

DESCRIPTION

METHOD FOR PRODUCING *cis*-5-HYDROXY-2-PIPERIDINECARBOXYLIC
ACID DERIVATIVE, AND METHOD FOR PURIFYING *cis*-5-HYDROXY-2-
5 PIPERIDINECARBOXYLIC ACID

TECHNICAL FIELD

[0001]

The present invention relates to an industrial method for producing a
10 derivative of *cis*-5-hydroxy-2-piperidinecarboxylic acid (which is also called *cis*-5-hydroxypipicolic acid). The present invention also relates to a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid.

BACKGROUND ART

15 [0002]

cis-5-Hydroxy-2-piperidinecarboxylic acid is a useful intermediate for
pharmaceuticals. However, since this compound has 2 asymmetric carbons, 4 kinds
of isomers are present, and selective synthesis of a single kind of isomer, or single
kind of diastereomer, is very difficult. Thus, improvement of the purity by
20 separation of unnecessary isomers, or purification, by chemical conversion is
necessary.

[0003]

For example, methods for obtaining *cis*-5-hydroxy-2-piperidinecarboxylic
acid by introduction of a hydroxyl group to 2-piperidinecarboxylic acid by
25 hydroxylation reaction using a microorganism or enzyme have been reported.
However, it has been reported that a compound having a substituted hydroxyl group
at another position, such as a 3-position-hydroxylated compound, may be produced

as a by-product in addition to the compound of interest, *cis*-5-hydroxy-2-piperidinecarboxylic acid (Non-patent Document 1). Separation of the by-product is not described in the document. A method in which 5-hydroxy-2-piperidinecarboxylic acid is synthesized from 5-hydroxylysine by enzymatic reaction has also been reported (Patent Document 1), but the document does not describe separation of the generated *cis/trans* isomers. In a report describing synthesis of 5-hydroxy-2-piperidinecarboxylic acid from 5-hydroxylysine by a method similar to the method of Patent Document 1 (Patent Document 2), isomers are separated using an ion-exchange column. Since this method requires use of excessive amounts of a filler and eluent with respect to the substrate, the method is not realistic from the viewpoint of industrial production.

Thus, there is no known method by which a necessary stereoisomer can be selectively obtained with high purity from *cis*-5-hydroxy-2-piperidinecarboxylic acid synthesized using a microorganism or enzyme.

[0004]

There are known chemical synthesis methods using, as a material, L-amino acid, in which the stereochemistry of one of the two asymmetric carbons, the 2-position carbon, can be fixed. For example, a method using L-pyroglutamic acid as a material (Patent Document 3) has been reported. This method requires use of an iridium catalyst, which is expensive, for formation of a piperidine ring, which is problematic. Methods using L-glutamic acid as a material (Non-patent Documents 2 and 3) and a method using a proline derivative as a material (Non-patent Document 4) have also been reported, but all of these methods require use of a diazo compound, which is highly risky, and also require a multistep complex process. Moreover, for separation of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid, the reaction is carried out via 5-oxo-2-piperidinecarboxylic acid to preferentially obtain the (2*S*,5*S*) compound by its reduction (Non-patent Document 4). This requires separation

using a silica gel column for removal of impurities such as isomers. Because of the load of this process, this method is not industrially satisfactory.

[0005]

It is also known that (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-

5 piperidinecarboxylic acid and its esters are lactonized to give benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (Non-patent Documents 4 and 5).

These documents on lactonization do not mention about impurities such as isomers, and the effects of purification by these methods have been unclear. Non-patent

Document 4 describes that benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-

10 carboxylate was obtained as an oily substance after purification with a silica gel column.

Non-patent Document 5 describes that crystallization of benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate occurs, but, since the

lactonization is carried out using *cis*-5-hydroxy-2-piperidinecarboxylic acid as a material, behavior of impurities such as isomers and the effect of purification are not

15 clear. There is a report describing separation of a *cis*/*trans* mixture of methyl *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate by acid treatment (Non-patent Document 3), but, since the compound was a carboxylic acid ester, it was impossible to separate lactone obtained from the *cis* isomer from the ester of the *trans* isomer by a simple method such as solvent extraction. Moreover, Non-patent Document 3

20 does not describe the yield in this process. When the present inventors studied this method, the yield of the lactone was low because of occurrence of a remarkable side reaction between lactone obtained from the *cis* isomer (benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate)) and the residual *trans* isomer ((2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid ester).

25 As described above, there are problems to be solved for industrial production of highly pure *cis*-5-hydroxy-2-piperidinecarboxylic acid by an inexpensive method.

PRIOR ART DOCUMENTS

[Patent Documents]

[0006]

Patent Document 1: JP 4590981 B

5 Patent Document 2: JP 2010-88395 A

Patent Document 3: WO 2010/126820

[Non-patent Documents]

[0007]

Non-patent Document 1: Adv. Synth. Catal., 2011, 353, 1375.

10 Non-patent Document 2: Chem. Commun., 1996, 349.

Non-patent Document 3: Tetrahedron Lett., 1988, 29, 2231.

Non-patent Document 4: Tetrahedron: Asym., 2006, 17, 2479.

Non-patent Document 5: Rec. Trav. Chim. Pays-Bas, 1959, 78, 648.

15 SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0008]

The present invention aims to provide a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid, which is a useful intermediate for pharmaceuticals, and a

20 method for producing its derivative.

MEANS FOR SOLVING THE PROBLEMS

[0009]

The present inventors intensively studied to solve the problems described

25 above. As a result, the present inventors solved the problems by a process of reacting low-purity *cis*-5-hydroxy-2-piperidinecarboxylic acid containing impurities with an acid halide and/or acid anhydride to induce a *cis*-5-hydroxy-2-

piperidinecarboxylic acid derivative, thereby solving the problems.

In a more specific mode, the present inventors discovered that impurities such as isomers can be separated by reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride to convert the *cis*-5-hydroxy-2-
5 piperidinecarboxylic acid into a lactone whose amino group is protected, and then performing crystallization and/or solvent extraction. That is, studies by the present inventors revealed that the *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative in the present invention can be selectively converted to lactone by reaction with an acid halide and/or acid anhydride, and that this lactone can be crystallized under specific
10 conditions. The present inventors then developed a method in which the lactone is crystallized to achieve removal of impurities such as isomers having low crystallizability.

In another specific mode, the present inventors discovered that impurities such as isomers having a carboxyl group can be removed by solvent extraction after
15 reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride, and then with an alcohol in the presence of an acid catalyst to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into an ester whose amino group is protected. That is, the present inventors studied to develop a method in which, in the presence of an acid catalyst, a *cis*-5-hydroxy-2-piperidinecarboxylic acid
20 derivative is selectively converted into lactone, and the lactone is reacted with an alcohol present in the reaction system to efficiently and selectively convert the *cis* isomer into an ester while suppressing a side reaction, followed by removing residual impurities such as isomers having a carboxyl group by solvent extraction.

The present inventors then discovered that highly pure *cis*-5-hydroxy-2-
25 piperidinecarboxylic acid can be obtained by hydrolyzing the resulting lactone and/or ester to give *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid, and then removing the protective group for the amino group, thereby completing the present

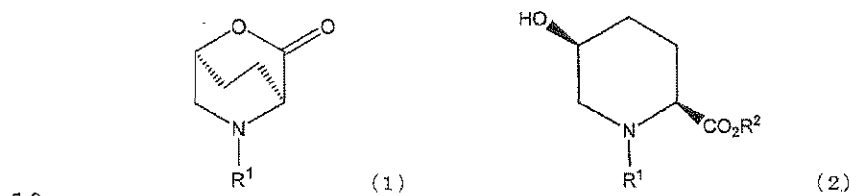
invention.

[0010]

That is, the present invention can be summarized as follows.

[1] A method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid
 5 derivative, the method comprising a step of converting *cis*-5-hydroxy-2-
 piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2)
 (wherein R¹ represents a protective group for an amino group, and R² represents a
 C₁-C₆ alkyl group).

[0011]



[0012]

[2] The method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid
 derivative according to [1], comprising a step of reacting *cis*-5-hydroxy-2-
 piperidinecarboxylic acid with an acid halide and/or acid anhydride to convert the
 15 *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (1).

[3] The method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid
 derivative according to [1], comprising a step of reacting *cis*-5-hydroxy-2-
 piperidinecarboxylic acid with an acid halide and/or acid anhydride, and then with an
 alcohol in the presence of an acid catalyst, to convert the *cis*-5-hydroxy-2-
 20 piperidinecarboxylic acid into a compound of Formula (2).

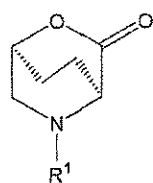
[4] A method for regenerating *cis*-5-hydroxy-2-piperidinecarboxylic acid,
 the method comprising the steps of:

converting *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of
 Formula (1) and/or Formula (2) (wherein R¹ represents a protective group for an

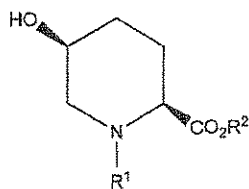
amino group, and R^2 represents a C_1 - C_6 alkyl group); and

converting the compound(s) of Formula (1) and/or Formula (2) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.

[0013]



(1)



(2)

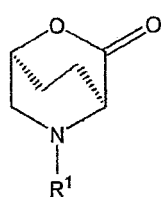
[0014]

[5] The method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative according to any one of [1] to [3], wherein the *cis*-5-hydroxy-2-piperidinecarboxylic acid is *cis*-5-hydroxy-2-piperidinecarboxylic acid synthesized by bacterial reaction and/or enzymatic reaction.

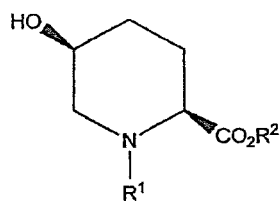
[6] The method for regenerating *cis*-5-hydroxy-2-piperidinecarboxylic acid according to [4], wherein the *cis*-5-hydroxy-2-piperidinecarboxylic acid is *cis*-5-hydroxy-2-piperidinecarboxylic acid synthesized by bacterial reaction and/or enzymatic reaction.

[7] A method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid, the method comprising a step of reacting a mixture containing *cis*-5-hydroxy-2-piperidinecarboxylic acid and an impurity with an acid halide and/or acid anhydride, or with an acid halide and/or acid anhydride and an alcohol, to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2), separating the compound(s) and then converting the separated compound(s) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.

[0015]



(1)



(2)

[0016]

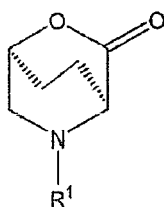
[8] The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to [7], wherein the impurity is 2-piperidinecarboxylic acid or an analogue thereof.

[9] The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to [8], wherein the 2-piperidinecarboxylic acid or an analogue thereof is *trans*-5-hydroxy-2-piperidinecarboxylic acid.

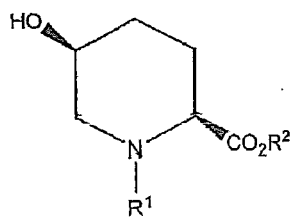
[10] The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to any one of [7] to [9], wherein the step of separating the compound(s) of Formula (1) and/or Formula (2) is carried out by crystallization or solvent extraction.

[11] The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to any one of [7] to [10], wherein the mixture containing *cis*-5-hydroxy-2-piperidinecarboxylic acid and an impurity is a mixture synthesized by bacterial reaction and/or enzymatic reaction.

[0016a] In one aspect, there is provided a method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative, (wherein R¹ represents a protective group for an amino group, and R² represents a C₁-C₆ alkyl group).



(1)



(2)

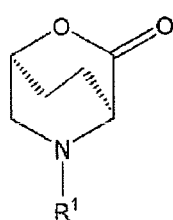
comprising a step of reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride to convert said *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (1); or

comprising a step of reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride, and then with an alcohol in the presence of an acid catalyst, to convert said *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (2).

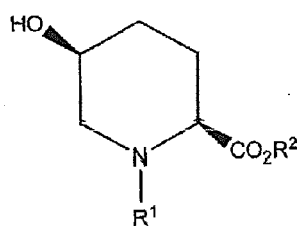
[0016b] In another aspect, there is provided a method for regenerating *cis*-5-hydroxy-2-piperidinecarboxylic acid, said method comprising the steps of:

converting *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2) according to the method described herein (wherein R^1 represents a protective group for an amino group, and R^2 represents a C_1 - C_6 alkyl group); and

converting the compound(s) of Formula (1) and/or Formula (2) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.



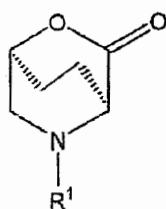
(1)



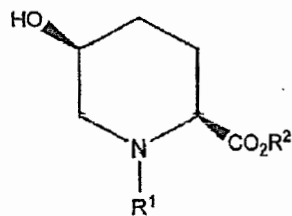
(2)

[0016c] In another aspect, there is provided a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid, said method comprising a step of reacting a mixture containing *cis*-5-hydroxy-2-piperidinecarboxylic acid and an impurity with an acid halide and/or acid anhydride, or with an acid halide and/or acid anhydride and an alcohol, to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s)

of Formula (1) and/or Formula (2), separating said compound(s) and then converting the separated compound(s) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.



(1)



(2)

5 EFFECT OF THE INVENTION

[0017]

By the method of the present invention, highly pure *cis*-5-hydroxy-2-piperidinecarboxylic acid and its derivatives can be provided.

10 BRIEF DESCRIPTION OF THE DRAWINGS

[0018]

Fig. 1 is a schematic diagram illustrating routes for purifying *cis*-5-hydroxy-2-

piperidinecarboxylic acid from *cis*-5-hydroxy-2-piperidinecarboxylic acid that may contain an impurity(s). The route 1 is a route in which *cis*-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid halide and/or acid anhydride to give a compound of Formula (1) directly or via *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid, and the compound is then converted into *cis*-5-hydroxy-2-piperidinecarboxylic acid. The route 2 is a route in which *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid catalyst to give a compound of Formula (1), and the compound is then converted into *cis*-5-hydroxy-2-piperidinecarboxylic acid by hydrolysis. The route 3 is a route in which *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid is reacted with an alcohol in the presence of an acid catalyst to give a compound of Formula (2), and the compound is then converted to *cis*-5-hydroxy-2-piperidinecarboxylic acid by hydrolysis.

Fig. 2. is a diagram showing the result of HPLC analysis of hydroxylysines obtained with recombinant lysine hydroxylases.

Fig. 3 is a diagram showing the result of HPLC analysis of hydroxylysines obtained with recombinant lysine hydroxylases.

DESCRIPTION OF THE EMBODIMENTS

[0019]

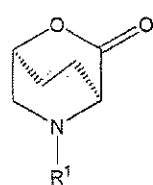
The present invention is described below in detail.

In the present invention, examples of the *cis*-5-hydroxy-2-piperidinecarboxylic acid include (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid, (2*R*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid, and mixtures thereof. The *cis*-5-hydroxy-2-piperidinecarboxylic acid may be a racemic compound. The *cis*-5-hydroxy-2-piperidinecarboxylic acid may be forming a salt with an acid or base.

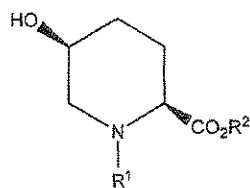
[0020]

The method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid

derivative of the present invention comprises a step of converting *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2).



(1)



(2)

[0021]

5 In Formula (1) and Formula (2), R¹ represents a protective group for an amino group, and specific examples of the protective group include the following groups. However, the protective group is not limited to these examples.

10 Examples of the protective group for the amino group include acyl groups such as formyl, acetyl, chloroacetyl, dichloroacetyl, trichloroacetyl, trifluoroacetyl, propionyl, benzoyl, and 4-chlorobenzoyl; alkoxycarbonyl groups such as methoxycarbonyl, ethoxycarbonyl, *tert*-butoxycarbonyl, benzyloxycarbonyl, and allyloxycarbonyl; arylalkyl groups such as benzyl, 4-methoxybenzyl, 4-bromobenzyl, and 1-phenethyl; and sulfonyl groups such as methanesulfonyl, *p*-toluenesulfonyl, and 2-nitrobenzenesulfonyl.

15 [0022]

Among these, acyl groups and alkoxycarbonyl groups are preferred since these groups can be easily removed; acetyl, chloroacetyl, trifluoroacetyl, benzoyl, *tert*-butoxycarbonyl, and benzyloxycarbonyl are more preferred; and acetyl, *tert*-butoxycarbonyl, and benzyloxycarbonyl are still more preferred since these are 20 industrially inexpensive. The protective group is especially preferably benzyloxycarbonyl since benzyloxycarbonyl can be removed by hydrogenation without leaving a non-volatile component, and the load of purification of *cis*-5-hydroxy-2-piperidinecarboxylic acid can therefore be reduced.

[0023]

In Formula (2), R² represents a C₁-C₆ alkyl, and specific examples of R² include primary alkyl groups such as methyl, ethyl, propyl, butyl, pentyl, and hexyl; secondary alkyl groups such as isopropyl, isobutyl, isopentyl, cyclopentyl, and cyclohexyl; and tertiary alkyl groups such as *tert*-butyl. Among these, methyl, ethyl, isopropyl, butyl, and *tert*-butyl are preferred, and ethyl and isopropyl are more preferred.

[0024]

In the present invention, the *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative may be the compound(s) of Formula (1) and/or Formula (2) itself/themselves.

Specific examples of the compound of Formula (1) include benzyl 5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate, *tert*-butyl 5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate, and 5-acetyl-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane.

Specific examples of the compound of Formula (2) include methyl *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate, ethyl *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate, isopropyl *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate, methyl *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate, ethyl *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate, and isopropyl *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate.

[0025]

The *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative may be a compound obtained by chemical conversion of a compound(s) of Formula (1) and/or Formula (2).

Specific examples of such a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative include carboxylic acids such as *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-

piperidinecarboxylic acid and *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylic acid; esters such as benzyl *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate and benzyl *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylate; carboxylic acid amides such as *cis-N*-(benzyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylic acid amide, *cis-N*-(*tert*-butyloxycarbonyl)-5-hydroxy-2-piperidinecarboxylic acid amide, benzyl *cis*-5-hydroxy-2-(methylcarbamoyl)piperidine-1-carboxylate, *tert*-butyl *cis*-5-hydroxy-2-(methylcarbamoyl)piperidine-1-carboxylate, benzyl *cis*-2-(1-*tert*-butyloxycarbamoylpyrrolidine-3-ylcarbamoyl)-5-hydroxypiperidine-1-carboxylate, *tert*-butyl *cis*-2-(1-benzyloxycarbamoylpyrrolidine-3-ylcarbamoyl)-5-hydroxypiperidine-1-carboxylate, benzyl *cis*-2-(1-*tert*-butyloxycarbamoylpiperidine-4-ylcarbamoyl)-5-hydroxypiperidine-1-carboxylate, and *tert*-butyl *cis*-2-(1-benzyloxycarbamoylpiperidine-4-ylcarbamoyl)-5-hydroxypiperidine-1-carboxylate.

[0026]

In one mode of the method of the present invention, *cis*-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid halide and/or acid anhydride to protect the amino group of the *cis*-5-hydroxy-2-piperidinecarboxylic acid, and subsequent lactonization of the resultant causes its conversion into a compound of Formula (1).

[0027]

The acid halide and/or acid anhydride is/are not limited as long as the acid halide and/or acid anhydride can protect the amino group and allow(s) the lactonization. Examples of the acid halide include acid chlorides and acid bromides. In view of ease of handling, acid chlorides are preferred. Examples of the acid anhydride include carboxylic acid anhydrides, and sulfonic acid anhydrides. In view of ease of handling and the cost, carboxylic acid anhydrides are preferred.

Specific examples of the acid halide and/or acid anhydride include acylating

agents such as formic acid-acetic anhydride, acetic anhydride, acetyl chloride, chloroacetyl chloride, dichloroacetyl chloride, trichloroacetyl chloride, trifluoroacetic anhydride, propionyl chloride, benzoyl chloride, 4-chlorobenzoyl chloride, acetyl bromide, propionyl bromide, and benzoyl bromide; alkoxycarbonylating agents such as di-*tert*-butyldicarbonate, benzyloxycarbonyl chloride, allyloxycarbonyl chloride, benzyloxycarbonyl bromide, and allyloxycarbonyl bromide; and sulfonylating agents such as methanesulfonyl chloride, *p*-toluenesulfonyl chloride, 2-nitrobenzenesulfonyl chloride, methanesulfonyl bromide, *p*-toluenesulfonyl bromide, and 2-nitrobenzenesulfonyl bromide.

10 Among these, acylating agents and alkoxycarbonylating agents are preferred. Acetic anhydride, chloroacetyl chloride, trichloroacetyl chloride, trifluoroacetic anhydride, di-*tert*-butyldicarbonate, benzyloxycarbonyl chloride, and allyloxycarbonyl chloride are more preferred since these can be easily removed after the protection of the amino group. Acetic anhydride, di-*tert*-butyldicarbonate, and benzyloxycarbonyl chloride are still more preferred since these are industrially inexpensive. Benzyloxycarbonyl chloride is especially preferred since it can be removed by hydrogenation without leaving a non-volatile component, and the load of purification of *cis*-5-hydroxy-2-piperidinecarboxylic acid can therefore be reduced. [0028]

20 In the present invention, two or more kinds of acid halide and/or acid anhydride may be used. In cases where two or more kinds of acid halide and/or acid anhydride are used, these may be added to the reaction system at once, but it is preferred to use different kinds of acid halide and/or acid anhydride for in each the process of amino group protection and the process of lactonization. For example, it is preferred to carry out a method in which benzyloxycarbonyl chloride is used for the protection of the amino group, and acetic anhydride is used for the lactonization. In cases where the protection of the amino group and the lactonization are carried out

in a single reactor, a single kind of acid halide and/or acid anhydride is preferably used for suppression of by-products having different protective groups. In cases where the protection of the amino group and the lactonization are carried out in separate reactors, two or more kinds of acid halide and/or acid anhydride are preferably used in view of optimization of the reactions and cost reduction. In particular, the protection of the amino group and the lactonization are preferably carried out in a single reactor since the number of reactors required for the production can be decreased, and the cost can therefore be reduced.

[0029]

The amount of the acid halide and/or acid anhydride to be used is usually 1 to 10 molar equivalents, preferably 1.2 to 5 molar equivalents, more preferably 1.5 to 3 molar equivalents relative to the total amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid and the amine compound to be protected. The acid halide and/or acid anhydride may be added dividedly in a plurality of times.

[0030]

In the reaction with the acid halide and/or acid anhydride, a base may be used, if necessary. Specific examples of the base include, but are not limited to, tertiary amines such as triethylamine, diisopropylethylamine, *N*-methylmorpholine, quinuclidine, and 1,4-diazabicyclo[2.2.2]octane; pyridines such as pyridine, 4-dimethylaminopyridine, and 2,6-lutidine; organic strong bases such as 1,8-diazabicyclo[5.4.0]undeca-7-ene and tetramethylguanidine; metal amides such as lithium diisopropylamide and sodium hexamethyldisilazide; alkyl metals such as *n*-butyllithium, *sec*-butyllithium, *tert*-butyllithium, and isopropylmagnesium bromide; metal hydrides such as sodium hydride and calcium hydride; metal alkoxides such as sodium methoxide, sodium ethoxide, sodium *tert*-butoxide, and potassium *tert*-butoxide; carbonates such as sodium hydrogen carbonate and potassium carbonate; phosphates such as potassium phosphate and sodium hydrogen phosphate;

hydroxides such as sodium hydroxide, potassium hydroxide, lithium hydroxide, and calcium hydroxide; and cyanides such as sodium cyanide and potassium cyanide.

Preferred bases vary depending on the protective reagent to be used. In cases where a preferred protective reagent, that is, acetic anhydride, di-*tert*-butyldicarbonate, or benzyloxycarbonyl chloride, is used, the base is preferably tertiary amines, pyridines, carbonates, or hydroxides. In cases where the especially preferred protective reagent, that is, benzyloxycarbonyl chloride, is used, the base is preferably a hydroxide, which is inexpensive.

In cases where a base is used, the amount of base is preferably added such that the pH of the reaction mixture becomes 7 to 12. In cases where water is used as the solvent, the pH of the reaction mixture herein means the pH of the layer containing water. In cases where a solvent other than water is used, the pH of the reaction mixture herein means the pH of the layer which contains water when an equal volume of water is added to the reaction mixture. In cases where water is used as the solvent, hydrolysis of the compound of Formula (1) may occur when the basicity of the reaction mixture is too strong. The pH is therefore more preferably 7 to 12, especially preferably 7 to 11.

[0031]

Examples of the reaction solvent include water; esters such as ethyl acetate and butyl acetate; alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-ethyl-1-hexanol, and 2-butanol; ethers such as diethyl ether, di-*n*-butyl ether, diisopropyl ether, di-*n*-butyl ether, *tert*-butyl methyl ether, tetrahydrofuran, and dioxane; ketones such as acetone, methyl ethyl ketone, and methyl isobutyl ketone; nitriles such as acetonitrile; amides such as dimethylformamide, dimethylacetamide, and *N*-methylpyrrolidinone; sulfones such as dimethylsulfoxide and sulfolane; hydrocarbons such as hexane, heptane, and toluene; and mixed solvents of two or more of these. Among these, water, and mixed solvents containing water and one

or more of the above solvents, are preferred. In particular, in cases where the *cis*-5-hydroxy-2-piperidinecarboxylic acid is synthesized by bacterial reaction and/or enzymatic reaction, the *cis*-5-hydroxy-2-piperidinecarboxylic acid is obtained as a solution whose solvent is mainly water. Therefore, in view of simplicity of the production process, it is preferred to use the solution as it is, or to concentrate part of the solution, for carrying out the reaction using water as a main solvent.

[0032]

The reaction temperature is usually -20°C to 100°C, preferably -10°C to 50°C. Since reaction at high temperature may cause decomposition of reagents and products, the reaction temperature is more preferably 0°C to 30°C.

[0033]

The protection of the amino group and the lactonization are preferably carried out by adding an acid halide and/or acid anhydride (the route 1 in Fig. 1). It is especially preferred to carry out the protection of the amino group and the lactonization using a single acid halide and/or acid anhydride in a single reactor in view of simplicity of the operation (the upper formula in the route 1 in Fig. 1).

[0034]

The protection of the amino group and the lactonization may also be carried out under different conditions (the route 2 in Fig. 1). That is, the amino group may be protected by addition of the acid halide and/or acid anhydride, and then an acid catalyst may be used to perform the lactonization. In cases where the protection of the amino group and the lactonization are carried out under different reaction conditions, it is preferred to suppress production of the compound of Formula (1) at the stage of protection of the amino group. The protection of the amino group can be selectively and effectively carried out by using the acid halide and/or acid anhydride in the same molar amount as that of the amino group, or by adding the acid halide and/or acid anhydride dividedly such that excessive presence of the acid halide

and/or acid anhydride relative to the amino group is avoided, and then stopping the reaction when the material *cis*-5-hydroxy-2-piperidinecarboxylic acid has almost disappeared. In cases where the protection of the amino group is carried out in a solvent containing water, the compound of Formula (1) generated by lactonization, if any, can be hydrolyzed by a method such as making the pH of the reaction mixture strongly basic, increasing the temperature of the reaction mixture to a temperature of not less than room temperature, performing the reaction for long time, or a combination of one or more of these methods, and, as a result, the amino-protected compound can be obtained. By subsequently reacting the amino-protected compound with an acid catalyst, the compound of Formula (1) can be obtained.

[0035]

The compound of Formula (1) produced can be isolated by a method such as the crystallization described below.

[0036]

In another mode of the method of the present invention, *cis*-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid halide and/or acid anhydride, and then with an alcohol in the presence of an acid catalyst, to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (2) (the route 3 in Fig. 1). By the reaction with an alcohol in the presence of an acid catalyst, the compound of Formula (1) produced by the reaction with an acid halide and/or acid anhydride is converted to the compound of Formula (2). In this process, in cases where compounds having a carboxyl group other than the compound of Formula (1), especially the 2-piperidinecarboxylic acid or an analogue thereof described later, are contained as impurities, these compounds are not converted to lactone like the compound of Formula (1). Therefore, the compound derived from *cis*-5-hydroxy-2-piperidinecarboxylic acid can be selectively converted to the compound of Formula (2), which is an ester, while the other compounds are left in the reaction system as

carboxylic acids. Thereafter, by extraction with a basic aqueous solution, the compound of Formula (2) can be separated into the organic layer, while the impurities other than the compound of Formula (2) can be separated into the aqueous layer.

5 [0037]

The alcohol used herein is not limited as long as it is a C₁-C₆ alcohol. Examples of the alcohol include primary alcohols such as methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol; secondary alcohols such as 2-propanol, 2-butanol, cyclopentanol, and cyclohexanol; and tertiary alcohols such as
10 *tert*-butanol. Among these, secondary alcohols are preferred, and 2-propanol is more preferred since it is inexpensive, has a low boiling point, and can be easily removed. In cases where a primary alcohol is used, a side reaction may occur to cause direct conversion of a carboxylic acid into its ester in the presence of an acid catalyst, resulting in low effect of removal of impurities. In cases where a tertiary
15 alcohol is used, reactivity with the compound of Formula (1) is low. Therefore, the compound of Formula (2), which is a secondary alcohol, may react with the compound of Formula (1) to partially form a dimer, resulting in low yield of the compound of Formula (2). Thus, in the present invention, a secondary alcohol is preferably used.

20 An ester of the above-described alcohol may be added in the presence of an acid catalyst and water so that hydrolysis reaction of the ester allows the alcohol to be present in the reaction system, and the alcohol may be reacted with the *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid in the presence of an acid catalyst.

The alcohol, or the ester which generates the alcohol, may be used in an
25 excess amount, and the molar amount of the alcohol or ester to be used is usually 1 to 500 times the molar amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid. In cases where the amount of the alcohol or ester is too small, a side reaction in which

the compound of Formula (2) reacts with the compound of Formula (1) may occur to form a dimer, while in cases where the amount of the alcohol or ester is too large, the effect of the acid catalyst decreases. Thus, the molar amount of the alcohol or ester is preferably 2 to 100 times, more preferably 3 to 50 times, especially preferably 5 to 20 times the molar amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid

[0038]

Examples of the acid catalyst include inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid, and polyphosphoric acid; sulfonic acids such as *p*-toluenesulfonic acid, methanesulfonic acid, and trifluoromethanesulfonic acid; and carboxylic acids such as acetic acid, formic acid, trifluoroacetic acid, trichloroacetic acid, benzoic acid, and oxalic acid. Among these, sulfonic acids are preferred since they have high solubility in organic solvents and sufficiently strong acidity for allowing the reaction to proceed. *p*-Toluenesulfonic acid and methanesulfonic acid are more preferred since they are industrially inexpensive.

The molar amount of the acid catalyst to be added is usually 0.001 to 10 times the molar amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid. In cases where the molar amount of the acid catalyst is too small, the reaction proceeds slowly, while the molar amount of the acid catalyst is too large, the load of the post treatment is large. The molar amount of the acid catalyst is therefore preferably 0.01 to 5 times, more preferably 0.02 to 1 times, especially preferably 0.05 to 0.5 times the molar amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid.

[0039]

Examples of the reaction solvent include organic acids such as formic acid and acetic acid; esters such as ethyl acetate and butyl acetate; alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-ethyl-1-hexanol, and 2-butanol; ethers such as diethyl ether, di-*n*-butyl ether, diisopropyl ether, di-*n*-butyl

ether, *tert*-butyl methyl ether, tetrahydrofuran, and dioxane; ketones such as acetone, methyl ethyl ketone, and methyl isobutyl ketone; nitriles such as acetonitrile; amides such as dimethylformamide, dimethylacetamide, and *N*-methylpyrrolidinone; sulfones such as dimethylsulfoxide and sulfolane; hydrocarbons such as hexane, heptane, and toluene; and mixed solvents of two or more of these. Among these, solvents which do not show coordination of a proton are preferred since they do not weaken the activity of the acid catalyst. More specifically, hydrocarbons are preferred. In addition, organic acids, which themselves are acids, and esters, which produce acids by hydrolysis, are preferred. Toluene is more preferred because of its low cost, high reaction rate, and high solubility of acid catalysts therein.

[0040]

The reaction temperature is usually 0°C to 150°C, preferably 20°C to 120°C. Reaction at higher temperature may cause a side reaction, but reaction at lower temperature is slow and takes a long time. The reaction temperature is therefore more preferably 40°C to 80°C.

[0041]

The compound of Formula (2) generated can be isolated by a method such as the extraction method using an organic solvent described later.

[0042]

The compounds of Formulae (1) and (2) can be converted to *cis-N*-protected-5-hydroxy-2-piperidinecarboxylic acid by treatment with a base.

[0043]

Examples of the base to be used herein include hydroxides such as sodium hydroxide, potassium hydroxide, lithium hydroxide, and calcium hydroxide; phosphates such as sodium phosphate, potassium phosphate, and calcium phosphate; carbonates such as lithium carbonate, sodium carbonate, potassium carbonate, magnesium carbonate, and calcium carbonate; and hydrogen carbonates such as

sodium hydrogen carbonate and potassium hydrogen carbonate. The base is preferably a hydroxide.

The molar amount of the base to be used is usually 0.1 to 10 times, preferably 0.5 to 5 times, more preferably 0.9 to 3 times the total molar amount of the compounds of Formulae (1) and (2).

[0044]

By carrying out deprotection of the amino group of the *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid, *cis*-5-hydroxy-2-piperidinecarboxylic acid (*cis*-5-hydroxypipericolic acid) can be produced. In particular, in cases where R¹ in the Formulae (1) and (2) is a benzyloxycarbonyl group, deprotection of the amino group can be carried out by hydrogenation reaction. The hydrogenation reaction can be carried out by, for example, using a palladium carbon catalyst. By such a reaction, the *cis*-5-hydroxy-2-piperidinecarboxylic acid can be regenerated.

[0045]

The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid of the present invention is a method in which highly pure *cis*-5-hydroxy-2-piperidinecarboxylic acid is obtained by purification from *cis*-5-hydroxy-2-piperidinecarboxylic acid containing an impurity(s).

[0046]

Examples of the impurity include 2-piperidinecarboxylic acid or an analogue thereof, amino acids, peptides, sugars, and fatty acids. Since, among these, 2-piperidinecarboxylic acid or an analogue thereof, amino acids, and peptides have an amino group(s) and carboxyl group(s), these can be hardly separated from the desired *cis*-5-hydroxy-2-piperidinecarboxylic acid of interest by an ordinary purification method such as ion-exchange resin purification. In particular, since 2-piperidinecarboxylic acid or an analogue thereof has the same skeleton as *cis*-5-hydroxy-2-piperidinecarboxylic acid, its separation is very difficult, and

establishment of removal process is very important.

Examples of the 2-piperidinecarboxylic acid or an analogue thereof include isomers of *cis*-5-hydroxy-2-piperidinecarboxylic acid; and 2-piperidinecarboxylic acid and analogues thereof. Examples of the isomers of *cis*-5-hydroxy-2-
 5 piperidinecarboxylic acid include stereoisomers such as *trans* isomers, and structural isomers having hydroxyl groups at different positions. It should be noted that optical isomers of *cis*-5-hydroxy-2-piperidinecarboxylic acid are not included in the impurity of the present invention.

Examples of the *trans* isomers include *trans*-5-hydroxy-2-
 10 piperidinecarboxylic acid, that is, (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid, (2*R*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid, and mixtures of these. Examples of the structural isomers include *cis*-2-hydroxy-2-piperidinecarboxylic acid, *trans*-2-hydroxy-2-piperidinecarboxylic acid, *cis*-3-hydroxy-2-piperidinecarboxylic acid, *trans*-3-hydroxy-2-piperidinecarboxylic acid, and *trans*-4-hydroxy-2-
 15 piperidinecarboxylic acid. It should be noted that *cis*-4-hydroxy-2-piperidinecarboxylic acid is not included in the impurity of the present invention.

Examples of the 2-piperidinecarboxylic acid and analogues thereof include 2-piperidinecarboxylic acid and 1,2,3,4-tetrahydro-2-pyridinecarboxylic acid.

Examples of the amino acids include essential amino acids such as proline,
 20 lysine, and isoleucine, and unnatural amino acids such as 3-hydroxyproline, 4-hydroxyproline, and 5-hydroxylysine.

Examples of the peptides include dipeptides, oligopeptides, and proteins.

Examples of the sugars include glucose, gluconic acid, sodium gluconate, potassium gluconate, and calcium gluconate.

25 Examples of the fatty acids include C₂-C₄ short-chain fatty acids, C₅-C₁₂ medium-chain fatty acids, long-chain fatty acids having not less than 13 carbon atoms, and glycerol esters.

[0047]

A mixture containing *cis*-5-hydroxy-2-piperidinecarboxylic acid and an impurity(s) is reacted with an acid halide and/or acid anhydride, or with an acid halide and/or acid anhydride and an alcohol, to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2). Subsequently, the compound(s) is/are separated by crystallization, solvent extraction, and/or the like and then treated with a base to convert the compound(s) into *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid. By subsequently carrying out deprotection of the amino group, *cis*-5-hydroxy-2-piperidinecarboxylic acid which the impurity has been removed can be obtained.

That is, *cis*-5-hydroxy-2-piperidinecarboxylic acid can be separated from the impurity utilizing specific lactonization of the *cis*-5-hydroxy-2-piperidinecarboxylic acid. Even in cases where the impurity other than the above-described impurities is contained, the *cis*-5-hydroxy-2-piperidinecarboxylic acid can be separated also from the impurity.

In the present description, the crystallization includes not only ordinary crystallization by adding a poor solvent, acid, base, or the like to a solution, or azeotropically removing a good solvent such as water, to decrease solubility of the desired product and recover the product as crystals; but also recrystallization in which crude crystals that have once been obtained are dissolved in an appropriate solvent and then recrystallized. The crystallization may be promoted by addition of seed crystals to the solution.

In the crystallization of the compound of Formula (1), crystallization is preferably carried out for a crude product of the compound of Formula (1) obtained by the reaction with the acid halide and/or acid anhydride. This is because a small amount of acidic component(s) generated from the acid halide and/or acid anhydride, and/or other components derived from reagents, may remain to induce crystallization.

[0048]

Examples of the crystallization solvent for the crystallization treatment include water; organic acids such as acetic acid and propionic acid; esters such as ethyl acetate, isopropyl acetate, and butyl acetate; alcohols such as methanol, ethanol, 5 1-propanol, 2-propanol, 1-butanol, 2-ethyl-1-hexanol, and 2-butanol; ethers such as diethyl ether, di-*n*-butyl ether, diisopropyl ether, methyl-*tert*-butyl ether, di-*n*-butyl ether, tetrahydrofuran, and dioxane; ketones such as acetone, methyl ethyl ketone, and methyl isobutyl ketone; nitriles such as acetonitrile; aliphatic hydrocarbons such as hexane and heptane; aromatic hydrocarbons such as toluene and xylene; and 10 mixed solvents of two or more of these. Among these, alcohols, aliphatic hydrocarbons, aromatic hydrocarbons, and mixed solvents of two or more of these are preferred since solubility of the compound(s) of Formula (1) and/or Formula (2) in these solvents is sufficiently low. Aliphatic hydrocarbons, aromatic hydrocarbons, and mixed solvents of two or more of these are more preferred, and 15 hexane, heptane, toluene, and mixed solvents of two or more of these are especially preferred.

[0049]

Examples of the organic solvent to be used for the extraction include water-insoluble solvents, for example, esters such as ethyl acetate, isopropyl acetate, and 20 butyl acetate; ethers such as diethyl ether, methyl-*tert*-butyl ether, di-*n*-butyl ether, diisopropyl ether, di-*n*-butyl ether, *tert*-butyl methyl ether, and tetrahydrofuran; aliphatic hydrocarbons such as hexane and heptane; and aromatic hydrocarbons such as toluene and xylene.

[0050]

25 The above-described method is especially useful in cases where *cis*-5-hydroxy-2-piperidinecarboxylic acid is to be purified from a mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic

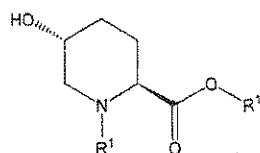
acid.

[0051]

That is, in a mode of the purification method of the present invention (the route 1 or route 2 in Fig. 1), a mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid halide and/or acid anhydride to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into the compound of Formula (1). The resulting compound is then separated by crystallization and/or extraction with an organic solvent, and then treated with a base to convert the compound into *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid. By subsequently carrying out deprotection of the amino group, *cis*-5-hydroxy-2-piperidinecarboxylic acid separated from the *trans* isomer can be obtained.

In such cases, the acid halide and/or acid anhydride is/are preferably used in an excessive amount over 1 molar equivalent relative to the total amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid and the *trans*-5-hydroxy-2-piperidinecarboxylic acid. The amount of the acid halide and/or acid anhydride is usually 1 to 10 molar equivalents, preferably 1.2 to 5 molar equivalents, more preferably 1.5 to 3 molar equivalents. In cases where both an acid halide and an acid anhydride are used, these are added such that their total amount exceeds 1 molar equivalent relative to the total amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid and the *trans*-5-hydroxy-2-piperidinecarboxylic acid.

By the reaction with the acid halide and/or acid anhydride, the *cis*-5-hydroxy-2-piperidinecarboxylic acid is converted to the compound of Formula (1). On the other hand, the *trans*-5-hydroxy-2-piperidinecarboxylic acid remains in the reaction system without being converted, or is converted to the mixed acid anhydride of *trans*-5-hydroxy-2-piperidinecarboxylic acid described below. This mixed acid anhydride can be converted into the *trans*-5-hydroxy-2-piperidinecarboxylic acid again by post treatment. Thus, its separation by crystallization reaction is possible.

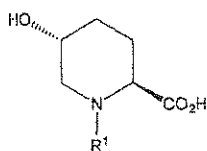


[0052]

In another mode of the purification method of the present invention (the route 3 in Fig. 1), a mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid is reacted with an acid halide and/or acid anhydride to protect the amino group. Subsequently, the *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid generated is reacted with an alcohol (or an ester that generates an alcohol) in the presence of an acid catalyst to allow conversion into the compound of Formula (2) via the compound of Formula (1). The resulting compound is separated by extraction with an organic solvent, and then treated with a base to allow conversion into *cis*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid. By subsequently carrying out deprotection of the amino group, *cis*-5-hydroxy-2-piperidinecarboxylic acid separated from the *trans* isomer can be obtained.

In such cases, the acid halide and/or acid anhydride is/are preferably used in an amount of about 1 molar equivalent relative to the total amount of the *cis*-5-hydroxy-2-piperidinecarboxylic acid and the *trans*-5-hydroxy-2-piperidinecarboxylic acid.

By performing the reaction with the acid halide and/or acid anhydride to protect the amino group, and then with the alcohol (or ester that generates an alcohol) in the presence of the acid catalyst, the *cis*-5-hydroxy-2-piperidinecarboxylic acid is converted into the compound of Formula (2), while the *trans*-5-hydroxy-2-piperidinecarboxylic acid is converted to the *trans*-*N*-protected-5-hydroxy-2-piperidinecarboxylic acid described below. Thus, their separation is possible by extraction with an organic solvent.



[0053]

The “*cis*-5-hydroxy-2-piperidinecarboxylic acid containing an impurity(s)” and “mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid” may be those synthesized by bacterial reaction and/or enzymatic reaction. For example, by the method described in Example 29 of JP 4590981 B, a “mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid” can be obtained. The “*cis*-5-hydroxy-2-piperidinecarboxylic acid containing an impurity(s)” and “mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid” may also be products recovered after separation of a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative by the method of the present invention.

[0054]

In cases where *cis*-5-hydroxy-2-piperidinecarboxylic acid is purified by the method of the present invention from the “*cis*-5-hydroxy-2-piperidinecarboxylic acid containing an impurity(s)” or “mixture of *cis*-5-hydroxy-2-piperidinecarboxylic acid and *trans*-5-hydroxy-2-piperidinecarboxylic acid” synthesized by bacterial reaction and/or enzymatic reaction, the obtained *cis*-5-hydroxy-2-piperidinecarboxylic acid is preferably further subjected to adsorption purification using activated carbon and/or crystallization using a solvent containing water to remove impurities such as a colored substance, to further increase purity of the *cis*-5-hydroxy-2-piperidinecarboxylic acid.

[0055]

As the activated carbon, an arbitrary known activated carbon may be used, and examples of the known activated carbon include coal-based, wood-based,

coconut shell-based, and resin-based activated carbons. The activated carbon may also be prepared by activating a material activated carbon such as a coal-based, wood-based, coconut shell-based, or resin-based material activated carbon by a method such as gas activation, steam activation, or chemical activation using zinc chloride, phosphoric acid, or the like.

Specific examples of the activated carbon include Calgon CPG, Calgon CAL, Calgon SGL, Diasorb W, Diahope MS10, Diahope M010, Diahope MS16, Diahope 6MD, Diahope 6MW, Diahope 8ED, Diahope ZGN4, and CENTUR, manufactured by Calgon Mitsubishi Chemical Corporation; GAC, GAC PLUS, GCN PLUS, C GRAN, RO, ROX, DARCO, CN, SX, SX PLUS, SA, SX, PK, and W, manufactured by Norit Japan Co., Ltd.; GW, GWH, GLC, 4GC, KW, PW, and PK, manufactured by Kuraray Chemical Co., Ltd.; HC-30S, GL-30S, 4G-3S, PS, and PC, manufactured by Tsurumicoal Co., Ltd.; P, W, CW, SG, SGP, S, GB, CA, and K, manufactured by Futamura Chemical Co., Ltd.; Shirasagi KL, Shirasagi W2C, Shirasagi WH2C, Shirasagi W5C, Shirasagi WH5C, Shirasagi WH5X, Shirasagi XS7100H-3, Carboraffin, Shirasagi A, Shirasagi C, and Shirasagi M, manufactured by Japan EnviroChemicals Ltd.; and Hokuetsu CL-K, Hokuetsu HS, and Hokuetsu KS, manufactured by Ajinomoto Fine-Techno Co., Inc.

By this operation, impurities showing absorption within the wavelength range of 400 nm to 800 nm can be removed.

[0056]

As the solvent containing water to be used for the crystallization, a water-soluble organic solvent can be used. Specific examples of the solvent include organic acids such as acetic acid and propionic acid; esters such as ethyl acetate; alcohols such as methanol, ethanol, 1-propanol, 2-propanol, and 1-butanol; ethers such as tetrahydrofuran and dioxane; ketones such as acetone, methyl ethyl ketone, and diethyl ketone; nitriles such as acetonitrile; amides such as dimethylformamide,

dimethylacetamide, and *N*-methylpyrrolidinone; sulfones such as dimethylsulfoxide and sulfolane; and mixed solvents of two or more of these. It is also preferred to further increase purity of the *cis*-5-hydroxy-2-piperidinecarboxylic acid by carrying out crystallization of the *cis*-5-hydroxy-2-piperidinecarboxylic acid using the above-described solvent. Among these, organic acids, alcohols, and ketones, whose solubility in water is higher, are preferred. Alcohols and ketones are more preferred, and ethanol and acetone are especially preferred.

EXAMPLES

10 [0057]

The present invention is described below in more detail by way of Examples. However, the present invention is not limited by these Examples.

[0058]

15 The quantitative analyses in the Examples were carried out by HPLC (High Performance Liquid Chromatography) under the following conditions.

<HPLC-1>

Column: Astec CLC-D (4.6 mm × 150 mm, 5 μm), manufactured by
SUPELCO

Mobile phase: 2 mmol/L aqueous copper sulfate solution

20 Flow rate: 1.0 mL/minute

Column temperature: 45°C

Detection wavelength: UV 254 nm

<HPLC-2>

25 Column: L-column (4.6 mm × 250 mm, 5 μm), manufactured by Chemicals Evaluation and Research Institute, Japan

Mobile phase:

A: 0.1 wt% aqueous trifluoroacetic acid solution

B: methanol

gradient (concentration of B): 0 minute, 20% → 2 minute, 20% → 10 minute, 80% → 20 minute, 80%

Flow rate: 1.0 mL/minute

5 Column temperature: 40°C

Detection wavelength: UV 200 nm

<HPLC-3>

Column: ZORBAX Eclipse Plus C18 (4.6 mm × 150 mm, 1.8 μm),
manufactured by Agilent Technologies

10 Mobile phase:

A: 0.1 wt% aqueous phosphoric acid solution

B: acetonitrile

gradient (concentration of B): 0 minute, 55% → 6 minute, 55% → 9 minute, 80% → 12 minute, 80%

15 Flow rate: 1.0 mL/minute

Column temperature: 40°C

Detection wavelength: UV 210 nm

<HPLC-4>

Column: COSMOSIL 5C₁₈-AR-II (4.6 mm × 150 mm), manufactured by
20 Nacalai Tesque, Inc.

Mobile phase: 50 mmol/L phosphate buffer (pH 2.7)

Flow rate: 1.0 mL/minute

Column temperature: 40°C

Detection wavelength: UV 340 nm

25 <HPLC-5>

Column: SUMICHIRAL OA-6100 (4.6 mm × 250 mm), manufactured by
Sumika Chemical Analysis Service, Ltd.

Mobile phase: 1 mmol/L copper sulfate

Flow rate: 1.0 mL/minute

Column temperature: 30°C

Detection wavelength: UV 254 nm

5 <HPLC-6>

Column: CLC-D (4.6 mm × 150 mm), manufactured by SUPELCO

Mobile phase: 2 mmol/L copper sulfate

Flow rate: 1.0 mL/minute

Column temperature: 30°C

10 Detection wavelength: UV 254 nm

[0059]

[Reference Example 1]

[Preparation Example of Recombinant *Escherichia coli* (*E. coli*) JM109/pKW32

15 (dpkA, aip, gdh, kr), in Which *N*-Methyl-L-amino Acid Dehydrogenase (Hereinafter Referred to as DpkA), L-Amino Acid Oxidase (Hereinafter Referred to as AIP), Glucose-1-dehydrogenase (Hereinafter Referred to as GDH), and Amino Acid Racemase (hereinafter referred to as KR) Are Co-expressed]

(1) Cloning of Genes

20 Based on a gene sequence of dpkA (SEQ ID NO:1) encoding DpkA (GenBank Accession No. BAD89743, SEQ ID NO:2) derived from *Pseudomonas putida* (*P. putida*), primers for amplifying the full-length sequence of the dpkA gene, dpkA_F (SEQ ID NO:9) and dpkA_R (SEQ ID NO:10), were designed and synthesized. PCR was carried out using chromosomal DNA of *P. putida* as a
25 template according to a conventional method, to obtain a DNA fragment of about 1.0 kbp.

A gene sequence of aip (SEQ ID NO:3) encoding a protein AIP (SEQ ID

NO:4), which has the amino acid sequence of L-amino acid oxidase derived from *Scomber japonicus* (GenBank Accession No.CAC00499) with the exception that the signal peptide is removed and methionine is added, was designed and artificially synthesized. Primers for amplifying the full-length sequence of the aip gene, aip_F (SEQ ID NO:11) and aip_R (SEQ ID NO:12), were designed and synthesized. PCR was carried out according to a conventional method, to obtain a DNA fragment of about 1.5 kbp.

Based on a gene sequence of gdh (SEQ ID NO:5) encoding a protein (SEQ ID NO:6) having the amino acid sequence of GDH derived from *Bacillus subtilis* (GenBank Accession No.NP_388275) with the exception that the 96th amino acid residue, glutamic acid, is substituted with alanine, primers for amplifying the full-length sequence of the gdh gene, gdh_F (SEQ ID NO:13) and gdh_R(SEQ ID NO:14), were designed and synthesized. PCR was carried out according to a conventional method, to obtain a DNA fragment of about 0.8 kbp.

Based on a gene sequence of kr (SEQ ID NO:7) encoding KR derived from *P. putida* (GenBank Accession No. NP_745855, SEQ ID NO:8), primers for amplifying the full-length sequence of the kr gene, kr_F (SEQ ID NO:15) and kr_R (SEQ ID NO:16), were designed and synthesized. PCR was carried out using chromosomal DNA of *P. putida* as a template according to a conventional method, to obtain a DNA fragment of about 1.2 kbp.

[0060]

(2) Preparation of Expression Plasmid

Each of the DNA fragments obtained in (1) was digested with restriction enzymes *EcoRI* and *XbaI*, and introduced downstream of the trc promoter in a *MunI/XbaI* digest of a plasmid pKW32, which is described in WO 2012/029819, using a Ligation-Convenience Kit (manufactured by Nippon Gene Co., Ltd.), to obtain pKW32dpkA, pKW32aip, pKW32gdh, and pKW32kr, respectively.

Subsequently, pKW32aip was digested with *SpeI* and *NdeI* to obtain a DNA fragment of about 2.4 kbp containing *aip*, and the resulting DNA fragment was introduced downstream of *dpkA* in the ring-opened (linear) plasmid of about 4.2 kbp obtained by digesting pKW32dpkA with *XbaI* and *NdeI*, to obtain pKW32 (*dpkA*, *aip*).

pKW32gdh was digested with *SpeI* and *NdeI* to obtain a DNA fragment of about 1.7 kbp containing *gdh*, and the resulting DNA fragment was introduced downstream of *aip* in the ring-opened (linear) plasmid of about 5.7 kbp obtained by digesting pKW32 (*dpkA*, *aip*) with *XbaI* and *NdeI*, to obtain pKW32 (*dpkA*, *aip*, *gdh*).

Finally, pKW32kr was digested with *SpeI* and *NdeI* to obtain a DNA fragment of about 2.1 kbp containing *kr*, and the resulting DNA fragment was introduced downstream of *gdh* in the ring-opened (linear) plasmid of about 6.5 kbp obtained by digesting pKW32 (*dpkA*, *aip*, *gdh*) with *XbaI* and *NdeI*, to obtain pKW32 (*dpkA*, *aip*, *gdh*, *kr*).

[0061]

(3) Preparation of Expressing Strain

Using the plasmid pKW32 (*dpkA*, *aip*, *gdh*, *kr*) obtained in (2), *E. coli* JM109 (manufactured by Takara Bio Inc.) was transformed according to a conventional method, to obtain recombinant *E. coli* JM109/pKW32 (*dpkA*, *aip*, *gdh*, *kr*).

[0062]

[Reference Example 2]

[Preparation Example of 5-Hydroxy-2-piperidinecarboxylic Acid Reaction Mixture]

To a 1-L jar fermenter (manufactured by ABLE Corporation, type BMJ), 45 g of 5-hydroxylysine hydrochloride (230 mmol, prepared according to a method described in Bull. Chem. Soc. Jpn., 1962, 35, 2006), 2.27 mL of Adekanol LG-109,

and 67.34 g (374 mmol) of glucose were added, and dissolved in water. Thereafter, 20 wt% aqueous sodium hydroxide solution was added dropwise thereto until the pH became 8. To the resulting liquid, bovine-liver-derived catalase (manufactured by Wako Pure Chemical Industries, Ltd.) was added at 2000 U/L; NADP⁺ (manufactured by Oriental Yeast Co., Ltd.) was added at 0.2 mmol/L; and wet cells of the recombinant *E. coli* JM109/pKW32 (dpkA, aip, gdh, kr) prepared in Reference Example 1 were added at 25 g/L; followed by adding water to the resulting mixture to a liquid volume of 566 mL. The reaction was allowed to proceed at 30°C, a stirring rate of 500 rpm, and an aeration rate of about 1 L/min for 43 hours. During the reaction, the pH was kept at 8 by adding 20 wt% aqueous sodium hydroxide solution dropwise. Exactly the same operations were carried out twice thereafter, and the reaction mixtures obtained by the 3 rounds of operations (using 135 g of 5-hydroxylysine hydrochloride, 690 mmol) were combined. The pH of the reaction mixture was adjusted to 2.5 using 6 mol/L sulfuric acid. Thereafter, 20 wt% aqueous sodium hydroxide solution was added dropwise to adjust the pH to 4, and the resulting liquid was centrifuged at 10,000 rpm for 20 minutes to remove insoluble impurities. The obtained supernatant was passed through a microfiltration membrane (manufactured by Asahi Kasei Corporation), and then through an ultrafiltration membrane (manufactured by Asahi Kasei Corporation), to remove impurities. Analysis of 5-hydroxy-2-piperidinecarboxylic acid contained in the resulting solution was carried out by HPLC under the conditions of <HPLC-1>. As a result, the amount of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid produced was 43.9g (302 mmol; yield, 44%), and the amount of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid produced was 29.7g (205 mmol; yield, 30%).

Example 1

[0063]

<1-1> Production of Benzyl (1*S*,4*S*)-5-Aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate

To 1153 g of a reaction mixture containing 20.7 g (143 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 18.0 g (124 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid [(2*S*,5*S*):(2*S*,5*R*) = 53.6:46.4 (molar ratio)] obtained according to the method of Reference Example 2, 10 mol/L aqueous sodium hydroxide solution (60 mL) was added to adjust the pH from 3.65 to 10.81. The resulting liquid was concentrated under reduced pressure, to obtain 422.4 g of a slurry solution. In each of 2 flasks, 211.2 g of the obtained slurry was placed, and the internal temperature was decreased to 5°C to 7°C, followed by carrying out the following operations for each flask.

[The following is description of the operations for one of the flasks. Each flask contained 10.4 g (72 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 9.0 g (62 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid.]

[0064]

To the flask, 18.8 mL (133 mmol) of benzyloxycarbonyl chloride was added dropwise, and 13.3 mL (133 mmol) of 10 mol/L aqueous sodium hydroxide solution was then added to adjust the pH from about 9 to 10, followed by allowing the reaction to proceed at a temperature within the range of 10°C to 20°C for 10 minutes. To the resulting liquid, 18.8 mL (133 mmol) of benzyloxycarbonyl chloride was added dropwise, and 13.5 mL (135 mmol) of 10 mol/L aqueous sodium hydroxide solution was then added to adjust the pH from 9 to 11, followed by allowing the reaction to proceed at a temperature within the range of 10°C to 20°C for 3 hours. To the resulting liquid, 19 mL of water was added, and 6.2 mL (44 mmol) of benzyloxycarbonyl chloride was then added dropwise, followed by allowing the liquid to gradually warm to room temperature. To the resulting liquid, 200 mL of toluene was added, and 4 mL of 10 mol/L aqueous sodium hydroxide solution (40

mmol) was then added to adjust the pH to 11, followed by separating the organic layer. To the aqueous layer, 100 mL of toluene was added to perform re-extraction, and the aqueous layer was separated to obtain the organic layer. This organic layer was combined with the previously obtained organic layer. The resulting mixture was washed with 10 mL of water, followed by separating the resulting organic layer. From the organic layer obtained from each of the two flasks, the solvent was removed by distillation under reduced pressure at 35°C, to obtain 64.9 g of a yellow oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 45 wt% benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (111 mmol; yield, 78% with respect to the (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid), 15 wt% (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid=benzyloxyformic acid=anhydride (23 mmol; yield, 19% with respect to the (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid), 36 wt% benzyl alcohol, and 5 wt% toluene. Thus, the conversion of the material 5-hydroxy-2-piperidinecarboxylic acid, which contained (2*S*,5*S*) and (2*S*:5*R*) at a ratio of (2*S*,5*S*): (2*S*:5*R*) = 53.6:46.4 (molar ratio), to benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate allowed effective removal of compounds having the stereochemistry of (2*S*,5*R*), and, by this, the purity of compounds having the stereochemistry of (2*S*,5*S*) could be increased to (2*S*,5*S*): (2*S*:5*R*) = 82.8:17.2 (molar ratio).

[0065]

The resulting crude product was cooled to 10°C to 15°C, and 36 mL of toluene and 36 mL of hexane were added thereto. As a result, precipitation of white solids occurred. The precipitated white solids were collected by filtration, and washed by sprinkling 100 mL of hexane thereon. The resulting white solids were dried under reduced pressure at room temperature, to obtain 20.7 g of white solids.

As a result of ^1H -NMR analysis, the white solids were found to be a mixture containing 92.5 wt% benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (yield, 51% with respect to the (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid) and 7.5 wt% (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid=benzyloxyformic acid=anhydride. By the crystallization, the purity of compounds having the stereochemistry of (2*S*,5*S*) could be increased to (2*S*,5*S*):(2*S*,5*R*) = 92.5:7.5 (molar ratio).

^1H -NMR (400 MHz, CDCl_3)

δ 1.77-1.85 (1H, m), 2.02-2.25 (3H, m), 3.52 (1H, d, $J=11.9$ Hz), 3.70 (1H, dt, $J=12.2, 3.3$ Hz), 4.70-4.86 (2H, m), 5.12-5.21 (2H, m), 7.32-7.40 (5H, m).

[0066]

<1-1'> Production of Benzyl (1*S*,4*S*)-5-Aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate

To 80 g of a reaction mixture containing 1.74 g (12.0 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 1.01 g (7.0 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid [(2*S*,5*S*):(2*S*,5*R*)=63.2:36.8 (molar ratio)] obtained according to the method of Reference Example 2, 10 mol/L aqueous sodium hydroxide solution was added to adjust the pH from 3.65 to 9.8. The resulting liquid was concentrated under reduced pressure, and 38 g of a solution was obtained. To the solution, 3.1 mL of 2-propanol was added. The internal temperature of the obtained solution was set to about 25°C. To the solution, 3.2 g (18.8 mmol) of benzyloxycarbonyl chloride was added dropwise, and 2.3 g (22.6 mmol) of 40 wt% aqueous sodium hydroxide solution was further added, followed by allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for 30 minutes. To the resulting liquid, 3.2 g (18.8 mmol) of benzyloxycarbonyl chloride was added dropwise, and 2.3 g (22.6 mmol) of 40 wt% aqueous sodium hydroxide solution was further added, followed by allowing the reaction to proceed at a temperature within

the range of 20°C to 25°C for 30 minutes. Thereafter, the operation of adding 1.6 g (9.4 mmol) of benzyloxycarbonyl chloride dropwise to the resulting liquid, adding 1.1 g (11.3 mmol) of 40 wt% aqueous sodium hydroxide solution thereto, and then allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for 30 minutes was repeated 3 times. Subsequently, the reaction mixture was cooled to 0°C to 5°C, and crystals precipitated in the reaction system were collected by filtration. The obtained crystals were dried under reduced pressure at 40°C to 45°C to obtain 2 g of white crystals.

As a result of ¹H-NMR analysis, the obtained crystals were found to be benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (80 wt%; yield, 56% with respect to the (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid).

[0067]

<1-1''> Production of Benzyl (1*S*,4*S*)-5-Aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate

To 160 g of a reaction mixture containing 3.26 g (22.5 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 2.24 g (15.4 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid [(2*S*,5*S*): (2*S*,5*R*)=59.4:40.6 (molar ratio)] obtained according to the method of Reference Example 2, 10 mol/L aqueous sodium hydroxide solution was added to adjust the pH from 3.65 to 9.5. The resulting liquid was concentrated under reduced pressure, and 74 g of a solution was obtained. To the solution, 6.0 mL of 2-propanol was added. The internal temperature of the obtained solution was set to about 25°C. To the solution, 6.4 g (37.6 mmol) of benzyloxycarbonyl chloride was added dropwise, and 4.5 g (45.2 mmol) of 40 wt% aqueous sodium hydroxide solution was further added, followed by allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for 30 minutes. To the resulting liquid, 6.4 g (37.6 mmol) of benzyloxycarbonyl chloride was added dropwise, and 4.5 g (45.2 mmol) of 40 wt% aqueous sodium hydroxide solution was

further added, followed by allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for additional 30 minutes. Thereafter, 3.2 g (18.8 mmol) of benzyloxycarbonyl chloride was added dropwise to the reaction mixture, and 2.3 g (22.6 mmol) of 40 wt% aqueous sodium hydroxide solution was further added dropwise thereto, followed by allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for 30 minutes. Thereafter, 1.9 g (11.1 mmol) of benzyloxycarbonyl chloride was added dropwise to the reaction mixture, and 1.4 g (14.0 mmol) of 40 wt% aqueous sodium hydroxide solution was added dropwise thereto, followed by allowing the reaction to proceed at a temperature within the range of 20°C to 25°C for 30 minutes. To the resulting reaction mixture, 71 mL of toluene was added, and liquid separation was carried out. To the extracted organic layer, 13 mL of water and 6.2 g of 40 wt% aqueous sodium hydroxide solution were added, and liquid separation was carried out again. The obtained aqueous layer, in an amount of 32 g, was divided into two aliquots, and subjected to the purification methods A and B.

[0068]

Purification Method A: Addition of Acetic Anhydride

To 16 g of the aqueous layer obtained in <1-1'>, 9.1 g (89 mmol) of acetic anhydride was added. The obtained crystals were collected by filtration, and dried under reduced pressure at 40°C to 45°C. The dried crystals appeared white, and their weight was 1.5 g.

The obtained crystals were analyzed by HPLC under the conditions of <HPLC-3>. As a result, the obtained crystals were found to be benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (100 wt%; yield, 50% with respect to the (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid).

[0069]

Purification Method B: Extraction under Acidic Conditions

To 16 g of the aqueous layer obtained in <1-1'>, 30 mL of water was added, and 5 mol/L hydrochloric acid was added thereto to adjust the pH in the system to 1.07. Thereafter, 55 mL of ethyl acetate was added to the resulting mixture, and liquid separation was carried out. Subsequently, 55 mL of ethyl acetate was added to the obtained aqueous layer, and liquid separation was carried out again. The obtained organic layers were combined, and concentrated under reduced pressure until the weight decreased to half of the original weight. To the obtained organic layer, 2.9 g (28.1 mmol) of triethylamine and 2.9 g (28.1 mmol) of acetic anhydride were added at 20°C to 25°C. After stirring the resulting mixture for 30 minutes, 0.57 g (5.6 mmol) of triethylamine and 0.57 g (5.6 mmol) of acetic anhydride were added thereto at 20°C to 25°C. After stirring the resulting reaction mixture for 30 minutes, 20 g of saturated aqueous sodium hydrogen carbonate solution was added thereto. After liquid separation, 71 mL of toluene was added to the obtained aqueous layer, and the operation of extraction was carried out again.

The obtained organic layers were combined, and concentrated under reduced pressure. Cooling of the obtained concentrate to 0°C to 5°C caused precipitation of crystals. The obtained crystals were collected by filtration, and then dried under reduced pressure at 40°C to 45°C. The dried crystals were white, and their weight was 2.4 g. The obtained crystals were analyzed by HPLC under the conditions of <HPLC-3>. As a result, the obtained crystals were found to be benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (98 wt%; yield, 70% with respect to the (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid).

[0070]

<1-2> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 20.0 g (92.5 wt%, 71 mmol) of the benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate obtained in <1-1> and 60 mL of ethanol were

added, and 76.5 mL of 1 mol/L aqueous sodium hydroxide solution was added thereto at room temperature, followed by stirring the mixture for 1 hour. The solvent was removed under reduced pressure at 35°C to 40°C, to obtain 61.2 g of a yellow oily substance. To the resulting substance, 100 mL of ethyl acetate was added, and the organic layer was separated. To the obtained aqueous layer, 14 mL of 5 mol/L hydrochloric acid was added to adjust the pH to 1.2. The aqueous layer was subjected to extraction using 200 mL of ethyl acetate twice, and the obtained organic layer was washed with 2 mL of water. After separation of the organic layer, the solvent was removed under reduced pressure at 35°C to 40°C, to obtain 24.0 g of a pale yellow oily substance.

As a result of ¹H-NMR analysis, this pale yellow oily substance was found to be (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (purity, 76 wt%; 65 mmol; yield, 92%). The peak for (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid was not found at all by ¹H-NMR.

¹H-NMR (400 MHz, CDCl₃, rotational isomer mixture)
 δ 1.27-1.39 (1H, m), 1.70-1.85 (1H, m), 1.98-2.06 (1H, m), 2.27-2.39 (1H, m), 2.80 (0.4H, t, *J*=11.8 Hz), 2.87 (0.6H, t, *J*=11.6 Hz), 3.61-3.72 (1H, m), 4.18-4.33 (1H, m), 4.81-4.88 (0.4H, m), 4.93-4.98 (0.6H, m), 5.12-5.21 (2H, m), 7.28-7.40 (5H, m).

[0071]

<1-3> Production of (2*S*,5*S*)-5-Hydroxy-2-piperidinecarboxylic Acid

To a flask, 18.0 g (64.5 mmol) of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <1-2>, 90 mL of ethanol, and 2.5 g of 10% palladium carbon (manufactured by N. E. Chemcat Corporation, PE-type, 55.3%, containing water) were added, and hydrogenation was carried out under normal temperature and pressure. Three hours later, 22 mL of water was added thereto, and hydrogenation was carried out under normal temperature and pressure. Disappearance of the starting material could be confirmed 8.5 hours later. The

palladium carbon was removed by filtration, and 100 mL of water was sprinkled thereon for washing. From the obtained filtrate, the solvent was removed under reduced pressure at 45°C to 55°C, to obtain 11.6 g of a white slurry.

The obtained slurry was analyzed by HPLC under the conditions of <HPLC-1>. As a result, the slurry was found to contain 5-hydroxy-2-piperidinecarboxylic acid with a purity of 65 wt% in an amount of 64.5 mmol and yield of 81%. The isomer ratio was as follows: (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid:(2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid=98.8:1.2. The material used in <1-1> had a purity of as low as (2*S*,5*S*):(2*S*,5*R*)=53.7:46.3 (molar ratio), and the purity was increased to (2*S*,5*S*):(2*S*,5*R*)=98.8:1.2 (molar ratio) by the method of the present invention.

[0072]

<1-4> Activated Carbon Treatment, and Crystallization

To a flask, 0.38 g of the slurry obtained in <1-3> (purity, 65 wt%; 1.7 mmol), 2.5 mL of water, 0.025 g of an activated carbon manufactured by Calgon Mitsubishi Chemical Corporation, CAL, and 0.025 g of an activated carbon manufactured by Calgon Mitsubishi Chemical Corporation, 6ED, were added. The resulting mixture was stirred at room temperature for 1 hour, and the active carbons were then removed by filtration, followed by concentrating the aqueous solution to 0.5 mL. While the concentrate was stirred, 5.0 mL of ethanol was added dropwise thereto, and the resulting mixture was cooled to 5°C. As a result, crystals gradually precipitated. While the temperature was kept at 5°C, the stirring was continued for 1 hour, and 2.0 mL of acetone was added dropwise thereto at the constant temperature of 5°C, followed by stirring the resulting mixture for additional 0.5 hour. Thereafter, the precipitated crystals were collected by filtration, and 1.0 mL of an ice-cooled ethanol/acetone solution (=5/2 volume ratio) was sprinkled thereon for washing. The crystals were then dried under reduced pressure at 60°C, to obtain 0.25 g (purity,

98 wt%; yield, 99.2%) of crystals.

The obtained crystals were analyzed by HPLC under the conditions of <HPLC-1>. Based on the result, the isomer ratio of 5-hydroxy-2-piperidinecarboxylic acid contained in the obtained crystals was as follows: (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid:(2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid=99.5:0.5.

Example 2

[0073]

<2-1> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid and (2*S*,5*R*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To 124 mL of a reaction mixture containing 0.723 g (4.98 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 0.830 g (5.72 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid obtained according to the method of Reference Example 2 [(2*S*,5*S*): (2*S*,5*R*)=46.5:53.5 (molar ratio)], 2.88 mL (36 mmol) of 50 wt% aqueous sodium hydroxide solution was added, and the resulting mixture was concentrated at a bath temperature of 40°C under a reduced pressure of 50 hPa, to obtain 132 g of a solution. In a water bath, 3.1 mL (22 mmol) of benzyloxycarbonyl chloride and 0.44 mL (5.5 mmol) of 50 wt% aqueous sodium hydroxide solution were dividedly added to the obtained solution while the pH was adjusted to about 9. After leaving the resulting solution to stand overnight, 60 mL of ethyl acetate and 0.88 mL (11 mmol) of 50 wt% aqueous sodium hydroxide solution were added thereto, followed by carrying out filtration through Celite. The organic layer was separated, and the aqueous layer was washed with ethyl acetate. To the aqueous layer, 2.9 mL of concentrated hydrochloric acid (33 mmol) was added to adjust the pH to 3. Thereafter, extraction with ethyl acetate was carried out 3 times. The solvent was removed from the resulting organic layer under reduced pressure at 35°C,

to obtain 2.44 g of a yellow oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 80 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (6.91 mmol; yield, 65%; (2*S*,5*S*): (2*S*,5*R*)=4:6 (molar ratio)), 12 wt% ethyl acetate, and 8 wt% acetic acid.

[0074]

<2-2> Production of Benzyl (2*S*,5*S*)-3-Oxo-2-oxa-5-azabicyclo[2.2.2]octane-5-carboxylate

To a flask, 2.35 g of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <2-1> [6.72 mmol, (2*S*,5*S*): (2*S*,5*R*)=4:6 (molar ratio)], 10 mL of toluene, and 116 mg (0.67 mmol) of *p*-toluenesulfonic acid monohydrate were added, and the reaction was allowed to proceed at 60°C for 2 hours. After cooling the resulting reaction product to room temperature, 30 mL of ethyl acetate, 5 mL of water, and 7 mL of 1 mol/L sodium hydroxide solution were added thereto. As a result, the pH of the aqueous layer became 9. After separating the aqueous layer, re-extraction was carried out with ethyl acetate. The obtained organic layer was washed with saturated aqueous sodium bicarbonate solution and saturated brine. The resulting organic layer was concentrated to obtain 0.44 g of a brown oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 75 wt% benzyl (2*S*,5*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (1.27 mmol; yield, 19%), 17 wt% ethyl acetate, and 9 wt% toluene.

In the material *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid, (2*S*,5*S*) and (2*S*,5*R*) were contained at (2*S*,5*S*): (2*S*,5*R*)=4:6 (molar ratio). By its conversion to benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate,

compounds having the stereochemistry of (2*S*,5*R*) could be effectively removed.

However, since the yield of benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate was 19%, the (2*S*,5*S*) isomer was obtained in a moderate yield of about 50%. It has been observed that a side reaction occurred between benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate generated by the reaction and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid left unreacted in the system, to cause esterification. This is assumed to be the cause of the moderate yield.

[0075]

10 <2-3> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.44 g (purity, 75 wt%; 1.27 mmol) of the benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate obtained in <2-2>, 2.6 mL of methanol, and 2.6 mL of 1 mol/L aqueous sodium hydroxide solution were added, and left to stand overnight. To the resulting reaction mixture, 1.3 mL of 2 mol/L hydrochloric acid was added to adjust the pH to 4, and extraction with ethyl acetate was then carried out. The resulting organic layer was concentrated to obtain 0.33 g of a brown oily substance. The substance was then purified by silica gel chromatography to obtain 0.25 g of (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.88 mmol; yield, 69%).

Example 3

[0076]

25 <3-1> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid and (2*S*,5*R*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.76 g of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid [5.24 mmol, (2*S*,5*S*): (2*S*,5*R*)=7:3

(molar ratio)], and 6.3 mL of 1 mol/L aqueous sodium hydroxide solution were added, and 0.93 mL (5.3 mmol) of benzyloxycarbonyl chloride was added to the resulting mixture under ice-cooling. The temperature of the mixture was then slowly increased to room temperature, and 2 mL of tetrahydrofuran and 5.2 mL of 1 mol/L aqueous sodium hydroxide solution were dividedly added thereto while the pH was adjusted to about 9. After leaving the resulting mixture to stand overnight, the organic layer was separated, and the aqueous layer was washed with ethyl acetate. To the aqueous layer, 3.2 mL of 2 mol/L hydrochloric acid was added to adjust the pH to 3, and the resulting mixture was extracted 3 times with ethyl acetate. The resulting organic layer was concentrated to obtain 0.79 g of a yellow oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 76 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (2.15 mmol; yield, 41%; (2*S*,5*S*): (2*S*,5*R*)=7:3 (molar ratio)), and 24 wt% ethyl acetate.

[0077]

<3-2> Production of Benzyl (2*S*,5*S*)-5-Aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate

To a flask, 0.79 g of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <3-1> (purity, 76 wt%; 2.15 mmol; (2*S*,5*S*): (2*S*,5*R*)=7:3 (molar ratio)), 5 mL of toluene, and 49 mg (0.28 mmol) of *p*-toluenesulfonic acid monohydrate were added, and the reaction was allowed to proceed at 60°C for 2 hours. After cooling the resulting reaction product to room temperature, 10 mL of ethyl acetate, 3 mL of water, and 1.2 mL of 1 mol/L sodium hydroxide solution were added thereto. As a result, the pH of the aqueous layer became 7. After separating the aqueous layer, re-extraction was carried out with ethyl acetate. The obtained organic layer was washed with saturated aqueous

sodium bicarbonate solution and saturated brine. The resulting organic layer was concentrated to obtain 0.34 g of a brown oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 62 wt% benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate (0.80 mmol; yield, 37%), 21 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.25 mmol; yield, 12%), and 17 wt% toluene.

In the material *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid, (2*S*,5*S*) and (2*S*,5*R*) were contained at (2*S*,5*S*):(2*S*,5*R*)=7:3 (molar ratio). By its conversion to benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate, compounds having the stereochemistry of (2*S*,5*R*) could be effectively removed. The total yield of benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate and (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid was 49%, and the (2*S*,5*S*) isomer was obtained in a moderate yield of about 70%.

Similarly to <2-1>, a side reaction occurred between benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate generated by the reaction and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid left unreacted in the system, to cause esterification. However, since the amount of (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid in the starting material was small, the (2*S*,5*S*) isomer could be selectively, and relatively efficiently recovered.

[0078]

<3-3> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.34 g of the oily substance obtained in <3-2> [containing 0.80 mmol benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate and 0.25 mmol (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid], 2 mL of methanol, and 1.9 mL of 1 mol/L aqueous sodium hydroxide solution were added,

and left to stand overnight. To the resulting reaction mixture, 2 mL of 2 mol/L hydrochloric acid was added to adjust the pH to 4, and extraction with ethyl acetate was then carried out. The resulting organic layer was concentrated to obtain 0.39 g of a brown oily substance.

5 As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 76 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (1.05 mmol; yield, 100%; (2*S*,5*S*):(2*S*,5*R*)=87:13 (molar ratio)), 23 wt% ethyl acetate, and 1 wt% acetic acid.

[0079]

10 <3-4> Production of (2*S*,5*S*)-5-Hydroxy-2-piperidinecarboxylic acid

To a flask, 0.39g of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <3-3> (purity, 77 wt%; 1.06 mmol), 2 mL of methanol, and 61 mg of 10% palladium carbon (manufactured by N. E. Chemcat Corporation, PE-type, 55.3%, containing water) were added, and hydrogenation was
15 carried out under normal temperature and pressure for 3 hours. The palladium carbon was removed by filtration through Celite, and washing with methanol-water was carried. The obtained filtrate was concentrated to obtain 0.21 g of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid as a pale yellow oily substance.

Example 4

20 [0080]

<4-1> Production of Benzyl (1*S*,4*S*)-5-Aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate and Ethyl (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate

To a flask, 0.26 g of (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained according to the method of <2-1> (purity, 82 wt%; 0.76 mmol; (2*S*,5*S*):(2*S*,5*R*)=5:5 (molar ratio)), 1 mL of ethyl acetate, and 5 μL

(0.08 mmol) of methanesulfonic acid were added, and the reaction was allowed to proceed at 60°C for 3 hours. After cooling the resulting reaction product to room temperature, 10 mL of ethyl acetate and 1 mL of 1 mol/L sodium hydroxide solution were added thereto. As a result, the pH of the aqueous layer became about 10.

5 After separating the aqueous layer, re-extraction was carried out with ethyl acetate. The obtained organic layer was dried over magnesium sulfate. The resulting organic layer was concentrated to obtain 0.12 g of a pale brown oily substance.

As a result of ¹H-NMR analysis, this oily substance was found to be a mixture containing 26 wt% benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-
10 carboxylate (0.11 mmol; yield, 15%), 52 wt% ethyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.20 mmol; yield, 26%), 13 wt% ethyl acetate, and 8 wt% toluene.

It is thought that, in the present Example, a small amount of ethanol generated from ethyl acetate reacted with benzyl (1*S*,4*S*)-5-aza-2-oxa-3-
15 oxobicyclo[2.2.2]octane-5-carboxylate, to generate ethyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate. In the material *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid, (2*S*,5*S*) and (2*S*,5*R*) were contained at (2*S*,5*S*):(2*S*,5*R*)=5:5 (molar ratio). As a result of ¹H-NMR analysis and HPLC analysis (under the conditions of <HPLC-2>), it could be confirmed that the
20 isomer ratio of the ethyl *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate was as follows: (2*S*,5*S*):(2*S*,5*R*)>10:1 (molar ratio). Thus, compounds having the stereochemistry of (2*S*,5*R*) could be effectively removed. Since the total yield of benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate and ethyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate was 41%, the yield
25 of the (2*S*,5*S*) isomer was about 80%. Thus, the (2*S*,5*S*) isomer could be selectively and efficiently recovered.

[0081]

<4-2> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.12 g of the oily substance obtained in <4-1> (containing 0.11 mmol of benzyl (1*S*,4*S*)-5-aza-2-oxa-3-oxobicyclo[2.2.2]octane-5-carboxylate and
 5 0.20 mmol of ethyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate), 1 mL of ethanol, and 0.66 mL of 1 mol/L aqueous sodium hydroxide solution were added, and left to stand overnight. The reaction mixture was then concentrated, and 0.7 mL of 1 mol/L hydrochloric acid was added thereto to adjust the pH to 3, followed by carrying out extraction with ethyl acetate. The
 10 resulting organic layer was concentrated under reduced pressure to obtain 0.11 g of a brown oily substance.

As a result of ¹H-NMR analysis, this oily substance was found to be a mixture containing 84 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.33 mmol, quantitative, (2*S*,5*S*): (2*S*,5*R*)=10:1 (molar ratio)), 11 wt% ethyl
 15 acetate, and 5 wt% toluene.

[0082]

<4-3> Production of (2*S*,5*S*)-5-Hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.11 g of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <4-2> (purity, 82 wt%, 0.33 mmol), 1 mL of
 20 methanol, and 18 mg of 10% palladium carbon (manufactured by N. E. Chemcat Corporation, PE-type, 55.3%, containing water) were added, and hydrogenation was carried out under normal temperature and pressure for 9 hours. The palladium carbon was removed by filtration through Celite, and washing with methanol-water was carried. The obtained filtrate was concentrated to obtain 58 mg of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid as a pale yellow oily substance
 25 ((2*S*,5*S*): (2*S*,5*R*)=10:1 (molar ratio), ¹H-NMR analysis).

Example 5

[0083]

<5-1> Production of Ethyl (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate

5 To a flask, 0.55 g of (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained according to the method of <2-1> (purity, 78 wt%; 1.52 mmol; (2*S*,5*S*): (2*S*,5*R*)=3:7 (molar ratio)), 2 mL of toluene, 0.5 mL of ethanol, and 10 μ L (0.15 mmol) of methanesulfonic acid were added, and the
10 reaction was allowed to proceed at 40°C for 5 hours.

 The reaction mixture was analyzed by HPLC under the conditions of <HPLC-2>, and the following result was obtained: ethyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate:ethyl (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate:(2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-
15 piperidinecarboxylic acid:(2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid=24:8:7:57.

 Under the assumption that the material and products have the same absorption coefficient, the degree of conversion of the (2*S*,5*S*) isomers was about 77%, and the degree of conversion of the (2*S*,5*R*) isomers was about 12%. Thus, the (2*S*,5*S*)
20 isomers showed a higher degree of esterification.

[0084]

<5-1'> Production of Isopropyl (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate

 To a flask, 0.62 g of (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-
25 piperidinecarboxylic acid and (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained according to the method of <2-1> (purity, 78 wt%; 1.72 mmol; (2*S*,5*S*): (2*S*,5*R*)=3:7 (molar ratio)), 2 mL of toluene, 0.5 mL of 2-

propanol, and 11 μL (0.17 mmol) of methanesulfonic acid were added, and the reaction was allowed to proceed at 50°C for 5 hours. After cooling the resulting reaction product to room temperature, 3 mL of toluene and 1.2 mL of 1 mol/L sodium hydroxide solution were added thereto. As a result, the pH of the aqueous layer became about 9. After separating the aqueous layer, re-extraction was carried out with toluene. The resulting organic layer was concentrated to obtain 0.17 g of a pale brown oily substance.

As a result of ^1H -NMR analysis, this oily substance was found to be a mixture containing 91 wt% isopropyl *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate (0.47 mmol; yield, 27%; (2*S*,5*S*):(2*S*,5*R*)=10:1 (molar ratio)), and 9 wt% toluene.

In the present Example, the material *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid contained (2*S*,5*S*) and (2*S*,5*R*) at (2*S*,5*S*):(2*S*,5*R*)=3:7 (molar ratio). Based on the result of ^1H -NMR analysis, the isomer ratio of the isopropyl *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate was follows: (2*S*,5*S*):(2*S*,5*R*)=10:1 (molar ratio). Thus, compounds having the stereochemistry of (2*S*,5*R*) could be effectively removed. Since the yield of the isopropyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate was 27%, the yield of the (2*S*,5*S*) isomer was about 80%. Thus, the (2*S*,5*S*) isomer could be selectively and efficiently recovered.

[0085]

<5-2> Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.17 g of the isopropyl (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylate obtained in <5-1'> (purity, 91 wt%; 0.47 mmol), 0.7 mL methanol, and 0.71 mL of 1 mol/L aqueous sodium hydroxide solution were added, and the reaction was allowed to proceed at 60°C for 4 hours. After washing the

reaction mixture with toluene, 0.45 mL of 2 mol/L hydrochloric acid was added thereto to adjust the pH to 3, and extraction with ethyl acetate was carried out. The resulting organic layer was concentrated to obtain 0.17 g of a brown oily substance. As a result of ¹H-NMR analysis, this oily substance was found to be a mixture containing 70 wt% *N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.41 mmol; yield, 88%; (2*S*,5*S*):(2*S*,5*R*)=7:1 (molar ratio)), 26 wt% ethyl acetate, and 4 wt% toluene.

[0086]

<5-3> Production of (2*S*,5*S*)-5-Hydroxy-2-piperidinecarboxylic Acid

To a flask, 0.17 g of the (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid obtained in <5-2> (purity, 70 wt%; 0.41 mmol; (2*S*,5*S*):(2*S*,5*R*)=7:1 (molar ratio)), 1 mL of methanol, and 22 mg of 10% palladium carbon (manufactured by N. E. Chemcat Corporation, PE-type, 55.3%, containing water) were added, and hydrogenation was carried out under normal temperature and pressure for 3 hours. The palladium carbon was removed by filtration through Celite, and washing with methanol-water was carried. The obtained filtrate was concentrated to obtain 89 mg of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid as a pale yellow oily substance.

[0087]

[Reference Example 3]

<3-1> Cloning of Lysine Hydroxylase Gene

Based on a gene sequence (hyl-1, SEQ ID NO:17) encoding an L-arginine-β hydroxylase VioC homologue Hyl-1 derived from the *Flavobacterium johnsoniae* NBRC14942 strain (GenBank Accession No. ABQ06186, SEQ ID NO:18), primers for amplifying the full-length sequence of the hyl-1 gene, hyl1_F (SEQ ID NO:29) and hyl1_R (SEQ ID NO:30), were designed and synthesized. Using chromosomal

DNA of *Flavobacterium johnsoniae* as a template, PCR was carried out according to a conventional method, to obtain a DNA fragment of about 1.0 kbp.

In addition, VioC homologues derived from the *Kineococcus radiotolerans* NBRC101839 strain, *Chitinophaga pinensis* NBRC15968 strain, *Chryseobacterium*
 5 *gleum* NBRC15054 strain, and *Niastella koreensis* NBRC106392 strain were designated Hyl-2 (GenBank Accession No.ABS05421, SEQ ID NO:20), Hyl-3 (GenBank Accession No.ACU60313, SEQ ID NO:22), Hyl-4 (GenBank Accession No.EFK34737, SEQ ID NO:24), and Hyl-5 (GenBank Accession No.AEV99100, SEQ ID NO:26), respectively. Based on gene sequences encoding the enzymes,
 10 (hyl-2 (SEQ ID NO:19), hyl-3 (SEQ ID NO:21), hyl-4 (SEQ ID NO:23), and hyl-5 (SEQ ID NO:25)), primers for amplifying the full-length sequence of each gene were designed and synthesized. Primers hyl2_f (SEQ ID NO:31) and hyl2_r (SEQ ID NO:32) for hyl-2, primers hyl3_f (SEQ ID NO:33) and hyl3_r (SEQ ID NO:34) for hyl-3, primers hyl4_f (SEQ ID NO:35) and hyl4_r (SEQ ID NO:36) for hyl-4, and
 15 primers hyl5_f (SEQ ID NO:37) and hyl5_r (SEQ ID NO:38) for hyl-5 were synthesized, and PCR was carried out using chromosomal DNA of each strain according to a conventional method. Each reaction produced a DNA fragment of about 1.0 kbp.

Each of the 5 kinds of DNA fragments obtained was digested with restriction
 20 enzymes *NdeI* and *XhoI*, and ligated into *NdeI/XhoI*-digested pET21a (Novagen) according to a conventional method, to obtain pEHYL1, pEHYL2, pEHYL3, pEHYL4, and pEHYL5, respectively.

A gene sequence (hyl-6, SEQ ID NO:27) encoding Hyl-6 (GenBank Accession No. ABS05421, SEQ ID NO:28), which was derived from a marine
 25 actinobacterium PHSC20C1, was artificially synthesized, and inserted into pJExpress401 (DNA2.0) to prepare a plasmid pJHYL6.

Subsequently, *E. coli* (*Escherichia coli*) BL21(DE3) (manufactured by

Invitrogen) was transformed with each of the resulting plasmids according to a conventional method, to obtain recombinant *E. coli* BL21(DE3)/pEHYL1, BL21(DE3)/pEHYL2, BL21(DE3)/pEHYL3, BL21(DE3)/pEHYL4, BL21(DE3)/pEHYL5, and BL21(DE3)/pJHYL6. In order to obtain bacterial cells expressing the genes, each type of recombinant *E. coli* was cultured at 30°C using liquid LB medium supplemented with ampicillin and a lac promoter inducer, and collected after 20 hours of the culture.

[0088]

<3-2> Confirmation of Lysine Hydroxylase Activity by Resting-cell Reaction

In a plastic tube, 5 mmol/L L-lysine, 10 mmol/L 2-oxoglutaric acid, 1 mmol/L L-ascorbic acid, 0.1 mmol/L iron sulfate, and recombinant *E. coli* obtained by the method according to Reference Example <3-1> were mixed to provide a reaction mixture such that the turbidity OD₆₀₀ was 10. The reaction was allowed to proceed in 0.5 mL of the prepared mixture at 30°C at pH 7.0 for 3 hours. The reaction product was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA), and hydroxylysine was analyzed by HPLC under the conditions of <HPLC-4>. As a result, as shown in Fig. 2 and Fig. 3, it could be confirmed that BL21(DE3)/pEHYL2 and BL21(DE3)/pJHYL6 produced a compound corresponding to the retention time of a standard product of 3-hydroxylysine, 8.04 minutes. It could also be confirmed that BL21(DE3)/pEHYL1, BL21(DE3)/pEHYL3, BL21(DE3)/pEHYL4, and BL21(DE3)/pEHYL5 produced a compound corresponding to the retention time of a standard product of 4-hydroxylysine, 8.16 minutes.

[0089]

<3-3> Synthesis of (2*S*,3*S*)-3-Hydroxylysine

To a 1-L jar fermenter, 35 mL of 1 mol/L potassium phosphate buffer (pH 7.0), 304 mL of desalted water, 1.28 g of L-lysine hydrochloride, 2.05 g of 2-oxoglutaric acid, 0.14 g of sodium L-ascorbate, 0.02 g of iron sulfate, 0.35 g of

Adekanol LG109, and 8 g of wet cells of recombinant *E. coli* BL21(DE3)/pEHYL2 obtained by the method according to Reference Example <3-1> were mixed together, and the reaction was allowed to proceed at 30°C, pH 7.0, a stirring rate of 500 rpm, and an aeration rate of 2.0 vvm for 17 hours. Completion of the reaction was
 5 judged by confirming disappearance of the peak for L-lysine by carrying out HPLC analysis under the conditions of <HPLC-5>. From the liquid after the completion of the reaction, bacterial cells and bacterial debris were removed by centrifugation and microfiltration, to obtain 390 g of a filtrate.

After passing 390 g of the filtrate through an ion-exchange resin column
 10 (DIAION (registered trademark) SK-1B (Type H), 60.0 g), washing was carried out with water, followed by elution with an aqueous solution containing 150 mmol of ammonia. The ammonia eluate was concentrated to obtain 1.0 g of (2*S*,3*S*)-3-hydroxylysine (6.17 mmol; yield, 88%).

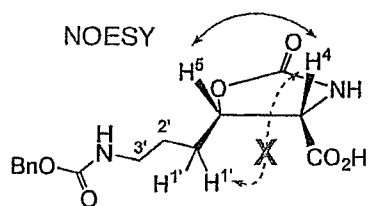
¹H-NMR (400MHz, D₂O) δ, 1.45-1.58 (2H, m), 1.63-1.73 (1H, m), 1.74-1.88 (1H,
 15 m), 2.93-3.04 (2H, m), 3.47 (1H, d, *J*=4.3 Hz), 3.89 (1H, dt, *J*=8.4, 4.5 Hz)
 [0090]

<3-4> Determination of Stereochemistry of (2*S*,3*S*)-3-Hydroxylysine

To a flask, 8.3 mg (0.051 mmol) of (2*S*,3*S*)-3-hydroxylysine obtained by the method according to Reference Example <3-3>, 0.26 ml of 1 mol/L aqueous sodium
 20 hydroxide solution, and 18 μL (0.13 mmol) of benzyloxycarbonyl chloride were added, and the resulting mixture was stirred at room temperature for 1 hour. To the mixture, 0.26 mL of 1 mol/L aqueous sodium hydroxide solution and 18 μL (0.13 mmol) of benzyloxycarbonyl chloride were further added, and the reaction was allowed to proceed overnight at room temperature. Subsequently, 0.5 mL of
 25 tetrahydrofuran was added to the reaction mixture, and the reaction was allowed to proceed at 60°C for additional 2 hours. After cooling the reaction mixture to room temperature, 95 mg of sodium hydroxide was added thereto, and the reaction was

allowed to proceed overnight at room temperature. The reaction mixture was washed twice with toluene-tetrahydrofuran (1:1), and 250 μ L of concentrated hydrochloric acid was added to the reaction mixture to make the reaction mixture strongly acidic. After 3 times of washing with ethyl acetate, the aqueous layer was extracted 4 times with 1-butanol. The 1-butanol layer was dried over anhydrous magnesium sulfate, and then concentrated, to obtain 14.6 mg of (4*S*,5*S*)-5-(3-benzyloxycarbonylaminoethyl)-2-oxo-4-oxazolidinonecarboxylic acid (0.045 mmol; yield, 89%).

The stereochemistry of the obtained (4*S*,5*S*)-5-(3-benzyloxycarbonylamino-2-oxo-4-oxazolidinyl)-2-oxo-4-oxazolidinecarboxylic acid was determined by NOESY measurement. The NOESY measurement was carried out using an AVANCE DRX500 spectrometer manufactured by Bruker (equipped with a CryoProbe) at 25°C with a mixing time of 0.8 msec. As a chemical shift reference, 3.31 ppm for methanol was used. The result of NOESY measurement was as follows.



[0092]

Since a cross peak was found between the 4-position hydrogen atom (H^4) and 5-position hydrogen atom (H^5), but was not found between the 4-position and the 1'-position, the substituents at the 4-position and the 5-position could be confirmed to have the *cis* configuration. Since the absolute configuration of the lysine used in the enzymatic reaction was *S*, it could be confirmed that the 5-(3-benzyloxycarbonylamino-2-oxo-4-oxazolidinecarboxylic acid obtained in the

present Reference Example has the stereochemistry of (4*S*,5*S*), and that 3-hydroxylysine as its material has the stereochemistry of (2*S*,3*S*).

¹H-NMR (400 MHz, MeOH-d₄) δ, 1.39-1.53 (3H, m, H^{1'}, H^{2'} x2), 1.59-1.68 (1H, m, H^{1'}), 3.02-3.08 (2H, m, H^{3'}), 3.60-3.64 (1H, m, H⁴), 3.93-4.00 (1H, m, H⁵), 4.90-5.02 (2H, m, Bn), 7.18-7.28 (5H, m, Bn).

[0093]

[Reference Example 4]

Production of (2*S*,3*S*)-3-Hydroxypipicollic Acid [(2*S*,3*S*)-3-Hydroxy-2-piperidinecarboxylic Acid]

In a plastic tube, 0.75 mL of 1 mol/L tris hydroxymethyl aminomethane buffer (pH 8.0), 9.21 mL of desalted water, 86 mg of the (2*S*,3*S*)-3-hydroxylysine obtained in Reference Example <3-3>, 0.083 mL of 50 mmol/L NADPH, 0.7 mL of 1.0 mol/L glucose, and 1.25 mL of a 100-g/L suspension of the recombinant *E. coli* JM109/pKW32 (dpkA, aip, gdh, kr) obtained in Reference Example 1 were mixed together, and the reaction was allowed to proceed at 30°C at pH 8.0 at a stirring rate of 1000 rpm for 20 hours. Completion of the reaction was judged by confirming disappearance of the peak for (2*S*,3*S*)-3-hydroxylysine by HPLC analysis under the conditions of <HPLC-6>. From the liquid after the completion of the reaction, bacterial cells and bacterial debris were removed by centrifugation, to obtain 10.5 g of a supernatant.

After passing 10.5 g of the supernatant through an ion-exchange resin column (DIAION (registered trademark) SK-1B (Type H), 4.0g), washing was carried out with water, followed by elution with an aqueous solution containing 16.4 mmol of ammonia. The ammonia eluate was concentrated to obtain 255 mg of a solid brown substance. As a result of NMR analysis, this solid substance was found to be a mixture containing 20 wt% (2*S*,3*R*)-3-hydroxypipicollic acid (0.35 mmol; yield,

66.3%) and 80 wt% tris hydroxymethyl aminomethane.

¹H-NMR (400 MHz, D₂O) δ, 1.38-1.56 (2H, m), 1.73-1.85 (2H, m), 2.71-2.79 (1H, m), 3.04-3.11 (1H, m), 3.23 (1H, d, *J*=7.6Hz), 3.79-3.86 (1H, m).

5 Example 6

[0094]

Production of (2*S*,5*S*)-*N*-Benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic Acid

To a flask, 78.7 mg (0.542 mmol) of (2*S*,5*S*)-5-hydroxy-2-piperidinecarboxylic acid and 2.8 mg (0.019 mmol) of (2*S*,5*R*)-5-hydroxy-2-piperidinecarboxylic acid obtained according to the method of Example 1, and 98.5 mg of (2*S*,3*S*)-3-hydroxy-2-piperidinecarboxylic acid obtained according to the method of Reference Example 4 (purity, 20 wt%, 0.136 mmol; containing 78.8 mg of tris hydroxymethyl aminomethane) were added, and 0.200 mL of water and 0.674 mL of 2 mol/L aqueous sodium hydroxide solution were added thereto at room temperature. After adding 0.190 mL (1.35 mmol) of benzyloxycarbonyl chloride dropwise to the resulting mixture, 0.500 mL of 2 mol/L aqueous sodium hydroxide solution was added thereto to adjust the pH from 9 to 10. The reaction was allowed to proceed at room temperature for 25 minutes, and 0.098 mL (0.697 mmol) of benzyloxycarbonyl chloride was then added dropwise to the resulting reaction mixture, followed by adding 0.400 mL of 2 mol/L aqueous sodium hydroxide solution thereto to adjust the pH from 10 to 11. The reaction was further allowed to proceed at room temperature for 40 minutes, and 0.098 mL (0.697 mmol) of benzyloxycarbonyl chloride was added dropwise thereto, followed by allowing the reaction to proceed at room temperature for 2 hours. To the resulting reaction mixture, 3 mL of toluene was added, and liquid separation was then carried out. To the extracted organic layer, 0.500 mL of water was added, and liquid separation was carried out again. The resulting organic layer was concentrated to obtain 330 mg of

a pale yellow oily substance. To this oily substance, 2 mL of ethanol, 1.1 mL of 1 mol/L aqueous sodium hydroxide solution, and 0.3 mL of water were added, and the reaction was allowed to proceed at room temperature for about 1 hour. The resulting reaction mixture was concentrated, and ethanol was evaporated.

5 Thereafter, 1 mL of ethanol, 0.3 mL of water, 0.1 mL of 1 mol/L aqueous sodium hydroxide solution, and 0.1 mL of 2 mol/L aqueous sodium hydroxide solution were added thereto, and the reaction was allowed to proceed at room temperature for 2 hours. The reaction mixture was concentrated, and ethanol was evaporated, followed by carrying out liquid separation using 3 mL of toluene. To the obtained
10 aqueous layer, 1 mL of water and 0.75 mL of 1 mol/L hydrochloric acid were added to adjust the pH of the aqueous solution to 1. The aqueous layer was subjected twice to extraction with 4 mL of ethyl acetate, and the resulting organic layer was concentrated to obtain 92.0 mg of a pale yellow oily substance.

As a result of ^1H -NMR analysis, this pale yellow oily substance was found to
15 contain 80.5 wt% (2*S*,5*S*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid (0.265 mmol; yield, 49%) and 19.5 wt% ethyl acetate. No peak was found at all for either (2*S*,5*R*)-*N*-benzyloxycarbonyl-5-hydroxy-2-piperidinecarboxylic acid or (2*S*,3*S*)-*N*-benzyloxycarbonyl-3-hydroxy-2-piperidinecarboxylic acid by ^1H -NMR.

20 INDUSTRIAL APPLICABILITY

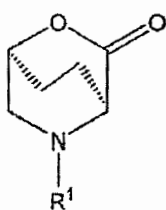
[0095]

The present invention can be used as a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid, which is useful as an intermediate for pharmaceuticals, and can also be used as a method for producing its derivatives.

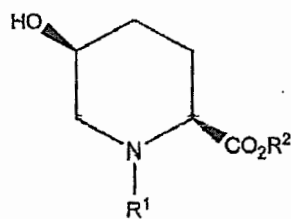
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Claims

1. A method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative, (wherein R^1 represents a protective group for an amino group, and R^2 represents a C_1 - C_6 alkyl group).



(1)



(2)

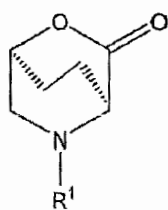
comprising a step of reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride to convert said *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (1); or

comprising a step of reacting *cis*-5-hydroxy-2-piperidinecarboxylic acid with an acid halide and/or acid anhydride, and then with an alcohol in the presence of an acid catalyst, to convert said *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound of Formula (2).

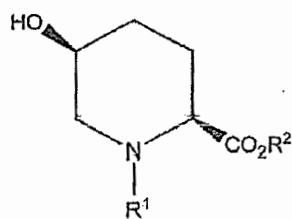
2. A method for regenerating *cis*-5-hydroxy-2-piperidinecarboxylic acid, said method comprising the steps of:

converting *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2) according to the method of claim 1 (wherein R^1 represents a protective group for an amino group, and R^2 represents a C_1 - C_6 alkyl group); and

converting the compound(s) of Formula (1) and/or Formula (2) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.



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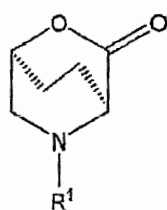


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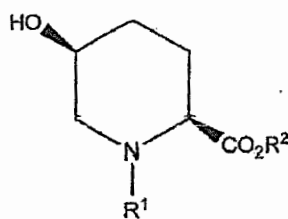
3. The method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative according to claim 1, wherein said *cis*-5-hydroxy-2-piperidinecarboxylic acid is *cis*-5-hydroxy-2-piperidinecarboxylic acid synthesized by bacterial reaction and/or enzymatic reaction.

4. The method for regenerating *cis*-5-hydroxy-2-piperidinecarboxylic acid according to claim 2, wherein said *cis*-5-hydroxy-2-piperidinecarboxylic acid is *cis*-5-hydroxy-2-piperidinecarboxylic acid synthesized by bacterial reaction and/or enzymatic reaction.

5. A method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid, said method comprising a step of reacting a mixture containing *cis*-5-hydroxy-2-piperidinecarboxylic acid and an impurity with an acid halide and/or acid anhydride, or with an acid halide and/or acid anhydride and an alcohol, to convert the *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2), separating said compound(s) and then converting the separated compound(s) into *cis*-5-hydroxy-2-piperidinecarboxylic acid.



(1)



(2)

6. The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to claim 5, wherein said impurity is 2-piperidinecarboxylic acid or an analogue thereof.

7. The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to claim 6, wherein said 2-piperidinecarboxylic acid or an analogue thereof is *trans*-5-hydroxy-2-piperidinecarboxylic acid.

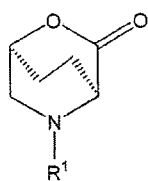
8. The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to any one of claims 5 to 7, wherein said step of separating the compound(s) of Formula (1) and/or Formula (2) is carried out by crystallization or solvent extraction.

9. The method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid according to any one of claims 5 to 8, wherein said mixture containing

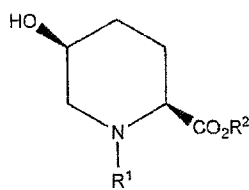
cis-5-hydroxy-2-piperidinecarboxylic acid and an impurity is a mixture synthesized by bacterial reaction and/or enzymatic reaction.

ABSTRACT

The present invention aims to provide a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid with high purity, and a method for producing its derivative. The present invention provides a method for producing a *cis*-5-hydroxy-2-piperidinecarboxylic acid derivative, which method comprises a step of converting *cis*-5-hydroxy-2-piperidinecarboxylic acid into a compound(s) of Formula (1) and/or Formula (2) (wherein R¹ represents a protective group for an amino group, and R² represents a C₁-C₆ alkyl group), and a method for purifying *cis*-5-hydroxy-2-piperidinecarboxylic acid.



(1)



(2)

[Fig. 1]

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Arg Val His Phe Ala Gly Glu His Thr Ala Phe Pro His Ala Trp Ile
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 Ser Gly Leu Gly Lys Ala Met Ala Ile Arg Phe Gly Lys Glu Gln Ala
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 Lys Val Val Ile Asn Tyr Tyr Ser Asn Lys Gln Asp Pro Asn Glu Val
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 Lys Glu Glu Val Ile Lys Ala Gly Gly Glu Ala Val Val Val Gln Gly
 50 55 60
 gat gtc acg aaa gag gaa gat gta aaa aat atc gtg caa acg gca att 240
 Asp Val Thr Lys Glu Glu Asp Val Lys Asn Ile Val Gln Thr Ala Ile
 65 70 75 80
 aag gag ttc ggc aca ctc gat att atg att aat aat gcc ggt ctt gca 288
 Lys Glu Phe Gly Thr Leu Asp Ile Met Ile Asn Asn Ala Gly Leu Ala
 85 90 95
 aat cct gtg cca tct cac gaa atg ccg ctc aag gat tgg gat aaa gtc 336
 Asn Pro Val Pro Ser His Glu Met Pro Leu Lys Asp Trp Asp Lys Val
 100 105 110
 atc ggc acg aac tta acg ggt gcc ttt tta gga agc cgt gaa gcg att 384
 Ile Gly Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg Glu Ala Ile
 115 120 125
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 Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Asn Val Ile Asn Met Ser
 130 135 140
 agt gtg cac gaa gtg att cct tgg ccg tta ttt gtc cac tat gcg gca 480
 Ser Val His Glu Val Ile Pro Trp Pro Leu Phe Val His Tyr Ala Ala
 145 150 155 160
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 165 170 175
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 180 185 190
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 Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Lys Gln Lys Ala Asp
 195 200 205
 gta gaa agc atg att cca atg gga tat atc ggc gaa ccg gag gag atc 672
 Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Glu Pro Glu Glu Ile
 210 215 220
 gcc gca gta gca gcc tgg ctt gct tcg aag gaa gcc agc tac gtc aca 720
 Ala Ala Val Ala Ala Trp Leu Ala Ser Lys Glu Ala Ser Tyr Val Thr
 225 230 235 240
 ggc atc acg tta ttc gcg gac ggc ggt atg aca caa tat cct tca ttc 768
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 245 250 255
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 Gln Ala Gly Arg Gly

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 35 40 45
 Lys Glu Glu Val Ile Lys Ala Gly Gly Glu Ala Val Val Val Gln Gly
 50 55 60
 Asp Val Thr Lys Lys Glu Glu Asp Val Lys Asn Ile Val Gln Thr Ala Ile
 65 70 75 80
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 85 90 95
 Asn Pro Val Pro Ser His Glu Met Pro Leu Lys Asp Trp Asp Lys Val
 100 105 110
 Ile Gly Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg Glu Ala Ile
 115 120 125
 Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Asn Val Ile Asn Met Ser
 130 135 140
 Ser Val His Glu Val Ile Pro Trp Pro Leu Phe Val His Tyr Ala Ala
 145 150 155 160
 Ser Lys Gly Gly Ile Lys Leu Met Thr Glu Thr Leu Ala Leu Glu Tyr
 165 170 175
 Ala Pro Lys Gly Ile Arg Val Asn Asn Ile Gly Pro Gly Ala Ile Asn
 180 185 190
 Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Lys Gln Lys Ala Asp
 195 200 205
 Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Glu Pro Glu Glu Ile
 210 215 220
 Ala Ala Val Ala Ala Trp Leu Ala Ser Lys Glu Ala Ser Tyr Val Thr
 225 230 235 240
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 245 250 255
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 acc gga cag gcc ccc ctg tat gcg gca cca ccg ttg tcg atg gac aac 96
 Thr Gly Gln Ala Pro Leu Tyr Ala Ala Pro Pro Leu Ser Met Asp Asn
 20 25 30
 ggc acc aac acc ctg acc gtg caa aac agc aat gcc tgg gtc gaa gtc 144
 Gly Thr Asn Thr Leu Thr Val Gln Asn Ser Asn Ala Trp Val Glu Val

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Phe	Thr	Leu	Ala	Arg	Asp	Ser	Arg	Leu	Ala	Asn	Ile	Thr	Val	Gly	Tyr
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Tyr Ala Tyr Gln Ile Pro Thr Ser Pro Leu Ile Val Glu Val Thr Pro
20 25 30
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Gln Glu Arg Asn Ile Leu Ser Asn Val Gly Ala Leu Leu Glu Lys Ala
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Phe Lys Ser Tyr Glu Asn Pro Asp Tyr Ile Glu Ala Leu His Leu Tyr
50 55 60
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Ser Phe Gln Leu Leu Pro Glu Arg Ile Ala Arg Ile Leu Ser Arg Phe
65 70 75 80
gga aca gat ttc tca gct gat cag tat ggc gct att att ttt aga ggt 288
Gly Thr Asp Phe Ser Ala Asp Gln Tyr Gly Ala Ile Ile Phe Arg Gly
85 90 95
ctt ctt gaa gtt gat cag gat cat ctg gga cca act cct gcg aat tgg 336

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115 120 125
ttg ctg cat ggt gca gtt cct tca aaa cca gta caa tat tat gcg cag 432
Leu Leu His Gly Ala Val Pro Ser Lys Pro Val Gln Tyr Tyr Ala Gln
130 135 140
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Arg Lys Gly Gly Gly Ile Leu His Ala Val Ile Pro Asp Glu Lys Met
145 150 155 160
gca gct acg caa aca ggt tcg gga tca aaa aca aat ttg tat gtt cat 528
Ala Ala Thr Gln Thr Gly Ser Gly Ser Lys Thr Asn Leu Tyr Val His
165 170 175
aca gaa gat gct ttt ctt tta cat cag gct gat ttt tta agt ttt cta 576
Thr Glu Asp Ala Phe Leu Leu His Gln Ala Asp Phe Leu Ser Phe Leu
180 185 190
tat ctg cga aat gaa gaa aga gtt cct tct aca ctt tac tca gta agg 624
Tyr Leu Arg Asn Glu Glu Arg Val Pro Ser Thr Leu Tyr Ser Val Arg
195 200 205
tcg cat ggt aag gtg aat aag ata atg gaa aag ctt ttt gat cca att 672
Ser His Gly Lys Val Asn Lys Ile Met Glu Lys Leu Phe Asp Pro Ile
210 215 220
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Tyr Gln Cys Pro Lys Asp Ala Asn Tyr Gln Glu Glu Ile Asn Asp Gly
225 230 235 240
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260 265 270
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275 280 285
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Ile Asn Ser Asp Tyr Ile Pro Asp Ser Gly Asp Val Ile Phe Val Asn
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<213> Flavobacterium johnsoniae NBRC14942

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Pro	Leu	Ala	Ser	Val	Leu	Tyr	Gly	Asn	Lys	Lys	Leu	Pro	Phe	Ile	Arg		
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Glu	Ala	Leu	Tyr	Asn	Leu	Thr	Glu	Phe	Trp	Asn	Glu	Ala	Lys	Glu	Leu		
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Ile	Asn	Ser	Asp	Tyr	Ile	Pro	Asp	Ser	Gly	Asp	Val	Ile	Phe	Val	Asn		
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Glu	Glu	Asn	Gly	Lys	Leu	Val	Pro	Cys	Glu	Arg	Arg	Gln	Met	Leu	Arg		
			325				330				335						
Met	Met	Ser	Lys	Thr	Ser	Leu	Ile	His	Ile	Arg	Ser	Met	Thr	His	Thr		
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Asp	Asp	Pro	Tyr	Phe	Val	Met	Glu	Glu	His	Leu	Gly	Lys	Val	Phe	Asp		
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Gln	Ala																
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gag ctg ccc gcg ccc cag cgg gcc gcg ctg gcc gcg ctg ggc gcg cgc																96	
Glu Leu Pro Ala Pro Gln Arg Ala Leu Ala Ala Leu Gly Ala Arg																	
	20											25					30
ctg acc gcg gac ccg gtg acc gag ccc gac gcc ttc ggc cgc cag gcg																144	

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Arg	Leu	Leu	Ala	Arg	Glu	Leu	Ser	Val	Glu	Val	Thr	Glu	Ala	Leu	Trp		
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gcg	ttc	gag	gaa	cgg	gga	tcg	gac	tcc	ggg	gtc	ctc	gtc	ctg	cgc	ggc	240	
Ala	Phe	Glu	Glu	Arg	Gly	Ser	Asp	Ser	Gly	Val	Leu	Val	Leu	Arg	Gly		
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Leu	Glu	Val	Gly	Glu	Leu	Pro	Pro	Thr	Pro	Ala	Asp	Asn	Thr	Gly	Gly		
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atc	ggc	ggg	cgc	acc	ctg	ctc	gcc	cgc	cag	cag	gcg	atc	gtc	agc	cac	336	
Ile	Gly	Gly	Arg	Thr	Leu	Leu	Ala	Arg	Gln	Gln	Ala	Ile	Val	Ser	His		
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Ala	Leu	Gly	His	Met	Val	Gly	Tyr	Ala	Ala	Glu	Gly	His	Gly	His	Leu		
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Gln	Gly	Ser	Arg	Val	Glu	Leu	Glu	Ala	His	Thr	Glu	Gln	Cys	Phe	Ser		
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Asp	Leu	Arg	Pro	Asp	Tyr	Val	Val	Leu	Gly	Cys	Leu	Arg	Gly	Asp	Ala		
			165					170					175				
gac	gcc	gcc	acc	tac	gcg	ttc	cgc	gcc	ctg	gac	ctg	ctg	gcc	cac	gtg	576	
Asp	Ala	Ala	Thr	Tyr	Ala	Phe	Arg	Ala	Leu	Asp	Leu	Leu	Ala	His	Val		
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Asp	Pro	Thr	Asp	Val	Met	Glu	Leu	Phe	Arg	Pro	Leu	Trp	Thr	Thr	Leu		
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gtc	gac	gag	tcc	ttc	gcc	gac	ttc	ctc	gac	acc	cgc	gag	gtg	cgc	ggg	672	
Val	Asp	Glu	Ser	Phe	Ala	Asp	Phe	Leu	Asp	Thr	Arg	Glu	Val	Arg	Gly		
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gac	cag	gac	ctc	atg	cac	ggc	atc	acc	aag	cac	gcc	cag	gcc	ctg	ctg	768	
Asp	Gln	Asp	Leu	Met	His	Gly	Ile	Thr	Lys	His	Ala	Gln	Ala	Leu	Leu		
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Glu	Arg	Val	Leu	Glu	Ile	Tyr	Val	Ala	His	Arg	His	Ala	Val	Val	Leu		
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cag	ccc	ggg	gac	gtg	ctg	ctg	ctg	gac	aac	ctg	cgc	gcc	atg	cac	ggc	864	
Gln	Pro	Gly	Asp	Val	Leu	Leu	Leu	Asp	Asn	Leu	Arg	Ala	Met	His	Gly		
		275					280					285					
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Arg	Ser	Pro	Phe	Ala	Pro	Arg	Phe	Asp	Gly	Thr	Asp	Arg	Phe	Ile	Ser		
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ggc	ggg	aac	cgc	gtc	gtg	cag	gcc	agc	ttc	agc	tga					996	
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35      40      45
Arg Leu Leu Ala Arg Glu Leu Ser Val Glu Val Thr Glu Ala Leu Trp
50      55      60
Ala Phe Glu Glu Arg Gly Ser Asp Ser Gly Val Leu Val Leu Arg Gly
65      70      75      80
Leu Glu Val Gly Glu Leu Pro Pro Thr Pro Ala Asp Asn Thr Gly Gly
85      90      95
Ile Gly Gly Arg Thr Leu Leu Ala Arg Gln Gln Ala Ile Val Ser His
100     105     110
Ala Leu Gly His Met Val Gly Tyr Ala Ala Glu Gly His Gly His Leu
115     120     125
Leu Gln Asp Met Val Pro Asn Ala Arg Leu Ala Ala Thr Gln Gln Ser
130     135     140
Gln Gly Ser Arg Val Glu Leu Glu Ala His Thr Glu Gln Cys Phe Ser
145     150     155     160
Asp Leu Arg Pro Asp Tyr Val Val Leu Gly Cys Leu Arg Gly Asp Ala
165     170     175
Asp Ala Ala Thr Tyr Ala Phe Arg Ala Leu Asp Leu Leu Ala His Val
180     185     190
Asp Pro Thr Asp Val Met Glu Leu Phe Arg Pro Leu Trp Thr Thr Leu
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Val Asp Glu Ser Phe Ala Asp Phe Leu Asp Thr Arg Glu Val Arg Gly
210     215     220
Pro Phe Ser Ile Leu Ser Gly Asp Val Asp Asp Pro Thr Met Leu Val
225     230     235     240
Asp Gln Asp Leu Met His Gly Ile Thr Lys His Ala Gln Ala Leu Leu
245     250     255
Glu Arg Val Leu Glu Ile Tyr Val Ala His Arg His Ala Val Val Leu
260     265     270
Gln Pro Gly Asp Val Leu Leu Leu Asp Asn Leu Arg Ala Met His Gly
275     280     285
Arg Ser Pro Phe Ala Pro Arg Phe Asp Gly Thr Asp Arg Phe Ile Ser
290     295     300
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<210> 21
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Leu Pro Arg Thr Met His Phe Ala Ala Glu Pro Pro Leu Gln Pro Leu
20      25      30
ata ata gat att act gaa gaa gaa aaa ctg gaa att acc tat atc ggg      144
Ile Ile Asp Ile Thr Glu Glu Glu Lys Leu Glu Ile Thr Tyr Ile Gly
35      40      45
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Ser	Met	Leu	His	Leu	Asn	Ala	Tyr	Thr	Leu	Leu	Pro	Glu	Arg	Ile	Ala	
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aag	gtg	ctg	agt	aat	ttc	ggg	aca	gac	ttt	tcc	gac	cag	caa	tac	gga	288
Lys	Val	Leu	Ser	Asn	Phe	Gly	Thr	Asp	Phe	Ser	Asp	Gln	Gln	Tyr	Gly	
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gct	gtc	gta	ttg	cgt	gga	ctg	ata	gaa	ata	ggg	cag	gat	gaa	tta	ggc	336
Ala	Val	Val	Leu	Arg	Gly	Leu	Ile	Glu	Ile	Gly	Gln	Asp	Glu	Leu	Gly	
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cca	acc	cca	cgt	tcc	tgg	cag	gaa	acc	gac	cat	gaa	aag	att	atg	gaa	384
Pro	Thr	Pro	Arg	Ser	Trp	Gln	Glu	Thr	Asp	His	Glu	Lys	Ile	Met	Glu	
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Tyr	Gly	Phe	Ile	Ser	Ser	Leu	Leu	His	Gly	Ala	Val	Pro	Ser	Lys	Pro	
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Val	Glu	Tyr	Phe	Ala	Gln	Arg	Lys	Gly	Gly	Gly	Leu	Met	His	Ala	Ile	
	145			150				155							160	
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Ile	Pro	Asp	Glu	Asn	Met	Ser	Phe	Thr	Gln	Thr	Gly	Ser	Gly	Ser	Arg	
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Thr	Asp	Leu	Phe	Val	His	Thr	Glu	Asp	Ala	Phe	Leu	His	Asn	Ala	Ala	
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gat	ttt	ctg	agt	ttt	ctt	ttc	ctg	cgg	aat	gaa	gaa	cgt	gtg	cct	tcc	624
Asp	Phe	Leu	Ser	Phe	Leu	Phe	Leu	Arg	Asn	Glu	Glu	Arg	Val	Pro	Ser	
	195						200					205				
acc	tta	tat	tct	atc	cgc	tct	cat	ggc	aga	ccg	gat	gcg	ata	tta	cag	672
Thr	Leu	Tyr	Ser	Ile	Arg	Ser	His	Gly	Arg	Pro	Asp	Ala	Ile	Leu	Gln	
	210				215						220					
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Glu	Leu	Phe	Lys	Pro	Ile	Tyr	Lys	Cys	Pro	Lys	Asp	Ala	Asn	Tyr	Ala	
	225			230						235					240	
tcc	gaa	gaa	gcc	ctg	gga	gat	gac	atc	cgt	act	tct	gtt	tta	tat	ggg	768
Ser	Glu	Glu	Ala	Leu	Gly	Asp	Asp	Ile	Arg	Thr	Ser	Val	Leu	Tyr	Gly	
			245				250						255			
agc	aga	tcc	gct	ccc	ttc	atg	cgc	ttt	gat	gct	gcg	gaa	cag	att	tat	816
Ser	Arg	Ser	Ala	Pro	Phe	Met	Arg	Phe	Asp	Ala	Ala	Glu	Gln	Ile	Tyr	
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Asn	Glu	Asp	Ala	Asn	Gln	Asp	Pro	Glu	Ala	Leu	His	Asn	Leu	Lys	Arg	
			275				280					285				
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Phe	Trp	Glu	Glu	Ala	Arg	Lys	Leu	Ile	Tyr	Asn	Asp	Phe	Val	Pro	Glu	
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Ser	Gly	Asp	Leu	Ile	Phe	Val	Asn	Asn	His	Leu	Cys	Ala	His	Gly	Arg	
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aat	gct	ttc	ctg	gca	ggc	ttc	aga	gag	gaa	aat	ggg	cag	ctg	gta	aaa	1008
Asn	Ala	Phe	Leu	Ala	Gly	Phe	Arg	Glu	Glu	Asn	Gly	Gln	Leu	Val	Lys	
			325				330						335			
tgc	gaa	cgc	cgt	ctt	atg	tta	cgt	atg	atg	agc	aaa	acc	agc	ctg	att	1056
Cys	Glu	Arg	Arg	Leu	Met	Leu	Arg	Met	Met	Ser	Lys	Thr	Ser	Leu	Ile	
			340				345						350			
aac	atc	cgt	gaa	gta	acc	cac	ccc	gaa	aac	cct	tat	ctc	atc	atg	gaa	1104
Asn	Ile	Arg	Glu	Val	Thr	His	Pro	Glu	Asn	Pro	Tyr	Leu	Ile	Met	Glu	
			355				360					365				
gag	cac	tac	gga	aaa	gta	tat	agc	gct	cac	ctg	gca	aac	ctt	taa		1149
Glu	His	Tyr	Gly	Lys	Val	Tyr	Ser	Ala	His	Leu	Ala	Asn	Leu			
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 35 40 45
 Lys Lys Leu Lys Arg Lys Tyr Lys Ser Tyr Asp Asp Pro Gly Phe Ile
 50 55 60
 Ser Met Leu His Leu Asn Ala Tyr Thr Leu Leu Pro Glu Arg Ile Ala
 65 70 75 80
 Lys Val Leu Ser Asn Phe Gly Thr Asp Phe Ser Asp Gln Gln Tyr Gly
 85 90 95
 Ala Val Val Leu Arg Gly Leu Ile Glu Ile Gly Gln Asp Glu Leu Gly
 100 105 110
 Pro Thr Pro Arg Ser Trp Gln Glu Thr Asp His Glu Lys Ile Met Glu
 115 120 125
 Tyr Gly Phe Ile Ser Ser Leu Leu His Gly Ala Val Pro Ser Lys Pro
 130 135 140
 Val Glu Tyr Phe Ala Gln Arg Lys Gly Gly Gly Leu Met His Ala Ile
 145 150 155 160
 Ile Pro Asp Glu Asn Met Ser Phe Thr Gln Thr Gly Ser Gly Ser Arg
 165 170 175
 Thr Asp Leu Phe Val His Thr Glu Asp Ala Phe Leu His Asn Ala Ala
 180 185 190
 Asp Phe Leu Ser Phe Leu Phe Leu Arg Asn Glu Glu Arg Val Pro Ser
 195 200 205
 Thr Leu Tyr Ser Ile Arg Ser His Gly Arg Pro Asp Ala Ile Leu Gln
 210 215 220
 Glu Leu Phe Lys Pro Ile Tyr Lys Cys Pro Lys Asp Ala Asn Tyr Ala
 225 230 235 240
 Ser Glu Glu Ala Leu Gly Asp Asp Ile Arg Thr Ser Val Leu Tyr Gly
 245 250 255
 Ser Arg Ser Ala Pro Phe Met Arg Phe Asp Ala Ala Glu Gln Ile Tyr
 260 265 270
 Asn Glu Asp Ala Asn Gln Asp Pro Glu Ala Leu His Asn Leu Lys Arg
 275 280 285
 Phe Trp Glu Glu Ala Arg Lys Leu Ile Tyr Asn Asp Phe Val Pro Glu
 290 295 300
 Ser Gly Asp Leu Ile Phe Val Asn Asn His Leu Cys Ala His Gly Arg
 305 310 315 320
 Asn Ala Phe Leu Ala Gly Phe Arg Glu Glu Asn Gly Gln Leu Val Lys
 325 330 335
 Cys Glu Arg Arg Leu Met Leu Arg Met Met Ser Lys Thr Ser Leu Ile
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 355 360 365
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ctg ctt cct acg gtc ata gaa gtc act tct cag gag aga aga atg ata      96
Leu Leu Pro Thr Val Ile Glu Val Thr Ser Gln Glu Arg Arg Met Ile
      20      25      30
aaa gat gca gcg ctg cat ctt cag aaa aaa tat ggc act tat gaa aac      144
Lys Asp Ala Ala Leu His Leu Gln Lys Lys Tyr Gly Thr Tyr Glu Asn
      35      40      45
cgg gat ttt ata aaa cat gtt cat caa ctg gcc tct tat ttt ctt ccg      192
Arg Asp Phe Ile Lys His Val His Gln Leu Ala Ser Tyr Phe Leu Pro
      50      55      60
gaa agg att cta aat ata gca gct gat ttt gca agt gac ttt tct gaa      240
Glu Arg Ile Leu Asn Ile Ala Ala Asp Phe Ala Ser Asp Phe Ser Glu
      65      70      75      80
aat cag tat gga gcg ctg gtt ttt aca gga ttg atg gag ata gac cag      288
Asn Gln Tyr Gly Ala Leu Val Phe Thr Gly Leu Met Glu Ile Asp Gln
      85      90      95
gaa gaa ata ggt tct act cca ccc aac tgg caa tcg gca gat tat tca      336
Glu Glu Ile Gly Ser Thr Pro Pro Asn Trp Gln Ser Ala Asp Tyr Ser
      100      105      110
aag ttt aat tta tat ggt ttt gcg tgt gcg ctt att cat ggg gca ctt      384
Lys Phe Asn Leu Tyr Gly Phe Ala Cys Ala Leu Ile His Gly Ala Leu
      115      120      125
ccc tca aag cct gta caa tat tat tca cag cgt aaa ggc ggt gga ttg      432
Pro Ser Lys Pro Val Gln Tyr Tyr Ser Gln Arg Lys Gly Gly Gly Leu
      130      135      140
atc cac gct att att cct gat gaa aaa atg aaa gaa aca cag aca gga      480
Ile His Ala Ile Ile Pro Asp Glu Lys Met Lys Glu Thr Gln Thr Gly
      145      150      155      160
tca gga tcc tca acg gat ctg tat gta cat aca gaa gat gct ttt ctg      528
Ser Gly Ser Ser Thr Asp Leu Tyr Val His Thr Glu Asp Ala Phe Leu
      165      170      175
aaa cat cag gct gac ttt tta agc ttt atg tat gtc cga aat gaa gag      576
Lys His Gln Ala Asp Phe Leu Ser Phe Met Tyr Val Arg Asn Glu Glu
      180      185      190
cag gta cct tca act ctt tat tct atc cgt tct cat gag tct att ggg      624
Gln Val Pro Ser Thr Leu Tyr Ser Ile Arg Ser His Glu Ser Ile Gly
      195      200      205
gaa aag tac agg aca ctt ttt gag cct att tat aaa atc cct aaa gat      672
Glu Lys Tyr Arg Thr Leu Phe Glu Pro Ile Tyr Lys Ile Pro Lys Asp
      210      215      220
gcc aat ctg gaa acg gga gaa aat gaa gaa gaa act ctg gat tct gta      720
Ala Asn Leu Glu Thr Gly Glu Asn Glu Glu Glu Thr Leu Asp Ser Val
      225      230      235      240
ttg tat gga aat acc aac ctt cct ttt atg cga ttt gat gcg gcg gaa      768
Leu Tyr Gly Asn Thr Asn Leu Pro Phe Met Arg Phe Asp Ala Ala Glu
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cag ctt ttc aat tcc agt atc aga cag tca gaa gaa gcg cag cat aca      816
Gln Leu Phe Asn Ser Ser Ile Arg Gln Ser Glu Glu Ala Gln His Thr
      260      265      270
ctg cat gag ttc tgg gaa gaa gcc aga cat ttg att tat tca gga ttt      864
Leu His Glu Phe Trp Glu Glu Ala Arg His Leu Ile Tyr Ser Gly Phe
      275      280      285
acg cct cag gcc gga gat gtt att ctg gtt aat aat cat tta tgt gct      912
Thr Pro Gln Ala Gly Asp Val Ile Leu Val Asn Asn His Leu Cys Ala
      290      295      300
cac gga aga tct gct ttc cgt gcg gga gta aga aat att gac ggt ata      960
His Gly Arg Ser Ala Phe Arg Ala Gly Val Arg Asn Ile Asp Gly Ile
      305      310      315      320
gaa cag ccg tgc gaa cga aga att atg ctt cgg atg atg agt aaa gtg      1008
Glu Gln Pro Cys Glu Arg Arg Ile Met Leu Arg Met Met Ser Lys Val

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gtc ata gaa gaa cat ctg ggt aaa aac ttt caa cat ttt taa						1098
Val Ile Glu Glu His Leu Gly Lys Asn Phe Gln His Phe						
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	20														
Lys Asp Ala Ala Leu His Leu Gln Lys Lys Tyr Gly Thr Tyr Glu Asn							40					45			
	35														
Arg Asp Phe Ile Lys His Val His Gln Leu Ala Ser Tyr Phe Leu Pro							55				60				
	50														
Glu Arg Ile Leu Asn Ile Ala Ala Asp Phe Ala Ser Asp Phe Ser Glu											75			80	
	65														
Asn Gln Tyr Gly Ala Leu Val Phe Thr Gly Leu Met Glu Ile Asp Gln															
		85					90						95		
Glu Glu Ile Gly Ser Thr Pro Pro Asn Trp Gln Ser Ala Asp Tyr Ser															
	100						105						110		
Lys Phe Asn Leu Tyr Gly Phe Ala Cys Ala Leu Ile His Gly Ala Leu															
	115						120						125		
Pro Ser Lys Pro Val Gln Tyr Tyr Ser Gln Arg Lys Gly Gly Gly Leu															
	130						135						140		
Ile His Ala Ile Ile Pro Asp Glu Lys Met Lys Glu Thr Gln Thr Gly															
	145					150					155				160
Ser Gly Ser Ser Thr Asp Leu Tyr Val His Thr Glu Asp Ala Phe Leu															
		165					170							175	
Lys His Gln Ala Asp Phe Leu Ser Phe Met Tyr Val Arg Asn Glu Glu															
	180						185						190		
Gln Val Pro Ser Thr Leu Tyr Ser Ile Arg Ser His Glu Ser Ile Gly															
	195						200						205		
Glu Lys Tyr Arg Thr Leu Phe Glu Pro Ile Tyr Lys Ile Pro Lys Asp															
	210					215					220				
Ala Asn Leu Glu Thr Gly Glu Asn Glu Glu Glu Thr Leu Asp Ser Val															
	225				230						235				240
Leu Tyr Gly Asn Thr Asn Leu Pro Phe Met Arg Phe Asp Ala Ala Glu															
		245								250				255	
Gln Leu Phe Asn Ser Ser Ile Arg Gln Ser Glu Glu Ala Gln His Thr															
	260						265						270		
Leu His Glu Phe Trp Glu Glu Ala Arg His Leu Ile Tyr Ser Gly Phe															
	275						280						285		
Thr Pro Gln Ala Gly Asp Val Ile Leu Val Asn Asn His Leu Cys Ala															
	290					295					300				
His Gly Arg Ser Ala Phe Arg Ala Gly Val Arg Asn Ile Asp Gly Ile															
	305				310					315					320
Glu Gln Pro Cys Glu Arg Arg Ile Met Leu Arg Met Met Ser Lys Val															
		325							330					335	
Ser Leu Ile Asp Met Arg Ala His Thr Leu Thr Glu Asp Pro Phe Phe															
	340						345						350		
Val Ile Glu Glu His Leu Gly Lys Asn Phe Gln His Phe															
	355					360							365		

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 <211> 1116

<212> DNA

<213> Niastella koreensis NBRC106392

<220>

<221> CDS

<222> (1)..(1113)

<400> 25

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tta ccg cca cct ttg agt cca ttg atc gtt gac gtt aca ccg aag gag	96
Leu Pro Pro Pro Leu Ser Pro Leu Ile Val Asp Val Thr Pro Lys Glu	
20 25 30	
cgt gcc tcc att tca aac gtg gcc aat atc tta tta aaa gcc ttt ggc	144
Arg Ala Ser Ile Ser Asn Val Ala Asn Ile Leu Leu Lys Ala Phe Gly	
35 40 45	
cat tat gaa cat cct gat ttc atc tcc gct ttg cac ctg aat gct ttt	192
His Tyr Glu His Pro Asp Phe Ile Ser Ala Leu His Leu Asn Ala Phe	
50 55 60	
cag tta tta ccg gaa cgt att gcg ggg ata ctg agc cgt ttt ggt acc	240
Gln Leu Leu Pro Glu Arg Ile Ala Gly Ile Leu Ser Arg Phe Gly Thr	
65 70 75 80	
gac ttc tcg cgc cac caa tac ggc gcg ttg gtg ttc aga ggc ctt aca	288
Asp Phe Ser Arg His Gln Tyr Gly Ala Leu Val Phe Arg Gly Leu Thr	
85 90 95	
gaa gta gat cag gag gcg ctt ggc cct acc ccg ccc tcg tgg aaa gaa	336
Glu Val Asp Gln Glu Ala Leu Gly Pro Thr Pro Pro Ser Trp Lys Glu	
100 105 110	
acc gat tac agc aag ctt gtt aaa tat gga ttt att tgc tcg ctg ctg	384
Thr Asp Tyr Ser Lys Leu Val Lys Tyr Gly Phe Ile Cys Ser Leu Leu	
115 120 125	
cat ggc gcc att cca tca aaa cca gta caa tat tat gcg cag cga aaa	432
His Gly Ala Ile Pro Ser Lys Pro Val Gln Tyr Tyr Ala Gln Arg Lys	
130 135 140	
ggc ggt ggt tta ctg cat gcc gtt att ccc gat gaa aaa atg agt cat	480
Gly Gly Gly Leu Leu His Ala Val Ile Pro Asp Glu Lys Met Ser His	
145 150 155 160	
acg caa acc ggc tcc ggc tcg cgc acc gat ctt ttt gtg cat acc gaa	528
Thr Gln Thr Gly Ser Gly Ser Arg Thr Asp Leu Phe Val His Thr Glu	
165 170 175	
gat gcg ttc tta ttt aac cag gcc gat ttt ctc agc ttc ctg ttc ctg	576
Asp Ala Phe Leu Phe Asn Gln Ala Asp Phe Leu Ser Phe Leu Phe Leu	
180 185 190	
cgg aat gaa gaa cag gtg cca tct acg tta tat tcg atc cgg tcg cat	624
Arg Asn Glu Glu Gln Val Pro Ser Thr Leu Tyr Ser Ile Arg Ser His	
195 200 205	
ggc gat acc aac gcc atc atg gcg gag ctg ttc aaa ccc att tat aag	672
Gly Asp Thr Asn Ala Ile Met Ala Glu Leu Phe Lys Pro Ile Tyr Lys	
210 215 220	
tgt ccg aag gat gcg aat tat gcc gac gat gaa aat gcc ggc gag gaa	720
Cys Pro Lys Asp Ala Asn Tyr Ala Asp Asp Glu Asn Ala Gly Glu Glu	
225 230 235 240	
gtg acc act tct atc tta tac ggt aac cgc gaa cgg ccc ttt atc cgc	768
Val Thr Thr Ser Ile Leu Tyr Gly Asn Arg Glu Arg Pro Phe Ile Arg	
245 250 255	
ttc gat gcc gcg gaa cag atc tac aac gaa aag gcc gga caa acg ccg	816
Phe Asp Ala Ala Glu Gln Ile Tyr Asn Glu Lys Ala Gly Gln Thr Pro	
260 265 270	
gaa gcc atg cac aac ctg gtg cgt ttt tgg gac gaa gcc aaa caa ctt	864
Glu Ala Met His Asn Leu Val Arg Phe Trp Asp Glu Ala Lys Gln Leu	
275 280 285	
atc tac aat gat ttc gtg ccc gat tcg ggc gat ctc att ttt gta aac	912
Ile Tyr Asn Asp Phe Val Pro Asp Ser Gly Asp Leu Ile Phe Val Asn	

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290	295	300	
aac cat ttg tgc gcg cat ggc cgg aat tca ttt gtg gcc ggt tat cgt			960
Asn His Leu Cys Ala His Gly Arg Asn Ser Phe Val Ala Gly Tyr Arg			
305	310	315	320
aat gaa aac ggt cag ctg gta aaa tgt gaa cgc cgg ttg atg tta cgc			1008
Asn Glu Asn Gly Gln Leu Val Lys Cys Glu Arg Arg Leu Met Leu Arg			
	325	330	335
atg atg agc aag acc agc ctc atc aat att cag tcg gtg acc cag tta			1056
Met Met Ser Lys Thr Ser Leu Ile Asn Ile Gln Ser Val Thr Gln Leu			
	340	345	350
aac gac ccg tat ttc att atg gaa gaa cac tac ggc aaa ttg ttt cat			1104
Asn Asp Pro Tyr Phe Ile Met Glu Glu His Tyr Gly Lys Leu Phe His			
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tca caa caa taa			1116
Ser Gln Gln			
370			

<210> 26

<211> 371

<212> PRT

<213> Niastella koreensis NBRC106392

<400> 26

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Arg Ala Ser Ile Ser Asn Val Ala Asn Ile Leu Leu Lys Ala Phe Gly	
	35 40 45
His Tyr Glu His Pro Asp Phe Ile Ser Ala Leu His Leu Asn Ala Phe	
	50 55 60
Gln Leu Leu Pro Glu Arg Ile Ala Gly Ile Leu Ser Arg Phe Gly Thr	
65	70 75 80
Asp Phe Ser Arg His Gln Tyr Gly Ala Leu Val Phe Arg Gly Leu Thr	
	85 90 95
Glu Val Asp Gln Glu Ala Leu Gly Pro Thr Pro Pro Ser Trp Lys Glu	
	100 105 110
Thr Asp Tyr Ser Lys Leu Val Lys Tyr Gly Phe Ile Cys Ser Leu Leu	
	115 120 125
His Gly Ala Ile Pro Ser Lys Pro Val Gln Tyr Tyr Ala Gln Arg Lys	
	130 135 140
Gly Gly Gly Leu Leu His Ala Val Ile Pro Asp Glu Lys Met Ser His	
145	150 155 160
Thr Gln Thr Gly Ser Gly Ser Arg Thr Asp Leu Phe Val His Thr Glu	
	165 170 175
Asp Ala Phe Leu Phe Asn Gln Ala Asp Phe Leu Ser Phe Leu Phe Leu	
	180 185 190
Arg Asn Glu Glu Gln Val Pro Ser Thr Leu Tyr Ser Ile Arg Ser His	
	195 200 205
Gly Asp Thr Asn Ala Ile Met Ala Glu Leu Phe Lys Pro Ile Tyr Lys	
	210 215 220
Cys Pro Lys Asp Ala Asn Tyr Ala Asp Asp Glu Asn Ala Gly Glu Glu	
225	230 235 240
Val Thr Thr Ser Ile Leu Tyr Gly Asn Arg Glu Arg Pro Phe Ile Arg	
	245 250 255
Phe Asp Ala Ala Glu Gln Ile Tyr Asn Glu Lys Ala Gly Gln Thr Pro	
	260 265 270
Glu Ala Met His Asn Leu Val Arg Phe Trp Asp Glu Ala Lys Gln Leu	
	275 280 285
Ile Tyr Asn Asp Phe Val Pro Asp Ser Gly Asp Leu Ile Phe Val Asn	
	290 295 300
Asn His Leu Cys Ala His Gly Arg Asn Ser Phe Val Ala Gly Tyr Arg	
305	310 315 320
Asn Glu Asn Gly Gln Leu Val Lys Cys Glu Arg Arg Leu Met Leu Arg	

				325					330					335			
Met	Met	Ser	Lys	Thr	Ser	Leu	Ile	Asn	Ile	Gln	Ser	Val	Thr	Gln	Leu		
			340					345					350				
Asn	Asp	Pro	Tyr	Phe	Ile	Met	Glu	Glu	His	Tyr	Gly	Lys	Leu	Phe	His		
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Ser	Gln	Gln															
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<210> 27
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<220>
 <223> artificial synthesis

<220>
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 <222> (1)..(1065)

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agc tta cgt gtc gca cgc agc aaa gag agc gat att acg gtc cac gaa	96
Ser Leu Arg Val Ala Arg Ser Lys Glu Ser Asp Ile Thr Val His Glu	
20 25 30	
ctg cag agc agc ctg ttt acg ctg gat tct gct agc gcg gaa gcg atc	144
Leu Gln Ser Ser Leu Phe Thr Leu Asp Ser Ala Ser Ala Glu Ala Ile	
35 40 45	
cat acc gcg gct gag cgc att acc gcc cac ccg aac gaa aac ccg gac	192
His Thr Ala Ala Glu Arg Ile Thr Ala His Pro Asn Glu Asn Pro Asp	
50 55 60	
gat ttc ggc cgt cag gcg ctg gca gcg gcg ttt agc ttg ccg gaa gag	240
Asp Phe Gly Arg Gln Ala Leu Ala Ala Ala Phe Ser Leu Pro Glu Glu	
65 70 75 80	
gtg cgt gcg gcg gtc ttg aat ttt gcc gag gtg ggt agc gag agc ggc	288
Val Arg Ala Ala Val Leu Asn Phe Ala Glu Val Gly Ser Glu Ser Gly	
85 90 95	
atc atg gtt gtt cgt ggt ctg tac gtg gat gag gac ctg gcc gac acc	336
Ile Met Val Val Arg Gly Leu Tyr Val Asp Glu Asp Leu Ala Asp Thr	
100 105 110	
ccg ctg gat aac aag agc ggc ctg ggt gcg cgt acc gtt ttt gcg aaa	384
Pro Leu Asp Asn Lys Ser Gly Leu Gly Ala Arg Thr Val Phe Ala Lys	
115 120 125	
gag atg gcc atg ctg gcg cat ctg ctg ggc agc atg gtg gcg tac gag	432
Glu Met Ala Met Leu Ala His Leu Leu Gly Ser Met Val Ala Tyr Glu	
130 135 140	
gcg gaa ggc aac ggt cat ctg att caa gac atg gtg ccg aat ccg aag	480
Ala Glu Gly Asn Gly His Leu Ile Gln Asp Met Val Pro Asn Pro Lys	
145 150 155 160	
ctg gcg gtc acg caa caa agc cag ggt agc aag gtt gag ctg gaa gca	528
Leu Ala Val Thr Gln Gln Ser Gln Gly Ser Lys Val Glu Leu Glu Ala	
165 170 175	
cat acc gag cag tgc ttc agc gac ttc aaa ccg gat tat gtt att ctg	576
His Thr Glu Gln Cys Phe Ser Asp Phe Lys Pro Asp Tyr Val Ile Leu	
180 185 190	
ggt gct ctg cgt ggc gac gaa aac gcc aac acc tat gca ttc tcc ggt	624
Gly Ala Leu Arg Gly Asp Glu Asn Ala Asn Thr Tyr Ala Phe Ser Gly	
195 200 205	
cgc aaa ctg gtt cag cac atg tcc gcc gaa gaa gtg gcg aaa ctg cgc	672
Arg Lys Leu Val Gln His Met Ser Ala Glu Glu Val Ala Lys Leu Arg	
210 215 220	

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Gln Pro Leu Trp Ala Thr Thr Ile Asp Glu Ser Phe Gln Pro Tyr Ile
225                230                235                240
ccg aat ccg gac gac gtt cgc ggt ccg ttc ccg att ctg acg ggc cca      768
Pro Asn Pro Asp Asp Val Arg Gly Pro Phe Pro Ile Leu Thr Gly Pro
                245                250                255
gag gac gat ccg tac atc cgt gta gac cag gag ctg atg cac ggt atc      816
Glu Asp Asp Pro Tyr Ile Arg Val Asp Gln Glu Leu Met His Gly Ile
                260                265                270
acc gcg gac gct caa cgc ctg ctg cgc aag gtt gtg gat acg tat atc      864
Thr Ala Asp Ala Gln Arg Leu Leu Arg Lys Val Val Asp Thr Tyr Ile
                275                280                285
gag cac cgc gac gcg cat gtc ttg cag cct ggt gat ctg ctg atg ctg      912
Glu His Arg Asp Ala His Val Leu Gln Pro Gly Asp Leu Leu Met Leu
                290                295                300
gac aat ctg cgt gca atg cac ggt cgt agc atg ttc gct ccg cgt ttt      960
Asp Asn Leu Arg Ala Met His Gly Arg Ser Met Phe Ala Pro Arg Phe
305                310                315                320
gac ggc aag gat cgt ttc att gca cgt ggt ttc gtc gtg cgt gat cgt      1008
Asp Gly Lys Asp Arg Phe Ile Ala Arg Gly Phe Val Val Arg Asp Arg
                325                330                335
cgt aag ttg tgg ccg cag ctg ttg gaa gat cgt cgc acc ctg ggt gca      1056
Arg Lys Leu Trp Pro Gln Leu Leu Glu Asp Arg Arg Thr Leu Gly Ala
                340                345                350
gtc cac tcc taa
Val His Ser
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<210> 28

<211> 355

<212> PRT

<213> marine actinobacterium PHSC20C1

<400> 28

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20        25        30
Leu Gln Ser Ser Leu Phe Thr Leu Asp Ser Ala Ser Ala Glu Ala Ile
35        40        45
His Thr Ala Ala Glu Arg Ile Thr Ala His Pro Asn Glu Asn Pro Asp
50        55        60
Asp Phe Gly Arg Gln Ala Leu Ala Ala Ala Phe Ser Leu Pro Glu Glu
65        70        75        80
Val Arg Ala Ala Val Leu Asn Phe Ala Glu Val Gly Ser Glu Ser Gly
85        90        95
Ile Met Val Val Arg Gly Leu Tyr Val Asp Glu Asp Leu Ala Asp Thr
100       105       110
Pro Leu Asp Asn Lys Ser Gly Leu Gly Ala Arg Thr Val Phe Ala Lys
115       120       125
Glu Met Ala Met Leu Ala His Leu Leu Gly Ser Met Val Ala Tyr Glu
130       135       140
Ala Glu Gly Asn Gly His Leu Ile Gln Asp Met Val Pro Asn Pro Lys
145       150       155       160
Leu Ala Val Thr Gln Gln Ser Gln Gly Ser Lys Val Glu Leu Glu Ala
165       170       175
His Thr Glu Gln Cys Phe Ser Asp Phe Lys Pro Asp Tyr Val Ile Leu
180       185       190
Gly Ala Leu Arg Gly Asp Glu Asn Ala Asn Thr Tyr Ala Phe Ser Gly
195       200       205
Arg Lys Leu Val Gln His Met Ser Ala Glu Glu Val Ala Lys Leu Arg
210       215       220
Gln Pro Leu Trp Ala Thr Thr Ile Asp Glu Ser Phe Gln Pro Tyr Ile

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			245				250							255	
Glu	Asp	Asp	Pro	Tyr	Ile	Arg	Val	Asp	Gln	Glu	Leu	Met	His	Gly	Ile
		260					265						270		
Thr	Ala	Asp	Ala	Gln	Arg	Leu	Leu	Arg	Lys	Val	Val	Asp	Thr	Tyr	Ile
		275					280						285		
Glu	His	Arg	Asp	Ala	His	Val	Leu	Gln	Pro	Gly	Asp	Leu	Leu	Met	Leu
	290					295					300				
Asp	Asn	Leu	Arg	Ala	Met	His	Gly	Arg	Ser	Met	Phe	Ala	Pro	Arg	Phe
305					310					315				320	
Asp	Gly	Lys	Asp	Arg	Phe	Ile	Ala	Arg	Gly	Phe	Val	Val	Arg	Asp	Arg
			325					330					335		
Arg	Lys	Leu	Trp	Pro	Gln	Leu	Leu	Glu	Asp	Arg	Arg	Thr	Leu	Gly	Ala
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Val	His	Ser													
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41

<210> 31
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<220>
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<400> 31
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31

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<400> 32

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<210> 36
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aatagctcga gaaaatgttg aaagttttta cc 32

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ataatcatat ggaaactatc attgaatcc 29

<210> 38

<211> 33

<212> DNA

<213> Artificial Sequence

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<400> 38

aatagctcga gttggttgtga atgaaacaat ttg

33