbial cells, with a solvent such as ethyl acetate, toluene, tert-butyl methyl ether and hexane, dehydrating the extract and purifying the product by distillation, crystallization, silica gel column chromatography, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the DNA base sequence determined in Example 21 and the amino acid sequence deduced therefrom.

Fig. 2 shows a method of constructing the recombinant vector pNTFP of Example 22 and the recombinant vector pNTFPG of Example 23 and the structures of these.

BEST MODES FOR CARRYING OUT THE INVENTION

The following examples illustrate the present invention in further detail. These examples are, however, by no means limitative of the scope of the invention. In the following

description, "%" means "% by weight" unless otherwise specified.

(Example 1: Asymmetric reduction of 5-acetylfuro[2,3-c]pyridine using various microorganisms)

A liquid broth (pH 7) comprising 40 g of glucose, 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of manganese sulfate tetrahydrate and 100 mg of sodium chloride, per liter, was distributed in 5-ml portions into large size test tubes and steam-sterilized at 120 °C for 20 minutes. These liquid broth portions were each aseptically inoculated with one loopful of one of the microorganisms listed in Table 1 and Table 2, and shake culture was carried out at 30 °C for 24 to 72 hours. After cultivation, 0.5 ml of each culture was centrifuged and the cells collected were suspended in 0.5 ml of 100 mM phosphate buffer (pH 6.5) containing 8% of glucose. This cell suspension was added to a test tube containing 5 mg of 5-acetylfuro[2,3-c]pyridine placed therein in advance, and the reaction was allowed to proceed at 30 °C for 26 hours. After reaction, 5 ml of ethyl acetate was added to each reaction mixture and, after mixing up, a portion of the organic phase was analyzed under the following HPLC conditions.

[HPLC analysis conditions]

Column: Chiralpak AS, product of Daicel Chemical Industries; eluent: hexane/ethanol/diethylamine = 92/8/0.1; flow rate: 1 ml/min; detection: 254 nm; column temperature: room temperature; elution time: 5-acetylfuro[2,3-c]pyridine 8.8 minutes, 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine 11.7 minutes, 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine 17.5 minutes.

The yield, optical purity and absolute configuration of

the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine per reaction mixture are shown in Table 1 or Table 2.

Table 1

Microorganism			Yield (mg/ml)	Optical purity (%e.e.)	Absolute configur- ation
Ashbya	<i>gossypii</i>	IFO 0560	0.17	95.6	S
Candida	<i>fennica</i>	CBS 6087	0.43	69.7	S
Candida	<i>guilliermondii</i>	IFO 0454	0.86	95.3	S
Candida	<i>intermedia</i>	IFO 0761	2.81	99.2	S
Candida	<i>krusei</i>	IFO 0011	0.12	100.0	S
Candida	<i>magnoliae</i>	IFO 0705	2.41	59.4	S
Candida	<i>maltona</i>	CBS 5612	7.43	99.9	S
Candida	<i>versatilis</i>	IFO 1908	0.11	94.5	S
Candida	<i>mogii</i>	IFO 0436	2.73	99.1	S
Candida	<i>norvegensis</i>	IFO 1020	0.18	100.0	S
Candida	<i>parapsilosis</i>	IFO 0585	5.86	99.8	S
Candida	<i>pseudotropicalis</i>	IAM 4840	0.32	95.6	S
Candida	<i>rugosa</i>	IFO 0750	0.12	91.9	S
Candida	<i>oleophila</i>	CBS 2219	0.72	79.8	S
Candida	<i>stellata</i>	IFO 0701	0.11	100.0	S
Candida	<i>tropicalis</i>	IFO 0006	6.17	99.9	S
Candida	<i>bodinii</i>	IFO 10574	0.21	90.5	S
Candida	<i>saitoana</i>	IFO 0380	0.32	89.0	S
Candida	<i>albicans</i>	IFO 0759	4.12	98.1	S
Candida	<i>cariosilignicola</i>	IFO 1910	1.44	100.0	S
Candida	<i>solani</i>	IFO 0762	0.32	94.4	S
Candida	<i>tenuis</i>	IFO 0716	0.36	95.1	S
Cryptococcus	<i>albidus</i> var. <i>albidus</i>	IFO 0378	2.37	99.3	S
Cryptococcus	<i>humicola</i>	CBS 1896	0.33	87.6	S
Cryptococcus	<i>terreus</i>	IFO 0727	0.26	83.3	S
Clavispora	<i>lusitanae</i>	IFO 1019	0.60	98.4	S
Debaryomyces	<i>hansenii</i>	IFO 0082	0.48	94.2	S
Debaryomyces	<i>marama</i>	IFO 0668	0.12	64.8	S
Debaryomyces	<i>carsonii</i>	IFO 0946	0.40	83.8	S
Debaryomyces	<i>castellii</i>	IFO 1359	0.70	81.6	S
Dipodascus	<i>ovetensis</i>	IFO 1201	6.81	97.2	S
Dipodascus	<i>tetrasperma</i>	CBS 765.70	2.77	93.7	S
Galactomyces	<i>reessii</i>	CBS 179.60	0.42	86.9	S
Geotrichum	<i>candidum</i>	CBS 178.71	9.24	98.8	S
Geotrichum	<i>fragrans</i>	CBS 164.32	3.03	98.8	S
Geotrichum	<i>candidum</i>	CBS 187.67	0.26	64.2	S
Guilliermondii	<i>selenospora</i>	IFO 1850	0.34	56.9	S
Hanseniaspora	<i>valbyensis</i>	IFO 0115	0.36	92.9	S
Hansenula	<i>polymorpha</i> DL1	AKU 4752	0.38	95.2	S
Hyphopichia	<i>burtonii</i>	IFO 0844	6.25	96.7	S
Issatchenkia	<i>orientalis</i>	IFO 1279	0.20	97.5	S
Kluyveromyces	<i>lactis</i>	IFO 1090	0.15	83.0	S
Kluyveromyces	<i>thermotolerans</i>	IFO 0662	0.98	94.3	S

Table 2

Microorganism			Yield (mg/ml)	Optical purity (%e.e.)	Absolute configur- ation
<i>Kuraishia</i>	<i>capsulata</i>	IFO 0721	0.78	98.8	S
<i>Lodderomyces</i>	<i>elongisporus</i>	IFO 1676	3.05	99.3	S
<i>Metschnikowia</i>	<i>bicuspidata</i>	IFO 1408	0.15	72.8	S
<i>Metschnikowia</i>	<i>gruessii</i>	IFO 0749	0.28	50.7	S
<i>Ogataea</i>	<i>minuta</i> var. <i>minuta</i>	IFO 0975	0.59	98.4	S
<i>Ogataea</i>	<i>minuta</i> var. <i>nonfermentans</i>	IFO 1473	0.22	100.0	S
<i>Ogataea</i>	<i>polymorpha</i>	IFO 0799	0.16	94.7	S
<i>Pachysolen</i>	<i>tannophilus</i>	IFO 1007	0.26	67.3	S
<i>Pichia</i>	<i>rhodanensis</i>	IFO 1272	0.26	97.9	S
<i>Pichia</i>	<i>trehalophila</i>	IFO 1282	0.63	98.7	S
<i>Pichia</i>	<i>wickerhamii</i>	IFO 1278	0.47	98.0	S
<i>Rhodsporidium</i>	<i>diobovatum</i>	IFO 0688	0.42	100.0	S
<i>Rhodsporidium</i>	<i>sphaerocarpum</i>	IFO 1438	4.13	100.0	S
<i>Rhodsporidium</i>	<i>toruloides</i>	IFO 0559	6.45	99.9	S
<i>Rhodotorula</i>	<i>araucariac</i>	IFO 10053	2.52	74.6	S
<i>Rhodotorula</i>	<i>glutinis</i>	IFO 1099	2.54	100.0	S
<i>Rhodotorula</i>	<i>glutinis</i> var. <i>dairuensis</i>	IFO 0415	1.34	100.0	S
<i>Rhodotorula</i>	<i>graminis</i>	IFO 0190	1.95	99.5	S
<i>Saccharomycopsis</i>	<i>fibuligera</i>	IFO 0104	1.28	100.0	S
<i>Saccharomycopsis</i>	<i>malanga</i>	IFO 1710	0.61	100.0	S
<i>Schwanniomyces</i>	<i>occidentalis</i> var. <i>occidentalis</i>	IFO 0371	0.26	94.3	S
<i>Sporidiobolus</i>	<i>johnsonii</i>	IFO 6903	5.58	99.2	S
<i>Sporobolomyces</i>	<i>salmonicolor</i>	IAM 12249	2.99	97.4	S
<i>Sporobolomyces</i>	<i>roseus</i>	IFO 1106	0.34	85.1	S
<i>Schizoblastosporion</i>	<i>kobayashii</i>	IFO 1644	0.46	79.4	S
<i>Stephanosascus</i>	<i>ciferrii</i>	IFO 1854	0.50	95.2	S
<i>Torulaspora</i>	<i>glohosa</i>	IFO 0016	0.95	42.2	S
<i>Trigonopsis</i>	<i>variabilis</i>	IFO 0671	0.95	48.7	S
<i>Trichosporon</i>	<i>aquaticum</i>	ATCC 22310	0.25	70.3	S
<i>Trichosporon</i>	<i>curaneum</i>	IFO 1198	0.16	100.0	S
<i>Trichosporon</i>	<i>fermentans</i>	ATCC 10675	2.45	85.2	S
<i>Williopsis</i>	<i>saturnus</i> var. <i>suaveolens</i>	IFO 0809	0.11	83.3	S
<i>Williopsis</i>	<i>saturnus</i> var. <i>mrakii</i>	IFO 0895	3.05	98.5	S
<i>Yamadazyma</i>	<i>haplophila</i>	IFO 0947	1.19	96.8	S
<i>Zygosaccharomyces</i>	<i>bailii</i>	IFO 0488	0.10	61.6	S
<i>Zygosaccharomyces</i>	<i>rouxii</i>	IFO 0493	0.17	47.0	S
<i>Candida</i>	<i>etchellsii</i>	IFO 1942	0.24	87.0	R
<i>Candida</i>	<i>lactis-condensi</i>	IFO 1286	1.28	96.5	R
<i>Candida</i>	<i>maris</i>	IFO 10003	7.08	98.4	R
<i>Ogataea</i>	<i>wickerhamii</i>	IFO 1706	7.68	77.6	R
<i>Pichia</i>	<i>farinosa</i>	IFO 0602	7.15	39.2	R
<i>Pichia</i>	<i>membranaefaciens</i>	IFO 0460	0.11	77.3	R
<i>Pichia</i>	<i>naganishii</i>	IFO 1670	0.33	22.3	R
<i>Yamadazyma</i>	<i>farinosa</i>	IFO 0534	3.12	97.4	R

(Example 2: Asymmetric reduction of 5-acetylfuro[2,3-c]pyridine using various microorganisms)

A liquid broth (pH 7) comprising 10 g meat extract, 10 g peptone, 5 g of yeast extract and 3 g sodium chloride, per liter, was distributed in 5-ml portions into large size test tubes and steam-sterilized at 120 °C for 20 minutes. These liquid broth portions were each aseptically inoculated with one loopful of one of the microorganisms listed in Table 3, and shake culture was carried out at 30 °C for 24 to 72 hours. After cultivation, 2 ml of each culture was centrifuged and the cells collected were suspended in 0.5 ml of 100 mM phosphate buffer (pH 6.5) containing 8% of glucose. This cell suspension was added to a test tube containing 2.5 mg of 5-acetylfuro[2,3-c]pyridine placed therein in advance, and the reaction was allowed to proceed at 30 °C for 26 hours. After reaction, the reaction mixture was analyzed in the same manner as in Example 1. The yield, optical purity and absolute configuration of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine per reaction mixture are shown in Table 3.

Table 3

Microorganism	Yield (mg/ml)	Optical purity (% e.e.)	Absolute configuration
<u>Alcaligenes xylosoxidans</u> IFO 13495	0.05	93.1	S
<u>Alcaligenes xylosoxidans subsp. dentrificans</u> IFO 12669	0.13	97.3	S
<u>Bacillus megaterium</u>	0.13	94.6	S
<u>Bacillus amyloliquefaciens</u> IFO 3022	0.10	97.7	S
<u>Brevibacterium incertum</u> IFO 12145	0.20	98.4	S
<u>Cellulomonas fimi</u> IAM 12107	1.16	91.5	S
<u>Corynebacterium</u> sp. ATCC 21245	0.26	57.3	S
<u>Jensenia canicruria</u> IFO 13914	4.91	98.6	S
<u>Ochrobactrum</u> sp. IFO 12950	0.68	98.9	S
<u>Pseudomonas stutzeri</u> IFO 13596	3.99	99.9	S
<u>Pseudomonas chlororaphis</u> IFO 3904	0.21	95.9	S
<u>Pseudomonas mendocina</u> IFO 14162	0.65	99.2	S
<u>Rhodococcus erythropolis</u> IFO 12320	0.13	68.5	S
<u>Rhodococcus rhodochrous</u> IFO 3338	0.10	85.8	S
<u>Tsukamurella paurometabola</u> IFO 12160	0.16	98.7	S
<u>Brevibacterium iodinum</u> IFO 3558	0.15	42.3	R
<u>Corynebacterium acetoacidophilum</u> ATCC 21476	0.12	40.9	R

(Comparative Example 1: Asymmetric reduction of 5-acetylfuro[2,3-c]pyridine using various microorganisms)

- 5 A liquid broth (pH 7) comprising 40 g of glucose, 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10
- 10 mg of manganese sulfate tetrahydrate and 100 mg of sodium chloride, per liter, was distributed in 5-ml portions into large size test tubes and steam-sterilized at 120 °C for 20 minutes. These liquid broth portions were each aseptically inoculated with one loopful of one of the microorganisms listed in Table
- 15 4, and shake culture was carried out at 30 °C for 24 to 72 hours. After cultivation, 2 ml of each culture was centrifuged and the cells collected were suspended in 0.5 ml of 100 mM phosphate buffer (pH 6.5) containing 8% of glucose. This cell suspension

was added to a test tube containing 2.5 mg of 5-acetylfuro[2,3-c]pyridine placed therein in advance, and the reaction was allowed to proceed at 30 °C for 26 hours. After reaction, the reaction mixture was analyzed in the same manner as in Example 1. The yield, optical purity and absolute configuration of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine per reaction mixture are shown in Table 4.

Table 4

Microorganism	Yield (mg/ml)	Optical purity (% e.e.)	Absolute configu- ration
<u>Saccharomyces cerevisiae</u> IFO 0258	0.00	-	-
<u>Saccharomyces cerevisiae</u> ATCC 9017	0.01	-	-

10

From the above results, it is evident that Saccharomyces cerevisiae known to be capable of converting the monocyclic acetylpyridine to optically active hydroxyethylpyridine hardly reacts with the bicyclic acetylpyridine derivative, namely 5-acetylfuro[2,3-c]pyridine.

15

(Example 3: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using Candida maris IFO 10003)

20

A liquid broth (45 ml) comprising 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of manganese sulfate tetrahydrate and 100 mg of sodium chloride, per 900 milliliters, and one drop of Adekanol were placed in a 500-ml Sakaguchi flask and sterilized, 5 ml of a sterilized 40% aqueous solution of glucose was added, and the whole was aseptically inoculated with 1 ml of the culture of Candida maris IFO 10003 as obtained by the culture method described in Example 1, and shake culture was carried out at 30 °C for 24 hours. The

25

30

resulting culture was used as a seed yeast. A 5-liter jar fermenter was charged with 2.25 liters of the liquid broth having the above composition and 5 drops of Adekanol and, after sterilization, 250 ml of a sterilized 40% aqueous solution of glucose was added, and the whole was aseptically inoculated with 50 ml of the seed. Cultivation was carried out under the following conditions for 40 hours: cultivation temperature 30 °C, rate of stirring 350 rpm, aeration 0.75 L/min. When, during cultivation, the pH was found to have become lower than 5.5, the pH was adjusted to 5.5 by adding 5 N aqueous sodium hydroxide. After cultivation, 2 L of the culture containing microbial cells, 10 g of 5-acetylfuro[2,3-c]pyridine and 60 g of glucose were placed in a 5-liter jar fermenter, and the reduction reaction was carried out at 30 °C with stirring for 22.5 hours. During the reaction, the pH of the reaction mixture was maintained at pH 6 using 5 N aqueous sodium hydroxide. At 4.5 hours and 7.5 hours after the start of the reaction, 60 g of glucose and 80 g of glucose were added, respectively. After completion of the reaction, the reaction mixture was extracted with 1 liter of ethyl acetate and the aqueous phase was further extracted with 1 liter of ethyl acetate. The organic phases were combined and dehydrated over anhydrous sodium sulfate, and the solvent was then distilled off under reduced pressure. To the residue were added 30 ml of ethyl acetate and 500 mg of active carbon, and the mixture was stirred at room temperature for 2 hours. The active carbon was removed by filtration and the solvent was then distilled off under reduced pressure. The residue was crystallized from a mixed solution composed of ethyl acetate and methylcyclohexane to give 8.1 g of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine as white solid. Yield: 81%, optical purity: 98.7% e.e., melting point: 59.5 to 60.5 °C, specific rotation $[\alpha]_D^{20} = +37.0^\circ$ (CHCl₃, c = 0.56). ¹H-NMR δ (CDCl₃): 1.56 (3H, d, J = 6.35 Hz), 4.12 (1H, s), 5.00 (1H, q, J = 6.35 Hz), 6.80 (1H, d, J = 1.95 Hz), 7.54 (1H, s), 7.77 (1H, d, J = 1.95 Hz), 8.80 (1H, s).

(Example 4: Synthesis of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using Candida tropicalis IFO 0006)

5 A liquid broth (225 ml) comprising 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of
10 manganese sulfate tetrahydrate and 100 mg of sodium chloride, per 900 milliliters, and two drops of Adekanol were placed in a 2-liter Sakaguchi flask and sterilized, 25 ml of a sterilized 40% aqueous solution of glucose was added, and the whole was aseptically inoculated with 2.5 ml of the culture of Candida
15 tropicalis IFO 0006 as obtained by the culture method described in Example 1, and shake culture was carried out at 30 °C for 24 hours. After cultivation, 300 ml of the culture was centrifuged, and the cells collected were suspended in 100 ml of 100 mM phosphate buffer (pH 6.5). The cell suspension, 1
20 g of 5-acetylfuro[2,3-c]pyridine and 3 g of glucose were placed in a 500-ml Sakaguchi flask, and the reaction was carried out at 30 °C with stirring for 5 hours. During the reaction, the pH of the reaction mixture was maintained at pH 6.5 using 5 N aqueous sodium hydroxide. After completion of the reaction,
25 the reaction mixture was extracted with ethyl acetate and the aqueous phase was further extracted with ethyl acetate. The organic phases were combined and dehydrated over anhydrous sodium sulfate, and the solvent was then distilled off under reduced pressure. The residue was dissolved in toluene at room
30 temperature and the solution was cooled in ice water to cause crystallization, whereby 750 mg of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine was obtained as white solid. Yield 75%, optical purity 100% e.e.

35 (Example 5: Preparation of a cell-free extract from Candida

intermedia IFO 0761 and synthesis of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the same)

Candida intermedia IFO 0761 was cultured by the same method of cultivation as described in Example 3 except that the 5-liter jar fermenter cultivation conditions were modified as follows: rate of stirring 700 rpm, aeration 1.5 liters/min, cultivation time 16 hours. After cultivation, 940 ml of the culture obtained was centrifuged, the cells collected were washed twice with 400-ml portions of 100 mM phosphate buffer (pH 6.5), the wet cells were suspended in 200 ml of 100 mM phosphate buffer (pH 6.5) supplemented with β -mercaptoethanol to a concentration of 5 mM, and the cells were disrupted using a Bead-Beater (product of BioSpec Products). The cell debris was removed by centrifugation, ammonium sulfate was added to 80% saturation, and the supernatant was removed by centrifugation. The sediment obtained was suspended in 15 ml of 100 mM phosphate buffer (pH 6.5) supplemented with β -mercaptoethanol to 5 mM and the suspension was dialyzed overnight against 100 mM phosphate buffer (pH 6.5) supplemented with β -mercaptoethanol to 1 mM to give 64.5 ml of a cell-free extract. The cell-free extract (0.75 ml) was added to a test tube containing 15 mg of 5-acetylfuro[2,3-c]pyridine and 104.3 mg of NADH, and the reaction was allowed to proceed at 30 °C for 4 hours. After completion of the reaction, the conversion to the product and the optical purity of the product were determined in the same manner as in Example 1. The conversion was 60.7% and the optical purity was (S) 99.5% e.e.

(Example 6: Synthesis of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using a cell-free extract from Candida intermedia IFO 0761)

The cell-free extract (0.75 ml) from Candida intermedia IFO 0761 as obtained in Example 5 was added to a test tube containing 15 mg of 5-acetylfuro[2,3-c]pyridine and 126.4 mg

of NADPH, and the reaction was allowed to proceed at 30 °C for 4 hours. After completion of the reaction, the conversion to the product and the optical purity of the product were determined in the same manner as in Example 1. The conversion
5 was 51.1% and the optical purity was (S) 99.4% e.e.

(Example 7: Preparation of acetone-dried cells from Candida maris IFO 10003 and synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the same)
10

A liquid broth (45 ml) comprising 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate
15 heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of manganese sulfate tetrahydrate and 100 mg of sodium chloride, per 900 milliliters, and one drop of Adekanol were placed in a 500-ml Sakaguchi flask and sterilized, 5 ml of a sterilized 40% aqueous solution of glucose was added, and the whole was
20 aseptically inoculated with 1 ml of the culture of Candida maris IFO 10003 as obtained by the culture method described in Example 1, and shake culture was carried out at 30 °C for 48 hours. After cultivation, 40 ml of the culture was centrifuged, and the cells collected were washed twice with deionized water and suspended
25 in 40 ml of deionized water. Acetone (1.2 L) was added thereto with ice cooling and stirring, and the mixture was stirred on ice for 30 minutes. After filtration, the cells on the filter paper were washed with cooled acetone and then dried under reduced pressure to give 1.3 g of acetone-dried cells. The
30 acetone-dried cells (10 mg), 5 mg of 5-acetylfuro[2,3-c]pyridine, 0.275 mg of NAD⁺, 0.275 mg of NADP⁺, 5.5 mg of glucose, 30 U of glucose dehydrogenase (GLUCDH "Amano" II, trademark, product of Amano Pharmaceutical) and 0.5 ml of 100 mM phosphate buffer were added to a test tube, and the reduction reaction
35 was carried out at 30 °C for 24 hours. After completion of the

reaction, the conversion to the product and the optical purity of the product were determined in the same manner as in Example 1. The conversion was 90.8% and the optical purity was (R) 99.9% e.e.

5

(Example 8: Preparation of a cell-free extract from Candida maris IFO 10003 and synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the same)

10 Candida maris IFO 10003 was cultivated by the same method of cultivation as described in Example 3 except that the 5-liter jar fermenter cultivation conditions were modified as follows: rate of stirring: 300 rpm, aeration: 0.75 L/min, cultivation time: 76 hours. Cells were centrifugally
15 collected from 2,150 ml of the culture obtained and washed with 500 ml of 100 mM phosphate buffer (pH 6.5), and the wet cells were suspended in 430 ml of 100 mM phosphate buffer (pH 6.5) supplemented with β -mercaptoethanol to a concentration of 5 mM, and the cells were disrupted using a Bead-Beater (product
20 of BioSpec Products). The cell debris was removed by centrifugation, and the supernatant was dialyzed overnight against 100 mM phosphate buffer (pH 6.5) supplemented with β -mercaptoethanol to 5 mM to give 289 ml of a cell-free extract. The cell-free extract (24 ml) was added to a three-necked flask
25 containing 0.25 g of 5-acetylfuro[2,3-c]pyridine, 30 mg of NAD⁺, 2.5 g of glucose and 300 U of glucose dehydrogenase (GLUCDH "Amano" II, trademark, product of Amano Pharmaceutical), and the reduction reaction was carried out. The reaction was allowed to proceed at 30 °C with stirring while adjusting the
30 pH of the reaction mixture to 6.5 using 5 N aqueous sodium hydroxide. Portions of the reaction mixture were analyzed at intervals by HPLC and, each time when the substrate was found exhausted, 0.25 g of the substrate was added, and the reaction was allowed to proceed continuedly. While repeating this
35 procedure, the reaction broth was carried out for about 72 hours.

Then, the yield of 5-(1-hydroxyethyl)furo[2,3-c]pyridine was 2.2 g. The yield was 88% and the optical purity was (R) 100% e.e.

- 5 (Example 9: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using a cell-free extract from Candida maris IFO 10003)

To a three-necked flask containing 20 ml of the cell-free extract obtained in Example 8 were added 0.25 g of 5-
10 acetylfuro[2,3-c]pyridine, 40 mg of NAD⁺, 0.25 g of sodium formate and 120 U of formate dehydrogenase (product of Fluka), and the reduction reaction was carried out. The reaction was conducted at 30 °C with stirring while adjusting the pH of the reaction mixture to 6.5 with 5 N formic acid. Portions of the
15 reaction mixture was analyzed at intervals by HPLC and, each time when the substrate was found exhausted, 0.25 g of the substrate was added, and the reaction was allowed to proceed continually. While repeating this procedure, the reaction was carried out for about 93 hours. After completion of the
20 reaction, the yield of 5-(1-hydroxyethyl)furo[2,3-c]pyridine was 1.8 g. Yield: 72%, optical purity: (R) 100% e.e.

- (Example 10: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using acetone-
25 dried cells of Candida maris IFO 10003)

The acetone-dried cells (20 mg) of Candida maris IFO 10003 as obtained in Example 7, 25 mg of 5-acetylfuro[2,3-c]pyridine, 2.2 mg of NAD⁺, 0.4 ml of 100 mM phosphate buffer (pH 6.5) and 0.1 ml of isopropanol were added to a test tube and the reduction
30 reaction was carried out at 30 °C for 39.5 hours. After completion of the reaction, the conversion to the product and the optical purity of the product were determined by the same analytical methods as in Example 1. The conversion was 47.2% and the optical purity was (R) 100% e.e.

(Example 11: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using a cell-free extract from Candida maris IFO 10003)

The cell-free extract (0.4 ml) from Candida maris IFO 10003 as obtained in Example 8, 25 mg of 5-acetylfuro[2,3-c]pyridine, 2.2 mg of NAD⁺ and 0.1 ml of isopropanol were added to a test tube, and the reduction reaction was carried out at 30 °C for 39.5 hours. After completion of the reaction, the conversion to the product and the optical purity of the product were determined by the same analytical methods as in Example 1. The conversion was 51.8% and the optical purity was (R) 100% e.e.

(Reference Example 1: Synthesis of 5-acetyl-7-chlorofuro[2,3-c]pyridine from 7-chloro-5-(1-hydroxyethyl)furo[2,3-c]pyridine)

7-Chloro-5-(1-hydroxyethyl)furo[2,3-c]pyridine (1.0 g) obtained by the method described in J. Org. Chem., 63, 7851 (1998) was dissolved in 4.0 ml of methylene chloride. Thereto were added 3 ml of a saturated aqueous solution of sodium bicarbonate, 0.62 ml of a 1 M aqueous solution of sodium bromide and 0.62 ml of a 1 M solution of 2,2,6,6-tetramethyl-1-piperidinyloxy radical in methylene chloride, and the resulting mixture was vigorously stirred on ice. Thereto was added portionwise 5.74 ml of an aqueous solution of sodium hypochlorite saturated with sodium bicarbonate in advance, and the mixture was stirred on ice for 30 minutes. After completion of the reaction, ethyl acetate was added, and 10 ml of 10% aqueous sodium hydrogen sulfite was added with stirring. After phase separation, the organic phase was taken, washed with a saturated solution of sodium chloride, and dried over anhydrous sodium sulfate. The solvent was distilled off under reduced pressure and the residue was crystallized from toluene-methylcyclohexane to give 0.43 g of 5-acetyl-7-chlorofuro[2,3-c]pyridine. ¹H-NMR δ (CDCl₃): 2.77 (3H, s),

7.00 (1H, d, J = 1.95 Hz), 7.89 (1H, d, J = 1.96 Hz), 8.32 (1H, s).

(Example 12: Asymmetric reduction of 5-acetyl-7-

5 chlorofuro[2,3-c]pyridine using various microorganisms)

A liquid broth (pH 7) comprising 40 g of glucose, 3 g of yeast extract, 6.5 g of diammonium hydrogen phosphate, 1 g of potassium dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate, 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of manganese sulfate tetrahydrate and 100 mg of sodium chloride, per liter, was distributed in 5-ml portions into large size test tubes and steam-sterilized at 120 °C for 20 minutes. These liquid broth portions were each aseptically inoculated with one loopful of one of the microorganisms listed in Table 5, and shake culture was carried out at 30 °C for 24 to 72 hours. Then, 0.5 ml of each culture was centrifuged and the cells collected were suspended in 0.5 ml of 100 mM phosphate buffer (pH 6.5) containing 8% of glucose. This cell suspension was added to a test tube containing 5 mg of 5-acetyl-7-chlorofuro[2,3-c]pyridine obtained in Reference Example 1 placed therein in advance, and the reaction was allowed to proceed at 30 °C for 26 hours. After reaction, 5 ml of ethyl acetate was added to each reaction mixture, followed by mixing up. A portion of this was analyzed under the following HPLC conditions.

[HPLC analysis conditions]

Column: Chiralpak AS, product of Daicel Chemical Industries;
30 eluent: hexane/ethanol/diethylamine = 92/8/0.1; flow rate: 1 ml/min; detection: 254 nm; column temperature: room temperature; elution time: 5-acetyl-7-chlorofuro[2,3-c]pyridine 7.6 minutes, 5-(1-(R)-hydroxyethyl)-7-chlorofuro[2,3-c]pyridine 10.3 minutes, 5-(1-(S)-
35 hydroxyethyl)-7-chlorofuro[2,3-c]pyridine 15.7 minutes.

The yield, optical purity and absolute configuration of the product 5-(1-hydroxyethyl)-7-chlorofuro[2,3-c]pyridine per reaction mixture are shown in Table 5.

5 Table 5

Microorganism	Yield (mg/ml)	Optical purity (% e.e.)	Absolut e configu ration
<u>Candida maltosa</u> CBS 5612	2.33	97.4	S
<u>Candida parapsilosis</u> IFO 0585	0.32	70.3	S
<u>Cryptococcus albidus</u> var. <u>albidus</u> IFO 0378	0.62	96.5	S
<u>Dipodascus ovetensis</u> IFO 1201	2.07	97.2	S
<u>Geotrichum candidum</u> CBS 178.71	3.35	98.2	S
<u>Hyphopichia burtonii</u> IFO 0844	1.61	81.4	S
<u>Lodderomyces elongisporus</u> IFO 1676	1.45	95.8	S
<u>Rhodosporidium toruloides</u> IFO 0559	1.90	98.7	S
<u>Sporidiobolus johnsonii</u> IFO 6903	4.24	98.3	S
<u>Candida maris</u> IFO 10003	5.10	90.5	R
<u>Ogataea wickerhamii</u> IFO 1706	5.85	18.0	R
<u>Yamadazyma farinosa</u> IFO 0534	0.46	78.8	R

(Example 13: Asymmetric reduction of 5-acetyl-7-chlorofuro[2,3-c]pyridine using various microorganisms)

A liquid broth (pH 7) comprising 10 g meat extract, 10 g peptone, 5 g of yeast extract and 3 g sodium chloride, per liter, was distributed in 5-ml portions into large size test tubes and steam-sterilized at 120 °C for 20 minutes. These liquid broth portions were each aseptically inoculated with one loopful of one of the microorganisms listed in Table 6, and shake culture was carried out at 30 °C for 24 to 72 hours. Then, 2 ml of each culture was centrifuged and the cells collected were suspended in 0.5 ml of 100 mM phosphate buffer (pH 6.5) containing 8% of glucose. This cell suspension was added to a test tube containing 2.5 mg of 5-acetyl-7-chlorofuro[2,3-c]pyridine obtained in Reference Example 1 placed therein in advance, and the reaction was allowed to proceed at 30 °C for 26 hours. After reaction, the reaction mixture was analyzed

in the same manner as in Example 12. The yield, optical purity and absolute configuration of the product 5-(1-hydroxyethyl)-7-chlorofuro[2,3-c]pyridine per reaction mixture are shown in Table 6.

5

Table 6

Microorganism	Yield (mg/ml)	Optical purity (% e.e.)	Absolute configu- ration
<u>Jensenia canicruria</u> IFO 13914	4.37	99.0	S
<u>Pseudomonas stutzeri</u> IFO 13596	1.30	99.6	S
<u>Corynebacterium acetoacidophilum</u> ATCC 21476	0.24	64.0	R

(Example 14: Enzyme purification)

Hereafter, the reducing activity was measured by allowing
 10 the reaction to proceed at 30 °C for 3 minutes in 3.0 ml of a
 reaction mixture containing 1 mM 5-acetylfuro[2,3-c]pyridine
 (substrate), 0.25 mM NADH, 0.3% (vol/vol) dimethyl sulfoxide
 and 0.05 ml of the enzyme solution in 100 mM phosphate buffer
 (pH 6.5) and measuring the decrease in absorbance at the
 15 wavelength 340 nm. These conditions were employed as standard
 reaction conditions for reducing activity measurement. The
 enzyme activity oxidizing 1 μ mole of NADH to NAD⁺ in one minute
 under these reaction conditions was defined as 1 unit.

A liquid broth (45 ml) comprising 3 g of yeast extract,
 20 6.5 g of diammonium hydrogen phosphate, 1 g of potassium
 dihydrogen-phosphate, 0.8 g of magnesium sulfate heptahydrate,
 60 mg of zinc sulfate heptahydrate, 90 mg of iron sulfate
 heptahydrate, 5 mg of copper sulfate pentahydrate, 10 mg of
 manganese sulfate tetrahydrate and 100 mg of sodium chloride,
 25 per 900 milliliters, and one drop of Adekanol were placed in
 a 500-ml Sakaguchi flask and sterilized, 5 ml of a sterilized
 40% aqueous solution of glucose was added, and the whole was
 aseptically inoculated with 1 ml of the culture of Candida maris
 IFO 10003 as obtained by preculturing in the same medium, and
 30 shake culture was carried out at 30 °C for 24 hours. The culture

obtained was used as a seed yeast.

A 5-liter jar fermenter was charged with 2.25 liters of the liquid broth having the above composition and 5 drops of Adekanol and, after sterilization, 250 ml of a sterilized 40% aqueous solution of glucose was added, and the whole was aseptically inoculated with 50 ml of the seed yeast. Cultivation was carried out under the following conditions for 140 hours: cultivation temperature 30 °C, rate of stirring 300 rpm, aeration 0.3 L/min. When, during cultivation, the pH was found to have become lower than 5.5, the pH was adjusted to 5.5 with 5 N aqueous sodium hydroxide.

Ten liters of the culture obtained by the above cultivation method was centrifuged, the cells collected were washed twice with 5-liter portions of physiological saline, the wet cells were suspended in 1,200 ml of 100 mM Tris-hydrochloride buffer (pH 7.5) containing 5 mM β -mercaptoethanol and 0.1 mM PMSF and the cells were disrupted using a Dyno-mill (product of Dyno Mill). The cell debris was removed by centrifugation and 1,760 ml of a cell-free extract was obtained.

Protamine sulfate (3 g) was added to this cell-free extract and, after overnight stirring at 4 °C, the resulting precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 35% saturation and, after 1 hour of stirring at 0 °C, the resulting precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 65% saturation and, after 1 hour of stirring at 0 °C, the resulting precipitate was collected by centrifugation and suspended in 200 ml of 20 mM Tris-hydrochloride buffer (pH 7.5) containing 5 mM β -mercaptoethanol, and the suspension was dialyzed against 30 L of the same buffer.

The dialyzate was applied to a DEAE-TOYOPEARL 650M (product of Tosoh) column (340 ml) equilibrated beforehand with 20 mM Tris-hydrochloride buffer (pH 7.5) containing 5 mM β -mercaptoethanol to thereby allow the enzyme to be adsorbed, and

the active fraction was eluted by a linear concentration gradient of sodium chloride (from 0 mM to 50 mM). Ammonium sulfate was added to this active fraction to a final concentration of 0.5 M, the resulting mixture was applied to a Phenyl-TOYOPEARL 650 M (product of Tosoh) column (74 ml) equilibrated in advance with 20 mM Tris-hydrochloride buffer (pH 8.5) containing 5 mM β -mercaptoethanol and 0.5 M ammonium sulfate to thereby cause enzyme adsorption, the column was washed with the same buffer and the active fraction was eluted by a linear concentration gradient of ammonium sulfate (from 0.5 M to 0 M). The active eluate fractions were combined and an electrophoretically single purified enzyme specimen was thus obtained. The molecular weight of the band in SDS-PAGE was about 29,000. Upon gel filtration analysis using a TSK-G 3000 SW (product of Tosoh) column (7.8 mm I.D. $\times$ 30 cm) and 0.1 M phosphate buffer (pH 7.0) containing 0.1 M sodium sulfate as the eluent, the molecular weight was about 60,000. Hereinafter, this enzyme is referred to as FPDH.

(Example 15: Optimal temperature for the activity of FPDH)
Activity measurements were conducted under the standard reaction conditions for reducing activity measurement as described in Example 14 except that the temperature alone was varied from 20 to 80 $^{\circ}$ C. As a result, the optimal temperature was found to be 50 to 55 $^{\circ}$ C.

(Example 16: Optimal pH for the activity of FPDH)
Activity measurements were conducted under the standard reaction conditions for reducing activity measurement as described in Example 14 while varying the pH within the range of 3.5 to 9.0 using acetate buffer, phosphate buffer and Tris-hydrochloride buffer as the buffer solution. As a result, the optimal pH was found to be 5.0-6.0.

(Example 17: Behaviors of FPDH against inhibitors)

Activity measurements were conducted under the standard reaction conditions for reducing activity measurement as described in Example 14 adding various compounds and metal salts as possible inhibitors at the respective concentrations shown in Table 7. The results are shown in Table 7 in terms of relative activity with the activity obtained without inhibitor addition being taken as 100%. As a result, the enzyme activity was inhibited by the mercury ion.

10 Table 7

Inhibitor	Conc. (mM)	Relative activity (%)
DTNB	0.01	98
Iodoacetic acid	1.0	80
Dithiothreitol	1.0	93
2-Mercaptoethanol	1.0	94
N-ethylmaleimide	1.0	84
p-Chloromercuribenzoic acid	0.1	100
EDTA	1.0	98
1,10-Phenanthroline	1.0	98
Quercetin	0.01	78
Diphenylhydantoin	0.5	102
MgSO ₄	1.0	81
MnCl ₂	1.0	76
ZnSO ₄	1.0	74
CuSO ₄	1.0	103
CoCl ₂	1.0	85
HgCl ₂	1.0	0

(Example 18: Specificity of FPDH)

The reducing activity of FPDH was examined against various compounds. Activity measurements were conducted under the standard reaction conditions for reducing activity measurement using various carbonyl compounds shown in Table 8 and Table 9 as substrates in lieu of 5-acetylfuro[2,3-c]pyridine. In Table 8 and Table 9, the results are shown in terms of relative activity with the reducing activity when 5-acetylfuro[2,3-c]pyridine was used as the substrate being taken as 100%. As a result, FPDH had reducing activity against

ketones and aldehydes but the reducing activity thereof was very low against carbocyclic ketones and the ketone in position α to a carboxyl group.

5 Table 8

Substrate (1 mM)	Relative activity (%)
5-Acetylfuro[2,3-c]pyridine	100
1-Chloro-5-acetylfuro[2,3-c]pyridine	66
2-Acetylpyridine	111
3-Acetylpyridine	107
4-Acetylpyridine	130
Acetylpyrazine	133
2-Acetylpyrrole	100
2-Acetylthiophene	77
2-Acetylfuran	43
2-Acetylthiazole	86
Acetophenone	88
m-Nitroacetophenone	136
p-Nitroacetophenone	116
o-Chloroacetophenone	9
m-Chloroacetophenone	120
p-Chloroacetophenone	88
p-Fluoroacetophenone	88
2-Hydroxyacetophenone	54
2,3'-Dichloroacetophenone	19
Benzylacetone	96
Acetone	40
2-Butanone	94
2-Pentanone	67
2-Hexanone	38
2-Octanone	38
Methyl isopropyl ketone	43
Methyl isobutyl ketone	14
Acetoin	43
Diacetyl	107
Acetylacetone	123
Diethyl ketone	22
Chloroacetone	99
1-Acetylcyclopentanone	86
1-Acetylcyclohexanone	68
Methyl pyruvate	132
Ethyl pyruvate	133

Table 9

Substrate (1 mM)	Relative activity (%)
Methyl acetoacetate	128
Ethyl acetoacetate	141
Ethyl 2-chloroacetoacetate	88
Pyruvic acid	3
2-Ketobutyric acid	5
2-Keto-n-valeric acid	7
Oxalacetic acid	4
Cyclopentanone	3
Cycloheptanone	4
1-Tetralone	2
2-Tetralone	0
Camphorquinone	3
Cyclohexanone	4
Pyridine-2-aldehyde	54
Pyridine-3-aldehyde	47
Pyridine-4-aldehyde	64
Benzaldehyde	94
o-Nitrobenzaldehyde	9
m-Nitrobenzaldehyde	103
p-Nitrobenzaldehyde	162
o-Chlorobenzaldehyde	0
m-Chlorobenzaldehyde	111
p-Chlorobenzaldehyde	104
Acetaldehyde	36
Propionaldehyde	70
n-Butyr aldehyde	123
n-Hexyl aldehyde	71

Further, FPDH was examined for oxidation activity against various compounds. The oxidation activity was measured by allowing the reaction to proceed at 30 °C for 3 minutes in 3.0 ml of a reaction mixture containing 1 mM substrate, 0.25 mM NAD⁺, 0.3% (vol/vol) dimethyl sulfoxide and 0.05 ml of the enzyme solution in 100 mM phosphate buffer (pH 8.0) and measuring the increase in absorbance at the wavelength 340 nm. These conditions were employed as standard reaction conditions for oxidizing activity substrate measurement. The enzyme activity reducing 1 μ mole of NAD⁺ to NADH in one minute under these reaction conditions was defined as 1 unit. The data shown in Table 10 are relative activities with the oxidation activity

against the substrate 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine being taken as 100%. As a result, it was found that FPDH has oxidation activity against various compounds.

5 Table 10

Substrate (1 mM)	Relative activity (%)
(R)-5-(1-Hydroxyethyl)furo[2,3-c]pyridine	100
1-(2-Pyridyl)ethanol	23
1-(3-Pyridyl)ethanol	110
1-(4-Pyridyl)ethanol	24
Isopropanol	226
1-Buten-3-ol	261
4-Penten-2-ol	172
4-Phenyl-2-butanol	219
(R)-1-Phenylethanol	225

(Example 19: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using FPDH)

10 Stirred was 0.5 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.5 unit of FPDH obtained in Example 14, 5 mg of 5-acetylfuro[2,3-c]pyridine, 20 mg of glucose, 0.5 mg of NAD⁺ and 4 units of glucose dehydrogenase (product of Amano Pharmaceutical) at 30 °C for 17 hours. After reaction, the reaction mixture was analyzed in the same manner as in Example 15 1. As a result, the formation of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine with an optical purity of 100% e.e. with 100% conversion was confirmed.

20 (Example 20: Synthesis of 7-chloro-5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetyl-7-chlorofuro[2,3-c]pyridine using FPDH)

25 Stirred was 0.5 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.5 unit of FPDH obtained in Example 14, 5 mg of 5-acetyl-7-chlorofuro[2,3-c]pyridine, 20 mg of glucose, 0.5 mg of NAD⁺ and 4 units of glucose dehydrogenase (product of Amano

Pharmaceutical) at 30 °C for 3 hours. After reaction, the reaction mixture was analyzed in the same manner as in Example 12. As a result, the formation of 7-chloro-5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine with an optical purity of
 5 >99% e.e. with 66.9% of conversion was confirmed.

(Example 21: FPDH gene cloning)

(Chromosomal DNA preparation)

Chromosomal DNA was extracted from a culture of Candida
 10 maris IFO 10003 by the method described by Hereford (Cell, 18, 1261 (1979)).

(FDPH gene cloning by PCR)

Purified FPDH obtained as described in Example 14 was denatured in the presence of 8 M urea and then digested with
 15 Achromobacter-derived lysyl endopeptidase (product of Wako Pure Chemical Industries) and the peptide fragments obtained were sequenced by the Edman method. Considering the DNA sequences deduced from the amino acid sequences, two PCR primers were synthesized (primer 1: 5'-GGNGCNATHGTNAAYATGGG-3',
 20 primer 2: 5'-CCDATNGGRTGYTGNGTDAT-3').

A buffer solution (100 µl) for ExTaq containing the two primers (primer 1 and primer 2, 100 picomoles each), 660 ng of the chromosomal DNA, 20 nanomoles of each dNTP and 2.5 U of ExTaq (product of Takara Shuzo) was prepared, and 40 cycles of thermal
 25 denaturation (95 °C, 1 min), annealing (40 °C, 1 min) and elongation reaction (65 °C, 2 min) were conducted and, after cooling to 4 °C, an amplified DNA was confirmed by agarose gel electrophoresis.

(Subcloning of the PCR-amplified DNA)

30 The amplified DNA was subcloned into the pT7Blue vector (product of Novagen) and the base sequence thereof was determined. As a result, the amplified DNA was found to be comprised of 230 bases including the primer sequences. The sequence is the DNA sequence portion doubly underlined in the
 35 DNA sequence shown in Fig. 1. Hereinafter, this sequence is

referred to as "core sequence".

(Cloning of sequences adjacent to the core sequence by inverse PCR)

Based on the complementary sequence

5 GGAGCGGCCACATACGAGTGAATGG (primer 3) of a portion close to the 5' side of the core sequence and the sequence AGACACCATTGCTTGATATTTGCCC (primer 4) of a portion close to the 3' side, two PCR primers (primer 3 and primer 4) identical in sequence to those sequences were synthesized.

10 For preparing a template for inverse PCR, the chromosomal DNA of Candida maris IFO 10003 was first digested with the restriction enzyme PstI and the digest was self-circularized using T4 DNA ligase. A buffer solution (100 μ l) for ExTaq containing 660 ng of the self-circularization product, the two
15 primers (primer 3 and primer 4, 100 picomoles each), 20 nanomoles of each dNTP and 2.5 U of ExTaq (product of Takara Shuzo) was prepared, and 40 cycles of thermal denaturation (94 °C, 0.5 min), annealing (55 °C, 0.5 min) and elongation reaction (72 °C, 1 min) were conducted and, after cooling to 4 °C, an
20 amplified DNA was confirmed by agarose gel electrophoresis.

The amplified DNA was subcloned into the pT7Blue vector (product of Novagen) and the base sequence thereof was determined. Based on this result and the core sequence data, the whole base sequence of the DNA coding for FPDH was determined.
25 That whole base sequence and the deduced amino acid sequence encoded by said DNA are shown in Fig. 1. In Fig. 1, the singly underlined portions indicate those amino acid sequences which could have been determined by the Edman method with the peptide fragments formed upon digestion of purified FPDH with lysyl
30 endopeptidase. The amino acid sequence of FPDH derived from Candida maris IFO 10003 is shown under SEQ ID NO:1 in the sequence listing. The base sequence of the DNA coding for that FPDH is shown under SEQ ID NO:2 in the sequence listing.

35 (Example 22: Construction of a recombinant vector containing

the FPDH gene)

For causing FPDH expression in *Escherichia coli*, a recombinant vector to be used for transformation was constructed. First, a double-stranded DNA was prepared which had an NdeI site added to the initiation codon site of the structural gene for FPDH and a new termination codon and an EcoRI site added immediately behind the termination codon, in the following manner. Based on the base sequence determined in Example 21, a primer 5 (5'-CGCCATATGTCCTACAATTTTGCCAAC-3') with an NdeI site added to the initiation codon portion of the structural gene for FPDH and a primer 6 (5'-GCGGAATTCTTATTATCTTGCGGTATAACCACC-3') with a new termination codon and an EcoRI site added immediately behind the termination codon of the structural gene for FPDH were synthesized.

A buffer solution (100 μ l) for ExTaq containing the two primers (primer 5 and primer 6, 100 picomoles each), 132 ng of the *Candida maris* IFO 10003-derived chromosomal DNA, 20 nanomoles of each dNTP and 2.5 U of ExTaq (product of Takara Shuzo) was prepared, and 30 cycles of thermal denaturation (94 $^{\circ}$ C, 0.5 min), annealing (60 $^{\circ}$ C, 0.5 min) and elongation reaction (72 $^{\circ}$ C, 1 min) were conducted and, after cooling to 4 $^{\circ}$ C, an amplified DNA was confirmed by agarose gel electrophoresis. This amplified fragment was digested with NdeI and EcoRI and the digest was inserted into the plasmid pUCNT (WO 94/03613) at the NdeI-EcoRI site downstream from the lac promoter, whereby a recombinant vector, pNTFP, was obtained. The construction scheme for and the structure of pNTFP are shown in Fig. 2.

(Example 23: Construction of a recombinant vector containing both the FPDH gene and the glucose dehydrogenase gene)

A double-stranded DNA comprising the *Bacillus megaterium* IAM 1030-derived glucose dehydrogenase (hereinafter referred to as GDH) gene with the *Escherichia coli* Shine-Dalgarno sequence (9 nucleotides) added at 5 bases upstream of the initiation codon of that gene and, further, with an EcoRI

digested site added just before that sequence and an SalI digested site added just behind the termination codon was prepared in the following manner. Based on the information on the base sequence of the GDH gene, a primer 7 (5'-
 5 GCCGAATTCTAAGGAGGTAAACAATGTATAAAGATTAGAAGG-3') with the Escherichia coli Shine-Dalgarno sequence (9 nucleotides) added at 5 bases upstream of the initiation codon of the GDH structural gene and, further, with an EcoRI digested site added just before that sequence, and a primer 8 (5'-
 10 GCGGTCGACTTATCCGCGTCCTGCTTGG-3') with an SalI site added just behind the termination codon of the GDH structural gene were synthesized in the conventional manner. Using these two primers, a double-stranded DNA was synthesized by PCR using the plasmid pGDK1 (Eur. J. Biochem., 186, 389 (1989)) as the
 15 template. The DNA fragment obtained was digested with EcoRI and SalI, and the digested fragment was inserted into pNTFP constructed in Example 22 at the EcoRI-SalI site thereof to give a recombinant vector, pNTFPG. The construction scheme for and the structure of pNTFPG are shown in Fig. 2.

20

(Example 24: Construction of recombinant Escherichia coli)
Escherichia coli HB101 (product of Takara Shuzo) was transformed with the recombinant vector pNTFP obtained in Example 22 and the recombinant vector pNTFPG obtained in Example
 25 23 to give recombinant Escherichia coli HB101 (pNTFP) and HB101 (pNTFPG), respectively. The thus-obtained transformants, namely Escherichia coli HB101 (pNTFP) and Escherichia coli HB101 (pNTFPG), have been deposited with the Ministry of International Trade and Industry National Institute of
 30 Bioscience and Human Technology (address: 1-3 Higashi-1-chome, Tsukuba City, Ibaraki Prefecture, Japan) under the accession numbers FERM BP-7116 (deposited April 11, 2000) and FERM BP-7117 (deposited April 11, 2000), respectively.

Further, a recombinant vector, pSTVG, was constructed by
 35 inserting a DNA fragment of about 0.9 kb obtained by double

digestion of the plasmid pGDA2 (J. Biol. Chem., 264, 6381 (1989)) with EcoRI and PstI and containing the Bacillus megaterium-derived GDH gene into the plasmid pSTV28 (product of Takara Shuzo) at the EcoRI-PstI site thereof. Escherichia coli HB101 (pNTFP) rendered competent in advance by the calcium chloride method was transformed with that pSTVG with a high introduction percentage, whereby Escherichia coli HB101 (pNTFP, pSTVG) was readily obtained.

10 (Example 25: Expression of FPDH in recombinant Escherichia coli)

The recombinant Escherichia coli HB101 (pNTFP) obtained in Example 24 was cultured on 2 x YT medium (1.6% (w/v) Bacto Tryptone, 1.0% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl, pH 7.0) containing 120 μ g/ml of ampicillin. Cells were collected, suspended in 100 mM Tris-hydrochloride buffer (pH 7) and sonicated to give a cell-free extract. The FPDH activity of this cell-free extract was determined by the method described in Example 14. The result is shown in Table 11 in terms of specific activity.

Table 11

Strain	Specific FPDH activity (U/mg)
HB101 (pUCNT)	< 0.1
HB101 (pNTFP)	12.7

25 With Escherichia coli HB101 (pNTFP), a distinct increase in FPDH activity was observed as compared with the transformant obtained by using the vector plasmid alone, namely Escherichia coli HB101 (pUCNT).

30 (Example 26: Simultaneous expression of FPDH and GDH in recombinant Escherichia coli)

The recombinant Escherichia coli HB101 (pNTFPG) obtained in Example 24 and the recombinant Escherichia coli HB101 (pNTFP, pSTVG) were treated in the same manner as in Example 25, and

the cell-free extracts obtained were assayed for FPDH activity and GDH activity. The GDH activity was measured by adding 0.1 M glucose (substrate), 2 mM NADP⁺ (coenzyme) and the enzyme to 1 M Tris-hydrochloride buffer (pH 8.0) and measuring the increase in absorbance at the wavelength 340 nm at 25 °C. The enzyme activity capable of reducing 1 μ mole of NADP⁺ to NADPH in 1 minute under these reaction conditions was defined as 1 unit. The FPDH activity was determined in the same manner as in Example 14. The thus-measured FPDH activity and GDH activity of each cell-free extract are shown in Table 12 in terms of specific activity.

Table 12

Strain	Specific FPDH activity (U/mg)	Specific GDH activity (U/mg)
HB101 (pUCNT)	< 0.1	<0.01
HB101 (pNTFP)	12.7	<0.01
HB101 (pNTFPG)	7.5	86.7
HB101 (pNTFP, pSTVG)	11.3	1.13

Escherichia coli HB101 (pNTFPG) and Escherichia coli HB101 (pNTFP, pSTVG) each showed evident increases in FPDH and GDH activities as compared with the transformant obtained by using the vector plasmid alone, namely Escherichia coli HB101 (pUCNT).

(Example 27: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the recombinant Escherichia coli having the FPDH gene introduced therein, under the conditions resulting from the addition of isopropanol to the reaction system)

2 $\times$ YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant Escherichia coli HB101 (pNTFP) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. One milliliter of the culture obtained was adjusted to pH 7.0, 50 mg of 5-acetylfuro[2,3-c]pyridine, 150 μ l of isopropanol and 0.22 mg

of NAD⁺ were added thereto, and the mixture was stirred at 30 °C for 7.5 hours. After completion of the reaction, the conversion to and the optical purity of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same analytical methods as used in Example 1. The conversion was 95.7% and the optical purity was (R) 100% e.e.

(Example 28: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the recombinant *Escherichia coli* having the FPDH gene introduced therein, with GDH separately added to the reaction system)

2 × YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant *Escherichia coli* HB101 (pNTFP) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. To 20 ml of the culture obtained were added 540 units of glucose dehydrogenase (product of Amano Pharmaceutical), 1.0 g of 5-acetylfuro[2,3-c]pyridine, 3 mg of NAD⁺ and 3 g of glucose and, while adjusting the pH to 6.5 by dropwise addition of 2.5 M aqueous sodium hydroxide, the mixture was stirred at 30 °C for 29 hours. After completion of the reaction, the conversion to and the optical purity of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same analytical methods as used in Example 1. The conversion was 97.1% and the optical purity was (R) 100% e.e.

(Example 29: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the recombinant *Escherichia coli* under simultaneous expression of FPDH and GDH)

2 × YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant *Escherichia coli* HB101 (pNTFPG) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. To 20 ml of the culture obtained were added 1.0 g of 5-acetylfuro[2,3-c]pyridine, 3 mg

of NAD⁺ and 3 g of glucose and, while adjusting the pH to 6.5 by dropwise addition of 2.5 M aqueous sodium hydroxide, the mixture was stirred at 30 °C. At 6 hour during the reaction, 1.0 g of 5-acetylfuro[2,3-c]pyridine and 3.0 g of glucose were added. After 29 hours of stirring, the yield and optical purity of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same analytical methods as used in Example 1. The yield was 2.66 g and the optical purity was (R) 100% e.e.

10

(Example 30: Synthesis of 5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine from 5-acetylfuro[2,3-c]pyridine using the recombinant Escherichia coli under simultaneous expression of FPDH and GDH under the conditions resulting from the addition of butyl acetate to the reaction system)

15

2 × YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant Escherichia coli HB101 (pNTFPG) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. To 20 ml of the culture obtained were added 4.0 g of 5-acetylfuro[2,3-c]pyridine, 3 mg of NAD⁺, 6 g of glucose and 20 ml of butyl acetate and, while adjusting the pH to 6.5 by dropwise addition of 5 M aqueous sodium hydroxide, the mixture was stirred at 30 °C. At 9 hour during the reaction, 1.0 g of 5-acetylfuro[2,3-c]pyridine and 1.5 g of glucose were added. After 78 hours of stirring with timely addition of NAD⁺, the yield and optical purity of the product 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same analytical methods as used in Example 1. The yield was 4.98 g and the optical purity was (R) 100% e.e.

25
30

(Example 31: Synthesis of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine from 5-(1-hydroxyethyl)furo[2,3-c]pyridine using the recombinant Escherichia coli having the FPDH gene)

2 × YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant Escherichia

35

coli HB101 (pNTFP) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. One milliliter of the culture obtained was adjusted to pH 6.5, 10 mg of 5-(1-hydroxyethyl)furo[2,3-c]pyridine (optical purity 0% e.e.), 50
5 μ l of acetone and 0.1 mg of NAD⁺ were added thereto, and the mixture was stirred at 30 °C for 7 hours. After completion of the reaction, the yield of 5-acetylfuro[2,3-c]pyridine and the residual amount and optical purity of 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same
10 analytical methods as used in Example 1. The yield of 5-acetylfuro[2,3-c]pyridine was 4.71 mg and the residual amount of 5-(1-hydroxyethyl)furo[2,3-c]pyridine was 5.29 mg and the optical purity thereof was (S) 89.2% e.e.

15 (Example 32: Synthesis of 5-(1-(S)-hydroxyethyl)furo[2,3-c]pyridine from 5-(1-hydroxyethyl)furo[2,3-c]pyridine using the recombinant Escherichia coli having the FPDH gene)

2 $\times$ YT medium (50 ml) placed in a 500-ml Sakaguchi flask and sterilized was inoculated with the recombinant Escherichia
20 coli HB101 (pNTFP) obtained in Example 24, and shake culture was carried out at 37 °C for 18 hours. One milliliter of the culture obtained was adjusted to pH 6.5, 10 mg of 5-(1-hydroxyethyl)furo[2,3-c]pyridine (optical purity 0% e.e.) was added thereto, and the mixture was stirred at 30 °C for 7 hours.
25 After completion of the reaction, the yield of 5-acetylfuro[2,3-c]pyridine and the residual amount and optical purity of 5-(1-hydroxyethyl)furo[2,3-c]pyridine were determined by the same analytical methods as used in Example 1. The yield of 5-acetylfuro[2,3-c]pyridine was 4.98 mg and
30 the residual amount of 5-(1-hydroxyethyl)furo[2,3-c]pyridine was 5.02 mg and the optical purity thereof was (S) 99.2% e.e.

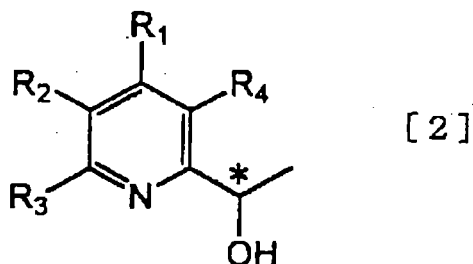
INDUSTRIAL APPLICABILITY

The invention makes it possible to produce an optically
35 active pyridineethanol derivative in high yields by

stereoselectively reducing acetylpyridine derivatives by causing an enzyme or enzyme source having asymmetric reduction activity to act thereon. It also provides a novel enzyme, a DNA coding for said enzyme, a recombinant vector having said DNA, and a transformant having said recombinant vector. By 5 using said enzyme and said transformant, it is possible to produce an optically active pyridineethanol derivative efficiently.

CLAIMS

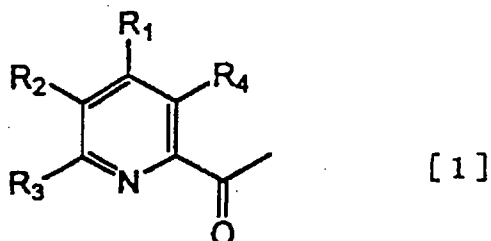
1. A method of producing an optically active
pyridineethanol derivative represented by the general formula
5 [2]:



wherein R_1 and R_2 are bound to each other to form a 5- to 8-membered
monocyclic heterocycle containing at least one hetero atom
10 selected from the group consisting of oxygen, sulfur and
nitrogen atoms, which heterocycle may optionally have a
substituent(s), or a polycyclic heterocycle resulting from the
condensation of such monocyclic heterocycle with another ring,
which polycyclic heterocycle may optionally have a
15 substituent(s),

R_3 and R_4 are the same or different and each represents a hydrogen
atom, a halogen atom, a hydroxyl group, an alkyl group
containing 1 to 12 carbon atoms, which may optionally have a
substituent(s), or an alkoxy group containing 1 to 12 carbon
20 atoms, which may optionally have a substituent(s),
and * indicates that the asterisked carbon atom is an asymmetric
one,

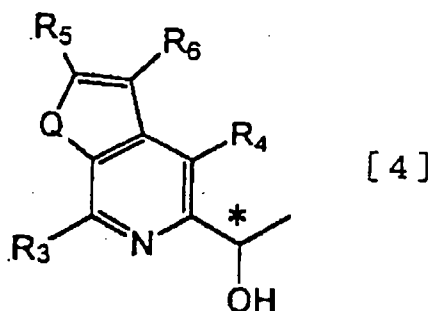
which method comprises stereoselectively reducing an
acetylpyridine derivative represented by the general formula
25 [1]:



wherein R_1 , R_2 , R_3 and R_4 are as defined above,
by causing an enzyme or enzyme source capable of asymmetrically
reducing the same to act thereon.

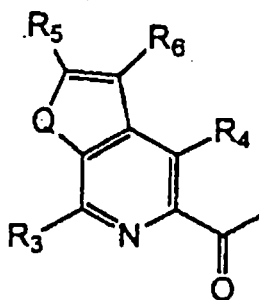
5

2. A method of producing an optically active
pyridineethanol derivative represented by the general formula
[4]:



10 wherein Q represents an oxygen or sulfur atom or a group of the
general formula $-N(D)-$, in which N is a nitrogen atom and D
represents a hydrogen atom or a monovalent protective group,
 R_3 , R_4 , R_5 and R_6 are the same or different and each represents
a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group
15 containing 1 to 12 carbon atoms, which may optionally have a
substituent(s), or an alkoxy group containing 1 to 12 carbon
atoms, which may optionally have a substituent(s),
and * indicates that the asterisked carbon atom is an asymmetric
one,

20 which method comprises stereoselectively reducing an
acetylpyridine derivative represented by the general formula
[3]:



[3]

wherein Q, R₃, R₄, R₅ and R₆ are as defined above,
by causing an enzyme or enzyme source capable of asymmetrically
reducing the same to act thereon.

5

3. The production method according to Claim 2,
wherein Q is an oxygen atom.

10

4. The production method according to Claim 2,
wherein Q is an oxygen atom,
R₃ is a hydrogen atom or a chlorine atom,
R₄ is a hydrogen atom,
R₅ is a hydrogen atom
and R₆ is a hydrogen atom or a methyl group.

15

5. The production method according to Claim 2,
wherein Q is an oxygen atom
and R₃, R₄, R₅ and R₆ each is a hydrogen atom.

20

6. The production method according to any of Claims 1
to 5,

wherein the reaction is carried out in the presence of
an enzyme capable of reducing the oxidized form nicotinamide
adenine dinucleotide and/or the oxidized form nicotinamide
adenine dinucleotide phosphate to the respective reduced forms
as well as a substrate for the reduction.

25

7. The production method according to Claim 6,
wherein said enzyme for reduction to the reduced form is

glucose dehydrogenase

and said substrate for reduction is glucose.

8. The production method according to Claim 6,
5 wherein said enzyme for reduction to the reduced form is
formate dehydrogenase
and said substrate for reduction is formic acid.

9. The production method according to any of Claims 1
10 to 8,

wherein said enzyme or enzyme source is derived from a
microorganism selected from the group consisting of
microorganisms of the genera Ashbya, Candida, Cryptococcus,
Clavispora, Debaryomyces, Dipodascus, Galactomyces,
15 Geotrichum, Guilliermondella, Hanseniaspora, Hansenula,
Hyphopichia, Issatchenkia, Kluyveromyces, Kuraishia,
Lodderomyces, Metschnikowia, Ogataea, Pachysolen, Pichia,
Rhodospiridium, Rhodotorula, Saccharomycopsis,
Schwanniomyces, Sporidiobolus, Sporobolomyces,
20 Schizoblastosporion, Stephanoascus, Torulasporea, Trigonopsis,
Trichosporon, Willopsis, Yamadazyma, Zygosaccharomyces,
Alcaligenes, Bacillus, Brevibacterium, Cellulomonas,
Corynebacterium, Jensenia, Ochrobactrum, Pseudomonas,
Rhodococcus and Tsukamurella.

25

10. The production method according to Claim 9,
wherein the product optically active pyridineethanol
derivative has the S absolute configuration

and said enzyme or enzyme source is derived from a
30 microorganism selected from the group consisting of
microorganisms of the genera Ashbya, Candida, Cryptococcus,
Clavispora, Debaryomyces, Dipodascus, Galactomyces,
Geotrichum, Guilliermondella, Hanseniaspora, Hansenula,
Hyphopichia, Issatchenkia, Kluyveromyces, Kuraishia,
35 Lodderomyces, Metschnikowia, Ogataea, Pachysolen, Pichia,

Rhodospiridium, Rhodotorula, Saccharomycopsis,
Schwanniomyces, Sporidiobolus, Sporobolomyces,
Schizoblastosporion, Stephanoascus, Torulaspora, Trigonopsis,
Trichosporon, Willopsis, Yamadazyma, Zygosaccharomyces,
5 Alcaligenes, Bacillus, Brevibacterium, Cellulomonas,
Corynebacterium, Jensenia, Ochrobactrum, Pseudomonas,
Rhodococcus and Tsukamurella.

11. The production method according to Claim 9,
10 wherein the product optically active pyridineethanol
derivative has the R absolute configuration
and said enzyme or enzyme source is derived from a
microorganism selected from the group consisting of
microorganisms of the genera Candida, Ogataea, Pichia,
15 Yamadazyma, Brevibacterium and Corynebacterium.

12. An enzyme having the following physical and chemical
properties (1) to (3):
(1) Activity: It stereoselectively reduces 5-
20 acetylfuro[2,3-c]pyridine, in the presence of reduced form
nicotinamide adenine dinucleotide as a coenzyme, to give 5-
(1-(R)-hydroxyethyl)furo[2,3-c]pyridine;
(2) Specificity: It has reducing ability against ketones and
aldehydes but is very low in reducing activity against
25 carbocyclic ketones and the α -position keto group of α -keto
acids;
(3) Molecular weight: It shows a molecular weight of about
60,000 in gel filtration analysis and a molecular weight of
about 29,000 in SDS polyacrylamide electrophoresis.

30

13. The enzyme according to Claim 12
which has the following physical and chemical properties
(4) to (6):
(4) Optimal temperature: 50 °C to 55 °C;
35 (5) Optimal pH: 5.0 to 6.0;

(6) Inhibitor: It is inhibited by the mercury ion.

14. An enzyme specified below under (a) or (b):

(a) An enzyme comprising an amino acid sequence shown under
5 SEQ ID NO:1 in the sequence listing;

(b) An enzyme comprising an amino acid sequence derived from
the amino acid sequence shown under SEQ ID NO:1 in the sequence
listing by deletion, substitution and/or addition of one or
several amino acids and having an activity by which 5-
10 acetylfuro[2,3-c]pyridine is stereoselectively reduced to
5-(1-(R)-hydroxyethyl)furo[2,3-c]pyridine.

15 15. The enzyme according to any of Claims 12 to 14
which is derived from a microorganism belonging to the
genus Candida.

16. The enzyme according to any of Claims 12 to 14
which is derived from Candida maris.

20 17. The enzyme according to any of Claims 12 to 14
which is derived from Candida maris IFO 10003.

18. The production method according to any of Claims 1
to 8,

25 wherein said enzyme is defined according to any of Claims
12 to 17

and the product optically active pyridineethanol
derivative has the R absolute configuration.

30 19. A DNA coding for the enzyme according to any of Claims
14 to 17.

20. A DNA comprising a base sequence shown under SEQ ID
NO:2 in the sequence listing.

21. A recombinant vector containing the DNA according to Claim 19 or 20.

5 22. The recombinant vector according to Claim 21 which is pNTFP.

23. The recombinant vector according to Claim 21 which comprises a DNA coding for glucose dehydrogenase.

10 24. The recombinant vector according to Claim 23, wherein said glucose dehydrogenase is derived from Bacillus megaterium.

15 25. The recombinant vector according to Claim 24 which is pNTFPG.

26. A transformant having the recombinant vector according to any of Claims 21 to 25.

20 27. The transformant according to Claim 26, wherein the host is Escherichia coli.

25 28. The transformant according to Claim 27 which is Escherichia coli HB101 (pNTFP).

29. The transformant according to Claim 27 which is Escherichia coli HB101 (pNTFPG).

30 30. A transformant having a first recombinant vector containing the DNA according to Claim 19 or 20 and a second recombinant vector containing a DNA coding for glucose dehydrogenase.

35 31. The transformant according to Claim 30, wherein said first recombinant vector is pNTFP

and said glucose dehydrogenase is derived from Bacillus megaterium.

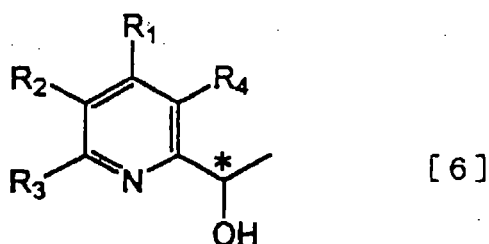
32. The transformant according to Claim 30 or 31,
5 wherein the host is Escherichia coli.

33. The production method according to any of Claims 1
to 5,

wherein said enzyme is the transformant according to any
10 of Claims 26 to 32

and said product optically active pyridineethanol
derivative has the R absolute configuration.

34. A method of producing an optically active
15 pyridineethanol derivative having the S absolute configuration
and represented by the general formula [6]:

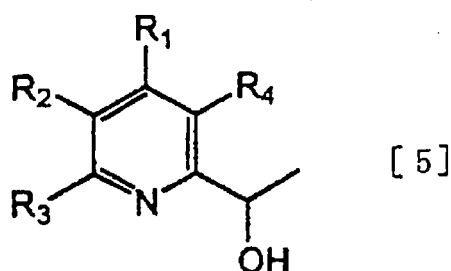


wherein R₁ and R₂ are bound to each other to form a 5- to 8-membered
20 monocyclic heterocycle containing at least one hetero atom
selected from the group consisting of oxygen, sulfur and
nitrogen atoms, which heterocycle may optionally have a
substituent(s), or a polycyclic heterocycle resulting from the
condensation of such monocyclic heterocycle with another ring,
25 which polycyclic heterocycle may optionally have a
substituent(s),

and R₃ and R₄ are the same or different and each represents a
hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group
containing 1 to 12 carbon atoms, which may optionally have a
30 substituent(s), or an alkoxy group containing 1 to 12 carbon

atoms, which may optionally have a substituent(s),
and * indicates that the asterisked carbon atom is an asymmetric
one,

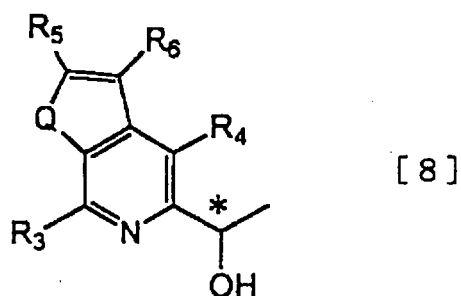
- which method comprises causing the enzyme according to
5 any of Claims 12 to 17 and/or the transformant according to any
of Claims 26 to 32 to act on a pyridineethanol derivative
represented by the general formula [5]:



- 10 wherein R_1 , R_2 , R_3 and R_4 are as defined above,
to thereby preferentially oxidize the R form of the
pyridineethanol derivative
and recovering the remaining S form of the
pyridineethanol derivative.

15

35. A method of producing an optically active
pyridineethanol derivative having the S absolute configuration
and represented by the general formula [8]:

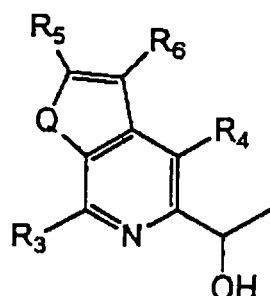


- 20 wherein Q represents an oxygen or sulfur atom or a group of the
general formula $-N(D)-$, in which N is a nitrogen atom and D
represents a hydrogen atom or a monovalent protective group,
 R_3 , R_4 , R_5 and R_6 are the same or different and each represents

a hydrogen atom, a halogen atom, a hydroxyl group, an alkyl group containing 1 to 12 carbon atoms, which may optionally have a substituent(s), or an alkoxy group containing 1 to 12 carbon atoms, which may optionally have a substituent(s),

5 and * indicates that the asterisked carbon atom is an asymmetric one,

which method comprises causing the enzyme according to any of Claims 12 to 17 and/or the transformant according to any of Claims 26 to 32 to act on a pyridineethanol derivative
10 represented by the general formula [7]:



[7]

wherein Q, R₃, R₄, R₅ and R₆ are as defined above,

to thereby preferentially oxidize the R form of the
15 pyridineethanol derivative

and recovering the remaining S form of the pyridineethanol derivative.

20 36. The production method according to Claim 35, wherein Q is an oxygen atom.

37. The production method according to Claim 35, wherein Q is an oxygen atom,

R₃ is a hydrogen atom or a chlorine atom,

25 R₄ is a hydrogen atom,

R₅ is a hydrogen atom

and R₆ is a hydrogen atom.