FUNGAL CELL WALL SYNTHESIS GENE

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ABSTRACT
A reporter system reflecting the transport process that transports GPI-anchored proteins to the cell wall was constructed and compounds inhibiting this process were discovered. Further, genes conferring resistance to the above compounds were identified and methods of screening for compounds that inhibit the activity of the proteins encoded by these genes were developed. Therefore, through the novel compounds, the present invention showed that antifungal compounds having a novel mechanism, i.e. inhibiting the process that transports GPI-anchored proteins to the cell wall, could be achieved.
FIG. 1
FIG. 3
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FIG. 7
FUNGAL CELL WALL SYNTHESIS GENE
CROSS-REFERENCES TO RELATED APPLICATIONS


BACKGROUND OF THE INVENTION

[0002] 1. Technical Field

[0003] The present invention relates to DNAs encoding proteins participating in fungal cell wall synthesis, proteins encoded by the DNAs, methods for examining whether or not a certain compound has an influence on the transport process involved in the transport of GPI-anchored proteins to the cell wall, and antifungal agents having an influence on the transport process involved in the transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the cell wall.

[0004] 2. Background Art

[0005] In recent years, management of opportunistic infections is gaining importance more than ever due to an increase in the number of elderly people and immunocompromised patients as a result of advanced chemotherapies, etc. Deep-seated mycosis due to Candida, Aspergillus, Cryptococcus, and such, account for a portion of such opportunistic infections, and the proportion is increasing year after year. The fact that opportunistic infections by many virulent bacteria occur one after another, shows that the problem of infectious diseases will not end as long as there are underlying diseases that diminish the immune functions of patients. Although new strategies for infectious diseases control, including the problem of resistant bacteria, will be one of the crucial issues in the soon-to-come aged society, extremely few effective therapeutic agents exist at present.

[0006] Up to now, therapeutic agents for fungal infections were developed based mainly on the strategy of creating novel compounds by chemically modifying known structure. However, due to problems such as the emergence of resistant bacteria, the development of new drugs based on new mechanisms is eagerly anticipated.

[0007] Considering such circumstances, the inventors focused on a novel approach in the area of antifungal agents in which the variety of therapeutic agents is still insufficient. Namely, the present inventors concentrated on influencing the onset, progress, and persistence of infections by preventing pathogens from showing pathogenicity. In order to avoid the establishment and progress of infection, the inventors thought that the most effective way would be to inhibit the adhesion onto the host, which is the first step in the establishment of infection, and the subsequent progression of colonization. In addition, a new unprecedented approach, namely, the inhibition of the expression of adhesion factors themselves, was also carried out.

[0008] In order to inhibit the expression of adhesion factors, the present inventors directed their attention to the hypothesis that cell wall glycoproteins such as adhesion factors are first GPI (Glycosylphosphatidylinositol)-anchored to the cell membrane, and then transported to the cell wall (FIG. 1). To date, 30 or more cell wall glycoproteins including adhesion ligands have been found to be transported via GPI-anchoring (referred to as GPI-anchored proteins). Hence, it was thought that if this transport step is inhibited, it may be quite possible to inhibit the expression of adhesion factors and major cell wall-constituting proteins at the cell wall (Ivanov K et al, Mol. Gen. Genet., 258: 53-59, 1998). GPI-anchored proteins have been reported to be present in Candida, which is a pathogenic fungi (Kapteyn J C et al, Eur. J. Cell Biol., 65:402-407, 1994).

[0009] The inventors initiated their research believing that novel antifungal agents that inhibit cell wall synthesis can be produced by inhibiting the process that transports GPI-anchored proteins existing in the cell membrane of a fungus to the cell wall.

BRIEF SUMMARY OF THE INVENTION

Disclosure of the Invention

[0010] An objective of this invention is to develop antifungal agents showing effects against the onset, progress, and persistence of infections by inhibiting the expression of cell wall glycoproteins, inhibiting the cell wall assembly and also adhesion onto cells, and preventing pathogens from showing pathogenicity.

[0011] In order to screen for compounds that inhibit the process that transports GPI-anchored proteins to the cell wall, the present inventors produced a reporter system that uses a fusion protein comprising a reporter enzyme and a transport signal existing in the C-terminals of one of the GPI-anchored proteins, CW2P (Van Der Vaart J M et al, J. Bacteriol., 177: 3104-3110, 1995).

[0012] When a DNA comprising a secretion signal gene-reporter enzyme gene-CW2P C-termius gene (present or absent) was constructed, and the fusion protein was expressed in Saccharomyces cerevisiae (hereinafter referred to as S. cerevisiae), it was demonstrated that activity of the reporter enzyme is detected in the cell wall when the CW2P C-termius is present, and in the culture supernatant when the CW2P C-termius is absent. Accordingly, it was predicted that if the process that transports GPI-anchored proteins to the cell wall is inhibited by a test sample, the activity of the reporter enzyme in the cell wall will be diminished, or the activity of the reporter enzyme will be found in the culture supernatant. Thus was initiated the screening for compounds that inhibit the process that transports GPI-anchored proteins to the cell wall using this reporter system. From the screening using this reporter system, several compounds that inhibit the process that transports GPI-anchored proteins to the cell wall were discovered. A representative example is the compound shown in formula (I).

[0013] The compound shown in the aforementioned formula (I) (hereinafter abbreviated as "compound (I)") inhib-
its the growth of _S. cerevisiae_ and _Candida albicans_ (hereinafter, referred to as _C. albicans_), and _C. albicans_ cultured in the presence of the aforementioned compound (la) shows a weak ability to adhere onto cells. Thus, the aforementioned compound (la) was confirmed to suit the initial objectives of the invention, which was to find a compound that inhibits the adhesion of fungi, due to suppressing the expression of the fungal adhesins, based on the inhibition of transport system of GPI-anchored proteins to the cell wall. Furthermore, observations using a transmission electron microscope confirmed that _C. albicans_ cultured in the presence of the aforementioned compound (la) has an abnormality in its cell wall synthesis.

Using the aforementioned compound (la), the present inventors proved that antifungal agents based on the mechanism that inhibits the process that transports GPI-anchored proteins to the cell wall, could be achieved.

Furthermore, to specify the target protein on which the aforementioned compound (la) acts, the present inventors searched for genes that confer resistance to the aforementioned compound (la).

A plasmid library of the _S. cerevisiae_ gene was introduced into _S. cerevisiae_, and by overexpression, plasmids were collected that showed resistance to the above-mentioned compound (la). The resistant gene was then cloned, the nucleotide sequence was determined, and the gene was named GWT1 (SEQ ID NO:1). In _S. cerevisiae_ overexpressing the GWT1 gene product, the aforementioned reporter enzyme that has the C-terminals of a GPI-anchored protein was transported to the cell wall, even in the presence of the aforementioned compound (la). Furthermore, observations under a transmission electron microscope confirmed that the cell wall is normal even in the presence of the aforementioned compound (la).

Moreover, when point mutations were randomly introduced to the genomic DNA of _S. cerevisiae_, and mutant strains R1 and R5 showing specific resistance to the aforementioned compound (la) were isolated, point mutations involving changes of the 405th codon of the GWT1 gene from GTC to ATC in the R1 mutant strain, and the 140th codon from GGG to AGG in the R5 mutant strain were discovered. Since resistance to the aforementioned compound (la) was seen when these mutant GWT1 genes were introduced to a GWT1 gene-disrupted strain, resistance to this compound was found to be explainable by the GWT1 gene alone. Therefore, this suggested that the aforementioned compound (la) directly acts on the GWT1 gene product to inhibit the function of the GWT1 protein.

By similar methods, the resistant genes of _C. albicans_ (SEQ ID NOs: 3 and 5) were cloned, the nucleotide sequences were determined, and the genes were named CoGWT1.

Furthermore, a database homology search using GWT1, revealed a homologue (SEQ ID NO:27) of _Schizosaccharomyces pombe_ (hereinafter, referred to as _S. pombe_). Furthermore, PCR with primers based on the sequence of the highly conserved region in the proteins encoded by the GWT1 genes of _S. cerevisiae_, _S. pombe_, and _C. albicans_, yielded homologues (SEQ ID NOs: 39 and 41) of _Aspergillus fumigatus_ (hereinafter, referred to as _A. fumigatus_). Furthermore, by performing PCR based on the sequence discovered from a database homology search with GWT1, revealed homologues (SEQ ID NOs: 54 and 58) of _Cryptococcus neoformans_ (hereinafter, referred to as _C. neoformans_).

More specifically, this invention relates to the following.

1. A DNA that encodes a protein having an activity to confer resistance to the compound shown in formula (la) on a fungus when the DNA is overexpressed in the fungus, wherein the DNA is selected from the group consisting of:
   (a) A DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40, or 59;
   (b) A DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54, or 58;
   (c) A DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54, or 58;
   (d) A DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40, or 59, wherein one or more amino acids have been added, deleted, substituted, and/or inserted;
   (e) A DNA that is amplified using SEQ ID NO: 29 and 31 or SEQ ID NO: 29 and 30 as primers.

2. A DNA that encodes a protein having an activity to decrease the amount of a GPI-anchored protein in the cell wall of a fungus due to a defect in the function of the DNA, wherein the DNA is selected from the group consisting of:
   (a) A DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40, or 59;
   (b) A DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54, or 58;
   (c) A DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54, or 58;
   (d) A DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40, or 59, wherein one or more amino acids have been added, deleted, substituted, and/or inserted; and
   (e) A DNA that is amplified using SEQ ID NO: 29 and 31 or SEQ ID NO: 29 and 30 as primers.

and wherein, “stringent conditions” refer to: for example, hybridization in 4xSSC at 65°C, then washing in 0.1xSSC for 1 hour at 65°C; or in a different method, “stringent conditions” are 4xSSC at 42°C in 50% formamide; or, hybridization in PERFECTHYB™ (TOYOBO) solution for 2.5 hours at 65°C, then washing in (i) 2xSSC, 0.05% SDS solution at 25°C for 5 minutes, (ii) 2xSSC, 0.05% SDS solution at 25°C for 5 minutes, and (iii) 0.1xSSC, 0.1% SDS solution at 50°C for 20 minutes;

A “defect in the DNA function” can occur, when the functional gene product of the DNA is not expressed or when the expression is diminished, for example by inserting a DNA that is irrelevant to the coding region of
the DNA, for example a selection marker, using the homologous recombination technique;  

... and a decrease in the protein derived from the GPI-anchored protein in the fungal cell wall is quantified by using any one of the following methods alone or in combination:  

... (i) a reporter system reflecting the process that transports GPI-anchored proteins to the cell wall, (ii) an enzyme linked immunosorbent assay (ELISA) that quantifies a GPI-anchored protein in the cell wall, (iii) measuring the activity of a GPI-anchored protein, such as adhesion onto animal cells, or (iv) observing the flocculent, fibrous structure of the outermost layer of the fungal cell by a transmission electron microscope.  

... A protein encoded by the DNA of 1 (paragraph [0022]) or 2 (paragraph [0023]).  

... A vector into which the DNA of 1 (paragraph [0022]) or 2 (paragraph [0023]) has been inserted.  

... A transformant harboring the DNA of 1 (paragraph [0022]) or 2 (paragraph [0023]), or the vector of 4 (paragraph [0025]).  

... The transformant of 5 (paragraph [0026]) which is a fungus that overexpresses the protein of 3 (paragraph [0024]).  

... A fungus, wherein the function of the protein of 3 (paragraph [0024]) is defective.  

... A method for producing the protein of 3 (paragraph [0024]), which comprises the steps of culturing the transformant of 5 (paragraph [0024]), and collecting the expressed protein from the transformant, or from the culture supernatant thereof.  

... An antibody that binds to the protein of 3 (paragraph [0024]).  

... A method of screening for a compound having an antifungal action, wherein the method comprises the steps of:  

... (a) contacting a test sample with the protein of 3 (paragraph [0024]);  

... (b) detecting the binding activity between the protein and the test sample; and  

... (c) selecting a compound having an activity to bind to the protein.  

... A method of screening for a compound that has an antifungal action, which comprises the steps of:  

... (a) contacting a test sample with a fungus that is overexpressing the protein of 3;  

... (b) detecting the amount of transport of a GPI-anchored protein to the cell wall; and  

... (c) selecting a compound that diminishes the amount of transport of the GPI-anchored protein to the cell wall detected in step (b) as compared to the amount of transport detected when the test sample was contacted with a fungus that is not overexpressing the protein of 3,  

... wherein, a decrease in the amount of GPI-anchored protein transported to the cell wall that results due to the test sample can be detected, for example, by detecting a decrease in growth rate, swelling, or temperature sensitivity of the cell, or by detecting a decrease of the protein derived from the GPI-anchored protein in the cell wall, but preferably, by detecting a decrease in the protein derived from the GPI-anchored protein at the cell wall;  

... and wherein a decrease of the protein derived from the GPI-anchored protein is quantified by using any one of the following methods alone or in combination:  

... (i) a reporter system reflecting the process that transports GPI-anchored proteins to the cell wall, (ii) an ELISA that quantifies one type of the GPI-anchored protein in the cell wall, (iii) measuring the activity of a GPI-anchored protein such as adhesion to animal cells, and (iv) observing the flocculent, fibrous structure of the outermost layer of a fungal cell by a transmission electron microscope.  

... A compound having an antifungal action that is isolated by the screening of 10 (paragraph [0031]) or 11 (paragraph [0032]).  

... An antifungal agent, comprising as an active ingredient a compound that inhibits the transport of GPI-anchored proteins to the cell wall of a fungus.  

... An antifungal agent, comprising as an active ingredient the antibody of 9 (paragraph [0030]) or the compound of 12 (paragraph [0033]).  

... The antifungal agent of 13 (paragraph [0034]), comprising as an active ingredient the compound represented by the general formula (I), a salt thereof, or a hydrate thereof, wherein in formula (I):  

... [R^1 and R^2 are identical to or different from each other and denote individually a hydrogen atom, halogen atom, hydroxyl group, nitro group, cyano group, trihalomethyl group, trichloromethyl group, a substituted or unsubstituted C_1-6 alkyl group, C_2-6 alkenyl group, C_2-6 alkynyl group, a substituted or unsubstituted C_1-6 alkoxy group, or a group represented by the formula:]  

... [wherein X stands for a single bond, carbonyl group, or a group represented by the formula —S(O)_2—;]  

... R^3 and R^6 are identical to or different from each other and denote a hydrogen atom or a substituted or unsubstituted C_1-6 alkyl group). Furthermore, R^1 and R^2 may together form a condensed ring selected from the group consisting of a substituted or unsubstituted benzene ring, a substituted or unsubstituted pyridine ring, a substituted or unsubstituted pyrrole ring, a substituted or unsubstituted thiophene ring, a substituted or unsubstituted furan ring, a substituted or unsubstituted
pyridazine ring, a substituted or unsubstituted pyrimidine ring, a substituted or unsubstituted pyrazine ring, a substituted or unsubstituted imidazole ring, a substituted or unsubstituted oxazole ring, a substituted or unsubstituted thiazole ring, a substituted or unsubstituted pyrazole ring, a substituted or unsubstituted isoxazole ring, a substituted or unsubstituted isothiazole ring, a substituted or unsubstituted cyclohexane ring, and a substituted or unsubstituted cyclopentane ring;

R³ and R₄ are identical to or different from each other and denote individually a hydrogen atom, halogen atom, hydroxyl group, nitro group, cyano group, carboxyl group, formyl group, hydroxylimino group, trifluoromethyl group, trifluoromethoxy group, C₁₋₆ alkyl group, C₁₋₆ alkoxy group, C₂₋₆ alkenyl group, C₂₋₆ alkenyl group, a group represented by the formula —C(O)NR³R⁴ (wherein R³ and R⁴ are identical to or different from each other and denote individually a hydrogen atom, or a C₁₋₆ alkyl group), the formula —CO₂R⁵ (wherein R⁵ has the same meaning as defined above), the formula —S(O)₂R⁶ (wherein R⁶ stands for an integer of 0 to 2 and R⁷ has the same meaning as defined above), the formula —S(O)₂N(R⁸)R⁹ (wherein R⁸ and R⁹ have the same meaning as defined above), a group of the formula

[0062] X² denotes a single bond, carbonyl group, or a group of the formula —S(O)₂—;
[0063] R⁵ and R⁶ are identical to or different from each other,
[0064] and denote a hydrogen atom, a substituted or unsubstituted C₁₋₆ alkyl group, or a substituted or unsubstituted C₆₋₁₄ aryl group), or a group of the formula

[0065] Z¹ Z² (wherein Z¹ denotes a single bond, oxygen atom, vinylene group, or ethynylene group;
[0066] Z² denotes a single bond, or a C₁₋₆ alkyl group substituted or unsubstituted with 0 to 4 substituents). R⁸ and R⁹ may together stand for a methylenedioxy group or 1,2-ethylenedioxy group, alternatively, R⁸ and R⁹ may together stand for the formation of a condensed ring selected from a group consisting of a substituted or unsubstituted benzene ring, substituted or unsubstituted pyridine ring, substituted or unsubstituted pyrrole ring, substituted or unsubstituted thiophene ring, substituted or unsubstituted furan ring, substituted or unsubstituted pyridazine ring, substituted or unsubstituted thiazole ring, substituted or unsubstituted pyrimidine ring, substituted or unsubstituted imidazole ring, substituted or unsubstituted oxazole ring, substituted or unsubstituted thiazole ring, substituted or unsubstituted pyrazole ring, substituted or unsubstituted isoxazole ring, substituted or unsubstituted isothiazole ring, substituted or unsubstituted cyclohexane ring, and substituted or unsubstituted cyclopentane ring, except in cases where both R¹ and R² stand for hydrogen atoms.]
[0071] wherein K denotes a sulfur atom, oxygen atom, or a group represented by the formula —NH—;

[0072] R⁰⁻ and R¹⁺ are identical to or different from each other and denote individually a hydrogen atom, halogen atom, hydroxyl group, nitro group, cyano group, trifluoromethyl group, trifluoromethoxy group, a group represented by the formula

[0073] wherein X⁵ denotes a single bond, carbonyl group, or a group represented by the formula —S(O)₂—;

[0074] R⁴⁻ and R⁵⁻ are identical to or different from each other and denote a hydrogen atom, or a substituted or unsubstituted C₁₋₆ alkyl group), or a group represented by the formula —X⁶⁻—R⁷⁺ (wherein X⁶ denotes a single bond, oxygen atom, or sulfur atom; R⁷ denotes a C₁₋₆ alkyl group, C₂₋₆ alkynyl group, C₂₋₆ alkenyl group, C₃₋₆ cycloalkyl group, or C₃₋₆ cycloalkenyl group). Alternatively, R⁴⁻ and R⁷⁺ may together form a methylenedioxy group, or a 1,2-ethylenedioxy group);

[0075] R⁴⁻ and R⁷⁺ are identical to or different from each other and denote individually a hydrogen atom, halogen atom, hydroxyl group, nitro group, cyano group, carboxyl group, formyl group, hydroxymino group, trifluoromethyl group, trifluoromethoxy group, C₁₋₆ alkyl group, C₁₋₆ alkynyl group, C₂₋₆ alkynyl group, C₂₋₆ cycloalkyl group, or a group represented by the formula

[0076] wherein Z¹⁺ denotes a single bond, vinylenic group, or ethynylene group;

[0077] Z²⁺ denotes a single bond, or a C₁₋₆ alkyl group that is substituted or unsubstituted with 0 to 4 substituents);

[0078] except in cases where (1) Ar stands for the aforementioned formula (IIId) wherein R¹⁻ and R²⁺ are both hydrogen atoms, (2) at least one of R³⁻ or R⁴⁻ denotes a hydrogen atom and the other is a hydrogen atom, methoxy group, hydroxyl group, methyl group, benzyloxy group, or a halogen atom, and Ar stands for the aforementioned formula (IIId) wherein R¹⁻ and R²⁺ both denote hydrogen atoms or methoxy groups, (3) at least one of R³⁻ or R⁴⁻ denotes a hydrogen atom and the other is a hydrogen atom, hydroxyl group, methoxy group, or benzyl group, and Ar stands for the aforementioned formula (IIId) wherein R¹⁻ and R²⁺ both denote hydroxyl groups or benzyl groups, or (4) Ar stands for the aforementioned formula (IIId) wherein R¹⁻ is a hydrogen atom and R²⁺ is a formyl group, hydroxymethyl group, or methoxycarbonyl group.]

[0079] 18. The compound of 17 (paragraph [0038]), or a salt or hydrate thereof, wherein Ar stands for the formula:

[0080] (wherein R¹ denotes a hydrogen atom, a substituted or unsubstituted C₁₋₆ alkyl group, or a benzyl group), and excluding the case when R⁴⁻ denotes a hydrogen atom.

[0081] 19. A compound represented by the general formula (IIlc2), or a salt or hydrate thereof, wherein in formula (IIlc2),

[0082] [R¹ and R² have the same meaning as defined above, except in cases wherein (1) R¹ denotes a group represented by the formula R¹ — O— (wherein R¹ has the same meaning as defined above), R² denotes a hydrogen atom, and R³ denote a hydrogen atom, (2) at least one of R⁴⁻ or R⁵⁻ denotes a hydrogen atom, and the other is a hydrogen atom, methoxy group, hydroxyl group, methyl group, benzyloxy group, or a halogen atom, and R⁷⁻ and R⁸⁻ both denote hydroxyl groups or methoxy groups, or (3) at least one of R¹⁻ or R⁴⁻ denotes a hydrogen atom, and the other is a hydrogen atom, hydroxyl group, methoxy group, or benzyl group, and R¹⁻ and R²⁺ both denote hydroxyl groups or benzyl groups]

[0083] 20. The antifungal agent of 17 (paragraph [0038]), having an antifungal action.

[0084] 21. The antifungal agent of 15 (paragraph [0038]), wherein at least one of R¹⁻ and R²⁺ denotes a group represented by the formula —C(O)NR⁷⁻R⁸⁻ (wherein R⁷⁻ and R⁸⁻ have the same meaning as defined above), the formula —CO₂R¹⁻ (wherein R¹⁻ has the same meaning as defined above), the formula —S(O)₂NR⁷⁻R⁸⁻ (wherein R⁷⁻ and R⁸⁻ have the same meaning as defined above), the formula —S(O)₂NR⁷⁻R⁸⁻ (wherein R⁷⁻ and R⁸⁻ have the same meaning as defined above), the formula

[0085] where Ar stands for the aforementioned formula (IIId) wherein R¹⁻ and R²⁺ both denote hydroxyl groups or benzyl groups, or (4) Ar stands for the aforementioned formula (IIId) wherein R¹⁻ is a hydrogen atom and R²⁺ is a formyl group, hydroxymethyl group, or methoxycarbonyl group.]

[0079] 18. The compound of 17 (paragraph [0038]), or a salt or hydrate thereof, wherein Ar stands for the formula:
[0085] (wherein \(X^2, R^3,\) and \(R^4\) have the same meaning as defined above), or a \(C_{1-6}\) alkoxy group substituted or unsubstituted with 0 to 4 substitutes, or \(R^2, R^3,\) and \(R^4\) together denote a methylenedioxy group, or a 1,2-ethyleneedioxy group.


[0087] 23. A method for treating a mycelic infection comprising administering a therapeutically effective dose of any one of the antifungal agents of 13 to 22 (paragraphs [0034-0043]) to a mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

[0088] FIG. 1 is a schematic diagram of the process that transports GPI-anchored proteins to the cell wall. A GPI (Glycosylphosphatidylinositol)-anchored protein is first anchored to the plasma membrane, and then transported to the cell wall.

[0089] FIG. 2 is a graph showing the activity of the aforementioned compound (la) in the S. cerevisiae reporter system. In the presence of the aforementioned compound (la) at a concentration of 0.39 to 1.56 \(\mu\)g/ml, cephalosporinase activity increased in the culture supernatant fraction and decreased in the cell wall fraction, and at a concentration of 3.13 \(\mu\)g/ml or more, growth inhibition was observed.

[0090] FIG. 3 is a graph showing the effect of the aforementioned compound (la) on the adhesion of C. albicans to animal cells. Even at a concentration of 1.56 \(\mu\)g/ml in which growth inhibition cannot be observed, adhesion of C. albicans to animal cells was inhibited to about a half.

[0091] FIG. 4 is a graph showing the effect of the aforementioned compound (la) on the amount of the Als1p antigen of C. albicans. In the presence of the aforementioned compound (la) at a concentration of 0.1 to 0.39 \(\mu\)g/ml, the amount of the Als1p antigen increased in the culture supernatant fraction and the amount of the antigen decreased in the cell wall fraction.

[0092] FIG. 5 is a photograph showing the Southern Blot of C. albicans genomic DNA analysis using the S. cerevisiae GWT1 gene as a probe. A single band was observed at 6.5 \(k\) bp with EcoRI, at 4.0 \(k\) bp with HindIII, at 2.0 \(k\) bp with EcoRI-HindIII, and at 2.5 \(k\) bp with EcoRI-PstI, and the homologue of the resistant gene to the aforementioned compound (la) in C. albicans was expected to exist as a single gene.

[0093] FIGS. 6A and 6B are graphs showing the activity of the aforementioned compound (la) in S. cerevisiae that overexpressed the GWT1 gene product. In S. cerevisiae CW63 strain (FIG. 6A), even at the concentration of the aforementioned compound (la) (0.39 to 1.56 \(\mu\)g/ml) in which cephalosporinase activity in the culture supernatant fraction is increased, and activity in the cell wall fraction is decreased, such an effect was not observed in S. cerevisiae CW63/GWT1 strain, and in S. cerevisiae CW63 strain, even at the concentration of the aforementioned (>3.13 \(\mu\)g/ml) in which growth is inhibited, growth inhibition was not observed in S. cerevisiae CW63/GWT1 strain (FIG. 6B).

[0094] FIG. 7 is a diagram in which the highly conserved regions in the proteins encoded by the GWT1 gene of S. cerevisiae, (SEQ ID NO:64 (F-domain) and SEQ ID NO:67 (R-domain)), S. pombe (SEQ ID NO:65 (F-domain) and SEQ ID NO:68 (R-domain)), and C. albicans (SEQ ID NO:66 (F-domain) and SEQ ID NO:69 (R-domain)) are aligned.

DETAILED DESCRIPTION OF THE INVENTION

[0095] The present invention will be described in detail below by explaining the meaning of the terms, symbols, and such mentioned in the present description.

[0096] In the present description, the structural formula of the compounds may represent a certain isomer for convenience, however, the present invention includes all geometrical isomers, optical isomers based on asymmetric carbon, stereoisomers, and tautomers that structurally arise from compounds, and mixtures of isomers, and it is not to be construed as being limited to the representation in the formula made for convenience, and may be any one or a mixture of isomers. Therefore, an optically active substance and a racemic substance having an asymmetric carbon atom in the molecule may exist, but in this invention there are no particular limitations and any one of them are included. Furthermore,
crystal polymorphism may exist, but similarly there are no limitations, and the crystal form may be any one form or may be a mixture, and may be either an anhydride or a hydrate. [0097] Furthermore, the compounds of the present invention include compounds exhibiting antifungal action after being metabolized, such as after being oxidized, reduced, hydrolyzed, or conjugated in vivo. Furthermore, the present invention includes compounds that produce the compounds of this invention after being metabolized, such as after being oxidized, reduced, and hydrolyzed in vivo.

[0098] The “Cₙ₋₃ alkyl group” in the present description means a straight chain or branched chain alkyl group, wherein the number of carbon ranges from 1 to 6, and specific examples include a methyl group, ethyl group, n-propyl group, i-propyl group, n-butyl group, i-butyl group, tert-butyl group, n-pentyl group, i-pentyl group, neopentyl group, n-hexyl group, 1-methylpropyl group, 1,2-dimethylpropyl group, 2-ethylpropyl group, 1-methyl-2-ethylpropyl group, 1-ethyl-2-methylpropyl group, 1,1,2-trimethylpropyl group, 1-methylbutyl group, 2-methylbutyl group, 1,1-dimethylbutyl group, 2,2-dimethylbutyl group, 2-ethylbutyl group, 1,3-dimethylbutyl group, 2-methylpentyl group, 3-methylpentyl group, and so on.

[0099] The “Cₙ₋₃ alkenyl group” in the present description means a straight chain or branched chain alkenyl group, wherein the number of carbon ranges from 2 to 6, and specific examples include a vinyl group, allyl group, 1-propenyl group, 2-propenyl group, 1-butenyl group, 1-buten-1-yl group, 1-buten-2-yl group, 1-buten-3-yl group, 2-buten-1-yl group, 2-buten-2-yl group, and so on.

[0100] The “Cₙ₋₃ alkyloxy group” in the present description means a straight chain or branched chain alkyloxy group, wherein the number of carbon ranges from 2 to 6, and specific examples include an ethoxy group, 1-propoxy group, 2-propoxy group, butoxy group, pentyl group, hexyl group, and so on.

[0101] The “Cₙ₋₁₄ aryl group” in the present description means an aryl group to which “Cₙ₋₃ alkyl group” defined above is bound, and specific examples include a methoxy group, ethoxy group, n-propoxy group, i-propoxy group, n-butoxy group, i-butoxy group, sec-butoxy group, t-butoxy group, n-pentoxy group, i-pent oxy group, sec-pent oxy group, t-pent oxy group, neopent oxy group, 1-methylbutoxy group, 2-methylbutoxy group, 1,1-dimethylpropoxy group, 1,2-dimethylpropoxy group, 1,3-dimethylpropoxy group, 2,2-dimethylbutoxy group, 1,1-dimethylbutoxy group, 2,2-dimethylbutoxy group, 1,3-dimethylbutoxy group, 2,3-dimethylbutoxy group, 3,3-dimethylbutoxy group, 1-ethylbutoxy group, 1,2,3-trimethylpropoxy group, 1,2,2-trimethylpropoxy group, 1-ethyl-1-methoxypropoxy group, 1-ethyl-2-methoxypropoxy group, and so on.

[0102] The “Cₙ₋₁₄ aryl group” in the present description refers to an aromatic ring group, wherein the number of carbon ranges from 6 to 14, and specific examples include a phenyl group, 1-naphthyl group, 2-naphthyl group, naphthyl group, indanenyl group, s-indanenyl group, acenaphthylene group, and so on.

[0103] The “halogen atom” of the present description means a fluorine atom, chlorine atom, bromine atom, and iodine atom.

[0104] “Substituted or unsubstituted” in the present description means “the substitutable site may have an arbitrary combination of one or more substituents” and specifically the substitutents are, for example, a hydrogen atom, halogen, nitro group, cyano group, hydroxyl group, mercapto group, hydroxalkyl group, carboxyl group, C₁₋₃ alkoxy carbonyl group, C₂₋₅ acylamino group, C₁₋₅ alkylamino group, pyridyl group, C₁₋₅ alkoxy sulfanyl group, C₁₋₅ alkylsulfanyl group, C₁₋₅ alkoxyaminoglyc group, C₁₋₅ alkylsulfamoyl group, C₁₋₅ alkoxy sulfanamoyl group, tetrahydropyranyl group, C₁₋₄ cyclicaromatic group, or the formula —X—hep —Rₙ (wherein X—hep denotes a single bond, oxygen atom, or sulfur atom; Rₙ denotes a C₁₋₃ alkyl group, C₂₋₅ alkenyl group, C₂₋₆ alkyloxy group, C₆₋₁₄ ary group, C₆₋₁₅ cy cloalkyl group, or C₉₋₁₅ cycloalkenyl group), and so on.

[0105] “May be substituted with 0 to 4 substituents” has the same meaning as “the substitutable site may have an arbitrary combination of 1 to 4 substituents” and the substituents have the same meaning as defined above.

[0106] “Salt” in the present invention refers to a pharmacologically acceptable salt, and there are no particular limitations as long as the salt has formed an addition salt with a compound of this invention, and a preferred example is a haloid acid salt such as hydrochloride, hydrobromide, hydroiodide, and hydrobromide; an inorganic acid salt such as a sulfite, nitrate, perchlorate, phosphate, carbonate, and bicarbonate; an organic carboxylate such as an acetate, oxalate, maleate, tartrate, and fumarate; an organic sulfonate such as a methanesulfonate, trifluoromethanesulfonate, ethanesulfonate, benzenesulfonate, toluenesulfonate, and camphorsulfonate; an amino acid salt such as an aspartate and glutamate; salts with an amine such as a trimethylammonium, trimethylamine, procaine, pyridine, and phenethylbenzylamine; alkaline earth metal salts such as magnesium and calcium; and so on.

[0107] Herein below, the following will be disclosed: 1. A method for obtaining DNAs encoding proteins participating in cell wall synthesis, 2. a method for examining whether or not a test sample influences the process that transports GPI-anchored proteins to the cell wall, and 3. a method for obtaining the aforementioned compound (la) of the present invention.

1. A Method for Obtaining DNAs Encoding Proteins Participating in Fungal Cell Wall Synthesis

[0108] Hereinafter, (1) a method for obtaining a DNA encoding a protein for acquiring resistance to the aforementioned compound (la) by overexpression in fungi; (2) a method for obtaining a DNA that hybridizes under stringent conditions with the DNA of SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5; (3) a method for obtaining a DNA that encodes a protein that participates in fungal cell wall synthesis, based on a homology search; and (4) a method for obtaining a fungus that overexpressed or lacked the protein for acquiring resistance to the aforementioned compound (la), will be described.

(1) A Method for Obtaining a DNA Encoding a Protein for Acquiring Resistance to the Aforementioned Compound (la) by Overexpression of the DNA in a Fungus

[0109] Herein, “fungus” means a fungus belonging to Division Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota. Preferable is a pathogenic fungus, Musc, Saccharomyces, Candida, Cryptococcus, Trichosporon, Malassezia, Aspergillus, Trichophyton, Microsporum, Sporothrix, Blastomyces, Coccidioides, Paracoccidioides, Penicillium, or Fusarium, and more preferable is C. albicans, C. glabrata, C. neoformans, or A. fumigatus. S. cerevisiae and S. pombe, for which genetic analyses are easy, are also preferred strains.
A plasmid library of a fungal gene is introduced into a fungus. The plasmid library of *S. cerevisiae* and *S. pombe* can be obtained from ATCC (Information for ATCC Number: 37323), and the plasmid library of *C. albicans* can be produced by the method according to Novaro-Garcia, F. et al., Mol. Cell. Biol., 15: 2197-2206, 1995. The obtained plasmid library is introduced to the fungus by the method according to Gietz, D. et al., Nucl. Acids Res. 20: 1425, 1992. Alternatively, a kit such as YEASTMAKER™ Yeast Transformation System (Clontech) may be used.

The fungus to which the plasmid library is introduced is cultured in the presence of the aforementioned compound (Ia). Specifically, an agar medium containing the aforementioned compound (Ia) at a concentration of 1.56 to 2.5 μg/mL, preferably 1.5 to 6.25 μg/mL, and more preferably 3.125 μg/mL is inoculated with the fungus into which a plasmid library has been introduced, is cultured for an appropriate length of time, at 30°C to 42°C for 2 to 5 days, or preferably at 37°C for 3 days. The colony formed upon proliferation is further cultured in a medium containing the aforementioned compound (Ia), and the plasmid is purified from the proliferated fungal cells. Purification of the plasmid can be performed by the method according to METHODS IN ENZYMOLOGY, Vol. 194: 169-182 (1991), for example. Preferably, the nucleotide sequence of the obtained plasmid is determined directly, but if necessary, cloning into an appropriate vector, for example pBlueScript II, and pUC19 suitable for nucleotide sequence determination, is done to determine the nucleotide sequence. A nucleotide sequence can be determined for example by the method accompanying the ABI377 System (PE Applied Biosystems) manual.

In the Examples of the present invention, all 27 of the independently obtained colonies of *S. cerevisiae*, and 28 colonies out of 30 colonies of *C. albicans* contained the DNAs of this invention. Only one gene that confers resistance to the aforementioned compound (Ia) exists in these fungi and this can be obtained by the aforementioned method.

A method for obtaining a DNA that Hybrdizes Under Stringent Conditions to the DNA of SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5

An example of a method for obtaining a DNA encoding a protein participating in fungal cell wall synthesis according to the present invention comprises designing a primer from the information of the nucleotide sequence of SEQ ID NO:1 using the genomic DNA of *S. cerevisiae* as a template, or designing a primer from the information of the nucleotide sequence of SEQ ID NO:3 or SEQ ID NO:5 using the genomic DNA of *C. albicans* as the template, then performing PCR, and cloning the amplified DNA into an appropriate vector, such as pBlueScript. The primer is designed as necessary according to the region to be amplified, and the length is preferably 15 bp or more, more preferably 20 bp or more, and in some cases sequences necessary for subsequent DNA construction, such as restriction enzyme sites, may be added. The conditions for PCR can be determined appropriately according to factors such as the length of primer, the length of the region to be amplified, and the amount of template DNA to be used. For example, a DNA encoding a protein participating in cell wall synthesis in a fungus can be obtained when using 200 ng of the genomic DNA of *C. albicans* as a template, and SEQ ID NO:21 and SEQ ID NO:22 as primers under conditions of 94°C for 4 minutes → 94°C for 30 seconds → 68°C for 5 minutes → 72°C for 4 minutes.

The DNA obtained by PCR may be used as a probe for obtaining other types of fungal DNA showing homology to the DNA encoding the protein participating in cell wall synthesis. Specifically, for example, to obtain a homologous gene of *C. albicans* encoding the protein participating in *S. cerevisiae* cell wall synthesis, DNA that hybridizes under stringent conditions can be cloned from the genomic library or cDNA library of *C. albicans*, using the genomic DNA of *S. cerevisiae* as a template, and using DNA that is obtained by PCR as a probe. Herein, stringent conditions refer to hybridization in 4×SSC at 65°C, then washing in 0.1×SSC at 65°C for 1 hour, for example. Furthermore, in another the stringent conditions are 4×SSC at 42°C in 50% formamide. Alternatively, conditions such as hybridization in the PERFECTHYMTM (TOYOBO) solution at 65°C for 2.5 hours, then washing in 1), 2×SSC, 0.05% SDS solution at 25°C for 5 minutes, 2), 2×SSC, 0.05% SDS solution at 25°C for 15 minutes, and 3) 0.1×SSC, 0.1% SDS solution at 50°C for 20 minutes, are also allowed.

The Examples of this invention demonstrate from Southern Blot analysis that there is only one gene in *C. albicans* that hybridizes with the DNA of SEQ ID NO:1, and shows the cloning of this gene. From the above-mentioned method, DNA that hybridizes with SEQ ID NO:1 or SEQ ID NO:3 can be obtained.

(3) A Method for obtaining a DNA that Encodes a Protein that Participates in Fungal Cell Wall Synthesis Based on a Homology Search

The present invention revealed the GWT1 homologues of *S. cerevisiae*, *C. albicans*, *S. pombe*, *A. fumigatus*, and *C. neoformans*. The region conserved among these genes is considered to be important for GWT1 gene products to exhibit their function, and may very well be conserved in other fungi.

Therefore, a DNA encoding a protein participating in fungal cell wall synthesis can be obtained by either carrying out hybridization upon constructing a probe based on the amino acid sequence of the conserved region, or by performing PCR by designing primers based on the sequence. The PCR primer may be of any sequence as long as it is designed to encode the conserved region, but is preferably SEQ ID NOs: 29 and 31 or preferably SEQ ID NOs: 29 and 30.

Furthermore, as another method, a DNA encoding a protein participating in fungal cell wall synthesis can be obtained by carrying out PCR with cDNA or genomic DNA upon finding a nucleotide sequence showing homology to GWT1 from gene fragments registered in databases, and then designing primers based on that nucleotide sequence.

Examples of PCR methods for obtaining a full-length gene based on the obtained sequence are techniques such as 3'-RACE, 5'-RACE, and inverse PCR, and it is also possible to select by hybridization a clone containing neighboring sequences. A full-length gene can be obtained by combining these techniques.

(4) A Method for Obtaining a Fungus that Overexpresses or Lacks a Protein for Acquiring Resistance to the Aforementioned Compound (Ia)

A Fungus, preferably *S. cerevisiae*, that overexpresses a protein for acquiring resistance to the aforementioned compound (Ia) of this invention can be obtained by the method of inserting an expression vector expressing the protein into a particular position on the fungal chromosome, for example an expression vector in which the DNA of SEQ ID NO:1 is connected downstream of a promoter, which can forcibly express the protein in fungi, preferably the promoter of budding yeast enolase gene (ENO1). The insertion method can be performed, for example, by the steps of, inserting a desired sequence into the multi-cloning site of pRK304 (Sikorski R S et al., Genetics. 122(1): 19-27, 1989), constructing a vector for integration, and introducing the vector into the
fungus. One can refer to METHODS IN ENZYMEOLOGY Vol. 194: 281-301 (1991) for details.

Furthermore, an overexpressed strain of *C. albicans* can be obtained by incorporating the gene of SEQ ID NO:3 or SEQ ID NO:5 into an expression vector for *C. albicans*, such as pCARS1 and pRM1 (Pla J et al, Yeast 12: 1677-1702, 1996), and then transforming *C. albicans* (Sanglard D et al, Antimicrobiol. Agents Chemother. 40: 2300-2305, 1996).

Fungi of this invention lacking a gene for acquiring resistance against the aforementioned compound (Ia), preferably *S. cerevisiae*, can be obtained by the following methods, but is not to be construed as being limited thereto.

PCR amplification is carried out using a marker gene, preferably his5 gene of *S. pombe*, as a template, and using primers that are designed so that PCR products that contain the gene to be deleted (30 bp or more, or preferably 40 bp or more). In the case of *S. cerevisiae*, the genetic sequence of SEQ ID NO:1, positioned on both ends can be obtained. The PCR products can be purified and introduced into fungi, then cultured in a selection medium corresponding to the marker gene, for example, his5, to obtain the deletion strain.

Furthermore, the deletion strain of *C. albicans* is obtained by the usual method using a hisG-URA3-hisG cassette (Fonzi W A et al, Genetics 134: 717-728, 1993) based on the nucleotide sequence information of SEQ ID NO:3 or SEQ ID NO:5.

2. A Method for Examining Whether or not the Test Sample Influences the Process that Transports GPI-Anchored Proteins to the Cell Wall

Whether or not the test sample inhibits the process that transports GPI-anchored proteins to the cell wall, or whether or not the test sample inhibits the expression of the GPI-anchored protein in the fungal surface can be examined by (1) a method using a reporter enzyme, (2) a method using an antibody that reacts with the surface glycoprotein of the fungal cell wall, (3) a method for examining the adhesion ability towards animal cells, and (4) a method for observing fungi using an optical microscope or an electron microscope.

By using the methods of (1) to (4) described below, preferably the methods of (1) to (4) in combination, the test sample is judged to inhibit the process that transports GPI-anchored proteins to the cell wall, or the expression of the GPI-anchored proteins at the fungal surface. Furthermore, it is judged that the test sample influences the process that transports GPI-anchored proteins to the cell wall when the degree of inhibition diminishes or the inhibition is no longer seen when the protein encoded by the DNA of the present invention is overexpressed in fungi.

Hereinafter, the methods of (1) to (4) will be described.

(1) A Method Using a Reporter Enzyme

The process that transports GPI-anchored proteins to the cell wall can be quantified by a tracer experiment such as labeling a GPI-anchored protein with a radioactive isotope, then upon fractionation of the fungal cell wall fraction, immunoprecipitating with an antibody against a GPI-anchored protein. Alternatively, the quantification can be more readily done by expressing the C-terminal sequence considered to function as a transport signal, which is commonly observed among GPI-anchored proteins, as a fusion protein with an easily measurable enzyme (reporter enzyme), fractionating the fungal cell wall fraction, and then using a reporter system that measures the enzyme activity of each fraction (Van Berkelen M A A et al, FEBS Letters, 349: 135-138, 1994). Hereafter, a method using the reporter enzyme will be explained, but the present invention is not to be construed as being limited thereto.

First, the reporter gene is constructed and is introduced into a fungus. The reporter gene is constructed by linking a promoter sequence that functions in fungi, followed by DNAs that respectively encode a signal sequence, a reporter enzyme, and a GPI-anchored protein C-terminal sequence so that the reading frames match. Examples of the promoter sequences are those of promoters such as GAL10, and ENO1. Examples of signal sequences are those of α-factor, invertase, lysozyme, and such. Examples of reporter enzymes are β-lactamase, lysozyme, alkaline phosphatase, β-galactosidase, and such. Green Fluorescence Protein (GFP), which can be detected easily, can be used, even though it does not have enzyme activity. Examples of GPI-anchored protein C-terminal sequences are α-agglutinin C-terminal sequence, CWP2 C-terminal sequence, and such. Furthermore, it is preferable to insert an appropriate selection marker such as LEU2, and URA3 into the vector containing the constructed reporter gene.

The constructed reporter gene is inserted into a fungus by an appropriate method, such as the lithium acetate method (Gietz D et al, Nucl. Acids Res. 20: 1425, 1992), and cultured, if necessary by a method suitable for the selection marker, such as Leu+ medium for LEU2, and Urn+ medium for URA3, and then fungi into which the DNA has been introduced are selected.

Whether or not a test sample influences the process that transports GPI-anchored proteins to the cell wall is examined by the following method.

The reporter gene-introduced fungi are cultured under appropriate conditions, for example at 30°C for 48 hours, in the presence of a test sample. After culturing, the culture supernatant is centrifuged, and the reporter enzyme activity of the culture supernatant fraction is measured. The remaining cell fraction is washed, then the cell wall components are separated by an appropriate method, such as degrading the cell wall glucan with glucanase, and then measuring the reporter enzyme activity of the cell wall fraction and the cytoplasmic fraction. The assay can be simply carried out by determining the amount of reporter enzyme in the cell fraction by centrifuging, then without washing the cells, determining the amount of reporter enzyme derived from the culture supernatant fraction that remains in the cell fraction by proportional calculation, and subtracting this from the amount of reporter enzyme of the cell fraction.

If an activity to increase the reporter enzyme activity within the culture supernatant fraction (activity per cell), or an activity to decrease the reporter enzyme activity in the cell wall fraction (activity per cell) is confirmed in the test sample, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

(2) A Method Using an Antibody that Reacts with the Surface Glycoprotein of a Fungal Cell Wall

Whether or not the test sample influences the expression of the GPI-anchored protein at the fungal surface layer can be detected by quantifying a GPI-anchored protein in the fungal cell wall using an antibody that reacts with the protein.

For example, as the antibody, the antigenic determinant is predicted from the amino acid sequence of a GPI-anchored protein, for example, α-agglutinin, Cwp2p, and Als1p (Chen M H et al, J. Biol. Chem., 270:26168-26177, 1995; Van Der Vaut J M et al, J. Bacteriol., 177:3104-3110, 1995; Hoyer L L et al, Mol. Microbiol., 15:53-54, 1995), the peptide of that region is synthesized, this is bound to an
antigenic substance, such as a carrier protein, and then polyclonal antibodies can be obtained by immunizing a rabbit and such, or a monoclonal antibody can be obtained by immunizing a mouse and such. Furthermore, a house rabbit polyclonal antibody against the Als1p peptide is preferable.

In an alternative method, a monoclonal antibody against a GPI-anchored protein may be obtained by immunizing a mouse and such with a fungus, preferably a fungus overexpressing the GPI-anchored protein, such as α-agglutinin, Cwp2p, and Als1p, and in some cases, by immunizing with the partially purified GPI-anchored protein, and selecting the clone yielded as a result of the fusion by ELISA, Western blot analysis, and such.

Whether or not the test sample influences the process that transports GPI-anchored proteins to the cell wall, and diminishes the amount of the protein derived from the GPI-anchored protein in the cell wall can be examined by the following method.

A fungus is cultured in the presence of a test sample under appropriate conditions, such as 30°C, for 48 hours. The cultured fungus is collected by centrifugation and the cells are disrupted, preferably using glass beads. The washed, disrupted cells are preferably subjected to centrifugal extraction with SDS, then the precipitate is washed. After the extraction, the disrupted cells are treated with an enzyme that degrades glucan, preferably glucanase, and the centrifuged supernatant thereof is the GPI-anchored protein sample.

The anti-Als1p peptide antibody is coated onto a 96-well plate by incubating at 4°C overnight. After washing with a washing solution, preferably PBS containing 0.05% Tween 20 (PBST), blocking is carried out with a reagent that blocks the non-specific adsorption sites of the 96-well plate, preferably a protein such as BSA, and gelatin, more preferably BlockAce. After washing again with a washing solution, preferably PBST, in some cases, after adding an appropriately diluted GPI-anchored protein sample, the reaction is carried out for an appropriate length of time, such as 2 hours at room temperature. After washing with a washing solution, preferably with PBST, an antibody against the enzyme-labeled C. albicans, preferably HRP-labeled anti-Candida antibody, is reacted for an appropriate length of time, such as 2 hours at room temperature. The method for labeling may be enzyme labeling or radioactive isotope labeling. After washing with a washing solution, preferably PBST, the amount of Als1p in the GPI-anchored protein sample is calculated by a method appropriate for the type of label, i.e., for an enzyme label, adding a substrate solution, and then upon stopping the reaction, measuring the absorbance at 490 nm.

A Method for Examining the Adhesion Ability Towards Animal Cells

Whether or not the test sample influences expression of a GPI-anchored protein on the fungal surface can be examined by measuring the activity of the GPI-anchored protein in the fungal cell wall, preferably by measuring the adhesion ability of fungi to animal cells, and such. Besides Als1p, Esp1p, and such participating in adhesion to animal cells, α-agglutinin participating in mating, Flo1p participating in yeast aggregation, and such are known as GPI-anchored proteins. Hereinafter, examination methods that use the adhesion ability of fungi to animal cells will be explained in detail, but this invention is not to be construed as being limited thereto.

As the fungus, a fungus having an adhesion ability towards cells is used, and preferably, the fungus is C. albicans. For mammalian cells, cells that adhere to the fungus are used, and preferably, are intestinal epithelial cells. The mammalian cells are cultured and are immobilized by an appropriate method such as ethanol immobilization. The test sample and the fungi, which have been incubated for an appropriate length of time, such as 48 hours at 30°C, are inoculated, then after culturing for a certain length of time, for example 1 hour at 30°C, the culture supernatant is removed, washed with a buffer, and is superposed onto an agar media, such as Sabouraud Dextrose Agar Medium (Difco). After culturing at 30°C overnight, the number of colonies is counted, and the adhesion rate is calculated.

If activity to lower the number of colonies formed by adhesion of fungi to cells is observed in a test sample compared to that of fungi that are not treated with the compound, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

A Method for Observing Fungi Using an Electron Microscope or an Optical Microscope

Whether or not the test sample influences the expression of the GPI-anchored proteins in the fungal surface can be examined by observing the structure of the fungal cell wall using an electron microscope.

In the presence of a test sample, a fungus such as C. albicans is cultured for a certain length of time, for example, 48 hours at 30°C, and the ultrafine morphological structure is observed with a transmission electron microscope. Herein, observation with a transmission electron microscope can be carried out, for example by the method according to the Electron Microscope Chart Manual (Medical Publishing Center). The flocculent fibrous structure of the outermost layer of the fungal cell that has a high electron density and is observable by transmission electron microscope image, is considered to be a surface glycoprotein layer having GPI-anchored proteins as its constituents, and is not influenced by other existing antifungal agents. When this flocculent fibrous structure of the outermost layer of a fungal cell, which has a high electron density, disappears leaving a slight layer with a high electron density, compared to that in the untreated cells, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

When images, in which fungal cells are largely swollen and budding (division) is inhibited, are observed under a transmission electron microscope in addition to an optical microscope, the test sample is judged to have an influence on the cell wall.

The compounds of the present invention represented by the formula (I)

(3) A Method for Examining the Adhesion Ability Towards Animal Cells

[0141] Whether or not the test sample influences expression of a GPI-anchored protein on the fungal surface can be examined by measuring the activity of the GPI-anchored protein in the fungal cell wall, preferably by measuring the adhesion ability of fungi to animal cells, and such. Besides Als1p, Esp1p, and such participating in adhesion to animal cells, α-agglutinin participating in mating, Flo1p participating in yeast aggregation, and such are known as GPI-anchored proteins. Hereinafter, examination methods that use the adhesion ability of fungi to animal cells will be explained in detail, but this invention is not to be construed as being limited thereto.

[0142] As the fungus, a fungus having an adhesion ability towards cells is used, and preferably, the fungus is C. albicans. For mammalian cells, cells that adhere to the fungus are used, and preferably, are intestinal epithelial cells. The mammalian cells are cultured and are immobilized by an appropriate method such as ethanol immobilization. The test sample and the fungi, which have been incubated for an appropriate length of time, such as 48 hours at 30°C, are inoculated, then after culturing for a certain length of time, for example 1 hour at 30°C, the culture supernatant is removed, washed with a buffer, and is superposed onto an agar media, such as Sabouraud Dextrose Agar Medium (Difco). After culturing at 30°C overnight, the number of colonies is counted, and the adhesion rate is calculated.

[0143] If activity to lower the number of colonies formed by adhesion of fungi to cells is observed in a test sample compared to that of fungi that are not treated with the compound, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

(4) A Method for Observing Fungi Using an Electron Microscope or an Optical Microscope

[0144] Whether or not the test sample influences the expression of the GPI-anchored proteins in the fungal surface can be examined by observing the structure of the fungal cell wall using an electron microscope.

[0145] In the presence of a test sample, a fungus such as C. albicans is cultured for a certain length of time, for example, 48 hours at 30°C, and the ultrafine morphological structure is observed with a transmission electron microscope. Herein, observation with a transmission electron microscope can be carried out, for example by the method according to the Electron Microscope Chart Manual (Medical Publishing Center). The flocculent fibrous structure of the outermost layer of the fungal cell that has a high electron density and is observable by transmission electron microscope image, is considered to be a surface glycoprotein layer having GPI-anchored proteins as its constituents, and is not influenced by other existing antifungal agents. When this flocculent fibrous structure of the outermost layer of a fungal cell, which has a high electron density, disappears leaving a slight layer with a high electron density, compared to that in the untreated cells, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

[0146] When images, in which fungal cells are largely swollen and budding (division) is inhibited, are observed under a transmission electron microscope in addition to an optical microscope, the test sample is judged to have an influence on the cell wall.

[0147] The compounds of the present invention represented by the formula (I)

[0148] (wherein the symbols have the same meaning as defined above) can be synthesized by utilizing conventional organic chemical reactions and such that have been known to date. For example, it can be synthesized by the following methods.
Production Method (1)

[0149]

In the above formulae, X is a leaving group such as a halogen group and acyl group. Other symbols in the formulae have the same meaning as defined above.

Process A1

[0151] A reaction for producing the Reissert compound (V). The compound can be produced based on the reaction conditions according to the literature, such as Org. Synth., VI, 115 (1988); Heterocycles, 36(11), 2489 (1993); J. Chem. Soc. (C), 666 (1969); or J. Heterocycl. Chem., 29(5), 1165 (1992). Specifically, the reagents used are, for example, a combination of benzoyl chloride and potassium cyanide.

Process A2

[0152] A process for alkylation. The compound (VI) can be produced by reacting the compound (V) with a substituted benzyl halide derivative, a substituted benzylmethanesulfonate derivative, or such in the presence of a base. Specific examples of the base include sodium hydride, sodium hydroxide.

Process A3

[0153] A process for hydrolysis reaction. The compound (I) can be produced by hydrolysis of the compound (VI) in the presence of a base.

Process B1

A process for conversion of the compound (V) to the compound (VII). The compound (VII) can be produced by reacting the compound (V) with a substituted benzaldehyde in the presence of a base and a phase-transfer catalyst. Examples of the base include sodium hydroxide and potassium hydroxide. Examples of the phase-transfer catalyst include triethylbenzylammonium chloride.

Process B2

A process for oxidation of the alcohol to the ketone. The ketone derivative (VIII) can be produced by using an oxidizing agent and a condition conventionally used for the oxidation reaction of an alcohol to a ketone. Specifically, the oxidizing agent is, for example, manganese dioxide, chromium dioxide, or benzoquinone.

Process B3

A process for reduction of the ketone to the methylene. The methylene derivative (I) can be produced by using a conventionally used combination of reducing agents for the reduction reaction of the ketone derivative (VIII) to the methylene derivative (I). Examples of the combination of the reducing agents include hydrazine hydrate and sodium hydroxide or potassium hydroxide, and triethylsilane and boron trifluoride, or trifluoromethanesulfonic acid.


Process C1

A process for halogenation or acylation of the hydroxy group. The compound (IX) can be produced by reacting a halogenating agent or an acylating agent with the compound (VII). Examples of the halogenating agent include thionyl chloride, concentrated hydrochloric acid, and phosphorus tribromide. Furthermore, examples of the acylating agent include acid halides such as acetyl chloride and acid anhydrides such as acetic anhydride.

Process C2

A process for reductive elimination reaction of the halogen group or the acyl group. The compound (1) can be produced by hydroelimination of the compound (IX), for example, by using a catalyst.

Examples of the catalyst include palladium-carbon.

Method C is a method for producing the compound (1) via Process A1, Process B1, Process C1, and Process C2.

Production Method (2)

The compound of the present invention represented by the formula (I) can also be synthesized by the following method.
In the formula, X is a leaving group such as a halogen group and acyl group. Other symbols in the formulae have the same meaning as defined above.

Process D1

A process for a Grignard reaction and a subsequent acid hydrolysis reaction. The compound (VIII) can be produced by reacting the compound (X) with a substituted or unsubstituted phenyl Grignard reagent, followed by hydrolysis in the presence of an acid.

Process D2

The methylene derivative (I) can be produced from the ketone derivative (VIII) by conditions similar to that of Process B3.

Method D is a method for producing the compound (I) via Process D1 and Process D2.

Process E1

A process for the reduction reaction from the ketone to the alcohol. The compound (VII) can be produced from the compound (VIII) using a reducing agent and conditions conventionally used for the reduction reaction of a ketone to an alcohol. Specific examples of the reducing agent include sodium borohydride and lithium aluminum hydride.

Process E2

Under conditions similar to that of Process C1, the halogenated or acylated derivative (IX) can be produced from the alcohol derivative (VII).

Process E3

Under conditions for reductive elimination reaction similar to that of Process C2, the compound (I) can be produced from the compound (IX).

Method E is a method for producing the compound (I) via Process D1, Process E1, Process E2, and Process E3.

Production Method (3)

The compound of the present invention represented by the formula (I) can also be synthesized by the following method.

The symbols in the formulae have the same meaning as defined above.

Process F1

A process for the chlorination reaction. The compound (XII) can be produced by reacting the compound (XI) with a chlorinating agent. Examples of the chlorinating agent include phosphorus oxychloride and thionyl chloride.

Process F2

A process for the coupling reaction with a Grignard reagent. The compound (I) can be produced by reacting the compound (XII) with a substituted or unsubstituted benzyl Grignard reagent in the presence of a catalyst, based on the reaction conditions according to the literature, such as Arch. Pharm., 314, 156 (1981). Examples of the catalyst include [1,1'-bis(diphenylphosphino)ferrocene]dichloro nickel(II).

Method F is a method for producing the compound (I) via Process F1 and Process F2.

Production Method (4)

The compound of the present invention of the formula (I), wherein R₁⁺ and R₂⁺ together form a condensed ring such as a benzene ring, pyridine ring, pyrrole ring, thiophene ring, furan ring, cyclohexene ring, or cyclopentane ring, can be synthesized by the following method.
The symbols in the formulae have the same meaning as defined above.

The production method in which the isoquinoline ring is formed is shown below as an example.

**Process G1**

A process for the condensation reaction and the subsequent reduction reaction. The compound (XIV) can be produced by a condensation reaction between the substituted or unsubstituted benzaldehyde derivative (XIII) and nitromethane, followed by reduction of the nitro group. Examples of the reagent used for the reduction of the nitro group include a combination of palladium-carbon and ammonia formate, and lithium aluminum hydride.

**Process G2**

An amide bond formation reaction. The compound (XV) can be produced by reacting the compound (XIV) and a substituted or unsubstituted phenylacetyl chloride with a coupling reagent for an amide bond formation reaction. Examples of the coupling reagents include a combination of N,N'-dicyclohexylcarbodiimide and N-hydroxysuccinimide, a combination of N,N'-dicyclohexylcarbodiimide and N-hydroxybenzotriazole, and 1,1'-carbonyldiimidazole.

**Process G3**

A process for the cyclization reaction. The compound (XV) can be produced based on the reaction conditions according to the literature, such as Organic Reaction, 6, 74 (1951); J. Heterocyclic Chem., 30, 1581 (1993). Examples of the reagent for this reaction include phosphorus oxychloride and polyphosphoric acid.

**Method G** is a method for producing the compound (I) via Process G1, Process G2, and Process G3.

**Production Method (5-1)**

Replacement of the substituent R^3a or R^4a of the compound (I) synthesized by the aforementioned production method.

**[0184]** Replacement of the substituent R^3a or R^4a of the compound (I) synthesized by the aforementioned production method.

**Method H**

- Replacement of the substituent with an amino group, amide group, sulfonamide group, etc.

**[0185]** Replacement of the substituent with an amino group, amide group, sulfonamide group, etc.

**Process G1**

- A reduction reaction of the nitro group. The compound (XVI) can be produced by reducing the compound (XVI) with a conventionally used method for reduction of a nitro group. Examples of the reduction methods include catalytic hydrogenation reduction by palladium-carbon, or palladium hydroxide, and reduction by iron-ammonium chloride, iron-hydrochloric acid, iron-acetic acid, etc.

**Process H2**

A process for the acylation or sulfonylation reaction. The compound (XVIII) can be produced by treating the compound (XVII) with a carboxylic acid or acid anhydride.

**[0188]** A process for the acylation or sulfonylation reaction. The compound (XVIII) can be produced by treating the compound (XVII) with an acid chloride or acid anhydride.

**Method H** is a method for producing the compound (XVIII) via Process H1 and Process H2.
The symbols in the formulae have the same meaning as defined above.

Method I

[0191] A process for the reductive amination reaction. The compound (XX) can be produced from the compound (XIX) and a substituted or unsubstituted aldehyde based on the reaction conditions according to the literature, such as J. Am. Chem. Soc., 93, 2897 (1971); Comprehensive Organic Syntheses, 8, 25 (1991); Tetrahedron, 40, 1783 (1984); and Tetrahedron, 41, 5307 (1985). Examples of the reductive amination reagent include sodium triacetoxyhydroborate, sodium cyanotrihydroborate, borane-pyridine complex, and palladium-carbon/hydrogen.

Process I2

[0192] A process for the acylation, sulfonylation, or reductive amination reaction. The compound (XXIa) or the compound (XXIb) can be produced from the compound (XX) using an acid chloride or an acid anhydride. The compound (XXIc) can be produced by carrying out a reductive amination reaction similarly to that of Process I1.

Production Method (5-2)

[0193] Method I is a method for producing the compound (XXIa), the compound (XXIb), or the compound (XXIc) via Process I1 and Process I2.

[0194] Replacement of the substituent $R^{1c}$ or $R^{4c}$ of the compound (1) synthesized by the aforementioned production method.

[0195] (5-2) Replacement of the substituent with a hydroxyl group, alkoxy group, etc.

Method J

[0196] The symbols in the formulae have the same meaning as defined above.

Process J1

[0197] The compound (XXIII) can be produced from the compound (XXII) by a demethylation reaction based on the reaction conditions according to the literature, such as Bull. Chem. Soc. Jpn., 44, 186 (1971); Org. Synth., Collect. Vol. V, 412 (1973); J. Am. Chem. Soc., 78, 1380 (1956); or J. Org. Chem., 42, 2761 (1977). Examples of the reagent used for the demethylation reaction include 47% aqueous hydrobromic acid solution, boron tribromide, pyridine hydrochloride, and iodo(trimethyl)silane.
Process J2

[0198] A process for the alkylation reaction. The compound (XXIV) can be produced by reacting the compound (XXIII) with a substituted or unsubstituted alkyl halide, a substituted or unsubstituted alkylmethane sulfonate, or such in the presence of a base.


Production Method (5-3)

[0200] Replacement of the substituent $R^{3s}$ or $R^{4s}$ of the compound (1) synthesized by the aforementioned production method

[0201] (5-3) Replacement of the substituent with a vinylene group, an ethynylene group, alkyl group, etc.

Method K

[0202] The symbols in the formulae have the same meaning as defined above.

Process K1

[0203] A process for the triflation reaction. The compound (XXV) can be produced by reacting the compound (XXIII) with trifluoromethane sulfonic acid anhydride in the presence of a base.

Process K2

[0204] A process for the coupling reaction with an alkyne. The compound (XXVI) can be produced by coupling the compound (XXV) with an alkyne derivative in the presence of a palladium phosphine complex, copper iodide, and a base. Examples of reagents that produce the palladium phosphine complex in the reaction system include a combination of palladium-carbon and triphenylphosphine, tetrakis(triphenylphosphine) palladium (0) and triphenylphosphine, dichlorobistriphenylphosphine palladium (II), palladium (II) acetate and tri(o-toly)phosphine, and palladium(II) acetate and 1,1'-bis(diphenylphosphino)ferrocene. Examples of the base include triethylamine, piperidine, pyridine, and potassium carbonate. Depending on the reaction, lithium chloride may be used.

Process K3

[0205] A process for the reduction reaction of the unsaturated hydrocarbon. The compound (XXVIIa) or the compound (XXVIIb) can be produced from the compound (XXVI), for example, by catalytic hydrogenation using a catalyst. Examples of the catalyst include palladium-carbon, palladium hydroxide, platinum oxide, and palladium-carbon-calcium carbonate.
The symbols in the formulae have the same meaning as defined above.

A process of the coupling reaction (Heck Reaction) with the alkene. The compound (XXVIIa) can be produced from the compound (XXVIII) using a catalyst (e.g. palladium complex and its ligand), based on the reaction conditions according to the literature, such as J. Org. Chem., 37, 2320 (1972); Org. Reactions, 23, 345 (1982); Comprehensive Organic Synthesis, Vol. 4, 833 (1991); Palladium Reagents and Catalysts, 125 (1995); Chem. Commun., 1287 (1984); Tetrahedron Lett., 26, 2667 (1985); and Tetrahedron Lett., 31, 2463 (1990). Examples of the combination of the catalysts used for this reaction (palladium complex and its ligand) include palladium (II) acetate and 1,1'-bis(diphenylphosphino)ferrocene, and palladium (II) acetate and tri(o-tolyl)phosphine. Examples of the tertiary base include triethylamine, disopropylethylamine, and 1,8-diazabicyclo[5.4.0]-7-undecene. X of the compound (XXVIII) denotes a leaving group, such as a halogen group and trifluoromethanesulfonyloxy group.

A process of the coupling reaction (Heck Reaction) with the alkene. The compound (XXVIIb) can be produced from the compound (XXVIIa) according to the conditions for a reduction reaction of an unsaturated hydrocarbon, similar to that of process K3.

Method L is a method for producing the compound (XXVIIa) by Process L1, followed by producing the compound (XXVIIb) by Process L2.

Various isomers of the compounds represented by the formula (I) of the present invention can be purified and isolated using ordinary separation techniques (for example, recrystallization, chromatography, and so on).
and can be produced by a conventional method. That is, base materials used for formulation can be selected from various materials ordinarily used for medicaments, quasi-drugs, cosmetics, and such. Specifically, the base materials to be used are, for example, animal fat and vegetable oil, mineral oil, ester oil, waxes, higher alcohols, fatty acids, silicone oil, surfactants, phospholipids, alcohols, polyhydric alcohols, water-soluble macromolecules, clay minerals, and purified water. As necessary, pH regulators, antioxidants, chelating agents, antiseptic and antifungal agents, coloring matters, fragrances, and such may be added, but the base materials of the external preparations of the present invention are not to be construed as being limited thereto. Furthermore, as necessary, components such as those that have a differentiation induction effect, blood flow accelerators, fungicides, antiphlogistic agents, cell activators, vitamins, amino acids, humectants, and keratolytic agents can be combined. The above-mentioned base materials is added to an amount that leads to the concentration usually used for external preparations. 

[0212] When the compounds of this invention or salts thereof, or hydrates thereof, is administered, there are no particular limitations on their form, and they can be administered orally or parenterally by a conventionally used method. They can be formulated into as dosage forms such as tablets, powder, fine granules, capsules, syrups, troches, inhalants, suppositories, injections, ointments, eye ointments, eye drops, nasal drops, ear drops, cataplasms, and lotions. The dose of the pharmaceutical compositions of this invention can be selected appropriately depending on the degree of the symptom, age, sex, weight, the dosage form, the type of salt, the specific type of disease, and such. 

[0213] A curative dose of the antifungal agent of this invention is administered to a patient. Herein, “curative dose” refers to the amount of the pharmaceutical agent that yields the desired pharmacological result and is effective for recovery or relief from the symptoms of a patient to be treated. The dose differs markedly depending on the weight of the patient, type of disease, degree of symptom, age of the patient, sex, sensitivity towards the agent, and such. Usually, the daily dose for an adult is approximately 0.05 to 1000 mg, preferably 0.1 to 500 mg, more preferably 0.1 to 100 mg, and is administered once or several times per day, or once to several times per several days. The dose for injections is normally, approximately 1 to 3000 μg/kg, and is preferably approximately 3 to 1000 μg/kg.

BEST MODE FOR CARRYING OUT THE INVENTION

Example A

[0214] The present invention is specifically illustrated below with reference to Examples, but it is not to be construed as being limited thereto.

Example A1

Construction of the Reporter Gene and Introduction Thereof into S. cerevisiae

[0215] (1) Construction of the Reporter Gene where Lysozyme is the Reporter Enzyme

[0216] A lysozyme gene comprising a promoter sequence was amplified by PCR using pEsh plasmid comprising the ENO1 promoter+secretion signal+lysozyme gene (Ichikawa K et al., Biosci. Biotech. Biochem., 57(10), 1686-1690, 1993) as template, and the oligonucleotides of SEQ ID NO:8 and SEQ ID NO:9 as primers, and this was subcloned into the Sall-EcoRI site of PCR-Script SK+(+) (a). Furthermore, a CWP2 gene was amplified by PCR using S. cerevisiae chromosomal DNA as template, and the oligonucleotides of SEQ ID NO:10 and SEQ ID NO:11 as primers, and this was subcloned into the EcoRI-HindIII site of pUC19 (b). Similarly, CYC1 terminator was amplified by PCR using pYES2 (INVITROGEN) as a template, and the oligonucleotides of SEQ ID NO:12 and SEQ ID NO:13 as primers, and this was subcloned into the newly introduced NotI-Kpn1 site of pUC19 (c).

[0217] Next, the lysozyme gene excised with Sall-EcoRI (a), and the CWP2 gene excised with EcoRI-HindIII (b) were inserted into the Sall-HindIII cleavage site of pEshII. Finally, pRLW63T was produced by excising a gene comprising the ENO1 promoter+secretion signal+lysozyme gene+CWP2 gene using BamHI-HindIII, inserting this into a pRS316 integration vector (Sikorski R S et al., Genetics. 122(1):19-27, 1989), and then inserting the CYC1 terminator excised with HindIII-Kpn1 (c) into the HindIII-Kpn1 cleavage site.

(2) Construction of the Reporter Gene where Cephalosporinase is the Reporter Enzyme

[0218] DNA comprising a promoter sequence and secretion signal portion was amplified by PCR using the above-mentioned pEshII as template, and the oligonucleotides of SEQ ID NO:14 and SEQ ID NO:15 as primers, and this was subcloned into the BamHI-NotI site newly introduced into pUC19 (d). Furthermore, a cephalosporinase gene was amplified by PCR using Citrobacter freundii chromosomal DNA as template, and the oligonucleotides of SEQ ID NO:16 and SEQ ID NO:17 as primers, and this was subcloned into the NspV-XbaI site newly introduced into pUC19 (e). Similarly, the CWP2 gene was amplified by PCR using the S. cerevisiae chromosomal DNA as template, and the oligonucleotides of SEQ ID NO:18 and SEQ ID NO:19 as primers, and this was subcloned into the XbaI-HindIII site of pUC19 (f).

[0219] After producing the full length ENO1 promoter+secretion signal portion by inserting the BamHI-Sall fragment of pEshII into the BamHI-Sall cleavage site of a plasmid into which (d) has been inserted, the cephalosporinase gene excised with NspV-XbaI, and the CWP2 gene excised with XbaI-HindIII were inserted into the NspV-HindIII cleavage site. Next, pRCW63T was produced by excising with EcoRI-HindIII, inserting this fragment into the above-mentioned pRS316, and then inserting the CYC1 terminator into the HindIII-Kpn1 cleavage site.

(3) Introduction of the Reporter Gene into S. cerevisiae

[0220] S. cerevisiae G2-10 strain was cultured by shaking in 10 ml of YPD medium at 30° C., then the cells were collected at the late logarithmic growth phase (2.5x10⁵ cells/ml). After washing with sterilized water, the above mentioned pRLW63T and pRCW63T were introduced by lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) (according to the YEASTMAKER™ Yeast Transformation System User Manual). pRLW63T and pRCW63T in which the URA3 gene was cloned with EcoRV and Apal, respectively, were used. After culturing in SD(Ura⁺) medium at 30° C. for 3 days, the grown colonies were cultured in YPD medium.

[0221] When the localizations of lysozyme and cephalosporinase activities were confirmed, both activities were
mainly localized in the cell wall, and the C-terminal sequence of CWP2 was confirmed to function as a transport signal to the cell wall.

Example A2
Screening of Pharmaceutical Agents by the S. cerevisiae Reporter System

[0222] Since sensitivity of the enzyme reaction is better with cephalosporinase compared to lysozyme, S. cerevisiae introduced with pRCW63T (S. cerevisiae CW63 strain) was used for the screening of compounds.

[0223] After stationary cultivation in YPD liquid medium at 30°C for 48 hours, the yeast cell culture was diluted 100 times with YPD liquid medium (3.5 x 10⁵ cells/ml) and 75 µl/well aliquots thereof were inoculated into a V-bottomed 96-well plate containing 25 µl/well of a diluted test sample, and this was subjected to stationary cultivation at 30°C for 48 hours. After centrifuging the plate, 25 µl of the supernatant was sampled and placed in a flat-bottomed 96-well plate, and this was used as the culture supernatant fraction.

[0224] The precipitated cells were suspended, and 75 µl/well aliquots of Zymolyase (Seikagaku Corporation) solution prepared with 2.4 M sorbitol were added and were allowed to react at 30°C for 1 hour. After centrifuging the plate, 10 µl of the supernatant was sampled and placed in a flat-bottomed 96-well plate, 15 µl of phosphate buffer was added, and this was used as the cell wall fraction.

[0225] The cephalosporinase activities in the medium and in the cell wall fraction were measured by adding 200 µM of nitrocefin solution to a pooled sample, and after a certain period of time, stopping the reaction with citric acid buffer, and then measuring the absorbance at 490 nm.

[0226] Furthermore, fungal growth in the presence of the test sample was determined by visual observation.

[0227] FIG. 2 showed that in the presence of the aforementioned compound (Ia) at a concentration of 0.39 to 1.56 µg/ml, cephalosporinase activity increases in the culture supernatant fraction, and the activity decreases in the cell wall fraction. In this manner, a compound that increases the cephalosporinase activity in the culture supernatant fraction, and in addition decreases the cephalosporinase activity in the cell wall fraction was considered to be a compound that inhibits the process that transports GPI-anchored proteins to the cell wall.

Example A3
Screening of Pharmaceutical Agents Using the Adhesion of Candida to Animal Cells

[0228] Three-milliliter aliquots of IEC-18 cells (1 x 10⁵ cells/ml in D-MEM medium (Nissui Pharmaceutical) containing 10% fetal calf serum and 2 mM glutamine) were placed in each well of a 6-well multi-well plate. The plate was incubated in a carbon dioxide gas incubator at 37°C for 3 days, the culture supernatant was removed, and ethanol immobilization was carried out.

[0229] C. albicans cultured in Sabouraud Dextrose Liquid Medium containing various concentrations of the test sample at 30°C for 48 hours was adjusted to 4 x 10⁷ cells/ml, and 1 ml was inoculated into each well of the plate in which the immobilized IEC-18 cells were cultured. After cultivation at 30°C for 1 hour, the culture supernatant was removed, washed with PBS, and then 2 ml of Sabouraud Dextrose Agar Medium (Difco) was superposed. After cultivation at 30°C overnight, the number of colonies (CFU) that had grown was counted and the adhesion rate was calculated.

[0230] FIG. 3 shows that even at a concentration of 1.56 µg/ml of the aforementioned compound (Ia), in which growth inhibition cannot be observed, adhesion of C. albicans to animal cells was inhibited to about a half. Compared to untreated C. albicans, a test sample that diminished CFU that adhered to cells was considered as a compound that inhibits the adhesion of C. albicans to animal cells.

Example A4
Screening of Pharmaceutical Agents Using the Amount of the GPI-Anchored Protein Quantified by ELISA

(1) Production of Anti-Als1p Peptide Antibody

[0231] A rabbit was immunized with the synthetic peptide of SEQ ID NO:20 conjugated with KLH. The obtained antiserum was affinity-purified, and the IgG fraction was used as the anti-Als1p peptide antibody.

(2) Screening of Pharmaceutical Agents by ELISA Using Anti-Als1p Peptide Antibody

[0232] C. albicans was cultured in Sabouraud Dextrose Liquid Medium (5 ml) containing various concentrations of the test sample at 30°C for 48 hours, and the cells were collected by centrifugation, washed, and then suspended in 300 µl of Tris-HCl buffer. The suspended cells were transferred to a microtube containing glass beads, and were disrupted by repeating 10 cycles of freezing and cooling on ice for 1 minute. The disrupted cells that were washed were extracted with 2% SDS at 95°C for 10 minutes, centrifuged, and then the precipitate was washed 5 times with phosphate buffer. To this precipitate, 0.5 ml of 5 µg/ml Zymolyase solution was added, reacted at 37°C for 1 hour, and the centrifuged supernatant was used as the GPI-anchored protein sample.

[0233] A 96-well plate was coated with 50 µl of anti-Als1p peptide antibody (40 µg/ml) at 4°C overnight. After washing 5 times with PBS containing 0.05% Tween 20 (PBST), blocking was carried out with 25% BlockAce at room temperature for 2 hours. After washing 3 times with PBST, 50 µl of the 2-fold serially diluted GPI-anchored protein sample was reacted at room temperature for 2 hours. After washing 5 times with PBST, 100 µl of 1000-fold diluted HRP-labeled anti-Candida antibody (ViroStat) was reacted at room temperature for 2 hours, then upon washing 5 times with PBST, 75 µl of substrate solution was added. After the reaction was stopped, absorbance at 490 nm was measured.

[0234] FIG. 4 shows that in the presence of the aforementioned compound (Ia) at a concentration of 0.1 to 0.39 µg/ml, the amount of Als1p antigen increases in the culture supernatant fraction, and the amount of antigen decreases in the cell wall fraction. In this manner, a compound that increased the amount of Als1p in the culture supernatant, or decreased the amount of Als1p in the cell wall fraction, as quantified by ELISA, compared to the amount of Als1p in C. albicans untreated with the compound, was considered to be a compound that inhibits the process that transports GPI-anchored proteins to the cell wall in C. albicans.

Example A5
Observation of the Cell Wall of C. albicans Cultured in the Presence of a Test Sample by an Electron Microscope

[0235] C. albicans which was cultured in Sabouraud Dextrose Liquid Medium (5 ml) containing various concentra-
tions of the test agent at 30°C for 48 hours, then centrifuged, and collected, was immobilized by potassium permanganate immobilization method, and the transmission electron microscope image thereof was observed.

[0236] The flocculent fibrous structure with high electron density was observed in the outermost layer of the cell, and was considered to be the surface layer glycoprotein layer having the GPI-anchored protein as its constituent. This flocculent fibrous structure was not influenced by other existing antifungal agents.

[0237] In C. albicans cultured in the presence of the aforementioned compound (Ia), the flocculent fibrous structure of the outermost layer of the cell having high electron density disappeared leaving a small amount of the layer with high electron density, compared to that in untreated cells. In this manner, when the flocculent fibrous structure of the outermost layer of the fungal cell having high electron density disappeared, the test sample was considered to be the compound influencing the process that transports GPI-anchored proteins to the cell wall.

Example A6
Screening of the Resistant Gene to the Aforementioned Compound (Ia) of S. cerevisiae

[0238] The plasmid library of the S. cerevisiae gene was obtained from ATCC (Information for ATCC Number: 37332).

[0239] S. cerevisiae G2-10 strain was cultured while shaking in 10 ml of YPD medium at 30°C, and cells were collected at the late logarithmic growth phase (1-2×10^7 cells/ml). After washing the cells with sterilized water, the plasmid library of the S. cerevisiae gene was introduced by the lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) according to YEASTMAKER™ Yeast Transformation System User Manual, and this was spread onto a SD(Leu^-) plate, and approximately 80,000 colonies were obtained. The colonies were collected and diluted, and were spread onto a SD(Leu^-) plate containing the aforementioned compound (Ia) at a concentration of 1.56 µg/ml and 3.125 µg/ml so that there were 570,000 colonies per plate. Subsequently, the resistant clone was obtained by incubating at 37°C for 72 hours.

[0240] When 27 clones were picked and plasmids were collected by the method according to METHODS IN ENZYMOLOGY, Vol. 194: 169-182 (1991), and the inserts were analyzed, all 27 contained the same fragment.

[0241] As a result of determining the nucleotide sequence using the ABI377 system (PE Applied Biosystems), the DNA of SEQ ID NO:1 was found to be the DNA that confers resistance to the aforementioned compound (Ia), and was named GWT1.

Example A7
Southern Blot Analysis of a C. albicans Homologue of the S. cerevisiae GWT1 Gene

[0242] A sample was prepared by treating 25 µg of the C. albicans genomic DNA with EcoRI (TakaRa), HindIII (TakaRa), BamHI (TOYOBO), or PstI (New England Biolabs) (including a combination of 2 types of enzymes) for 16 hours, then concentrating by ethanol precipitation, and dissolving in 25 µl of sterilized water. Twenty-five micrograms of genomic DNA digested with restriction enzymes was separated by 0.75% agarose gel electrophoresis method, and was transferred to a nylon membrane (GeneScreen PLUS/NEN).

[0243] A probe was produced by labeling 20 ng of the approximately 1.5 kb DNA fragment of SEQ ID NO: 1 with alpha32P-dCTP by the random primer method, and was purified using a GeneQuant column (Amersham-Pharimacia).

[0244] Hybridization was carried out by soaking the membrane in 10 ml of PERFECTHYB™ (TOYOBO) solution, preincubating at 65°C for 1 hour, then adding the labeled probe mentioned above, and incubating at 65°C for 2.5 hours. Washing was carried out with 1), 2xSSC, 0.05% SDS solution at 25°C for 5 minutes, 2) 2xSSC, 0.05% SDS solution at 25°C for 15 minutes, and 3) 0.1xSSC, 0.1% SDS solution at 50°C for 20 minutes. The washed membrane was wrapped with Saran Wrap, and contacted with an Imaging Plate (FUJI) for 12 hours at room temperature, the image that was transferred to the Imaging Plate was captured using BAS2000 (FUJI), and the image was analyzed. As a result, single bands were observed at 6.5 kb with EcoRI, 4.0 kb with HindIII, 2.0 kb with EcoRI-HindIII, and 2.5 kb with EcoRI-PstI (Fig. 5), and the homologue of the resistant gene to the aforementioned compound (Ia) of C. albicans was expected to exist as a single gene.

Example A8
Screening of the Resistant Gene to the Aforementioned Compound (Ia) of C. albicans

[0245] The genomic library of C. albicans was produced by the method according to Navaro-Garcia F et al, Mol. Cell. Biol., 15: 2197-2206, 1995. Specifically, the genomic DNA of C. albicans was partially digested with Sac3AL, then DNA fragments around 3 to 5 were collected, and these were inserted into the BamHI site of YEp352 shuttle vector.

[0246] S. cerevisiae G2-10 strain was cultured by shaking in 10 ml of YPD medium at 30°C, and cells were collected at the late logarithmic growth phase (2-5×10^7 cells/ml). After washing the cells with sterilized water, a genomic library of the C. albicans was introduced by the lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) according to YEASTMAKER™ Yeast Transformation System User Manual, and this was spread onto a SD(Ura^-) plate, and approximately 25,000 colonies were obtained. The colonies were collected and diluted, and were spread onto a SD plate containing the aforementioned compound (Ia) at a concentration of 1.56 µg/ml so that there were 500,000 colonies per plate. Subsequently, the resistant clones were obtained by incubation at 30°C for 6 hours, and then transferred to 37°C, and incubated for 66 hours.

[0247] When 30 clones were picked and plasmids were collected by the method according to METHODS IN ENZYMOLOGY, Vol. 194: 169-182 (1991), and the inserts were analyzed, 28 out of 30 contained the same fragment.

[0248] As a result of determining the nucleotide sequence using the ABI377 system (PE Applied Biosystems), the DNA of SEQ ID NO:3 was found to be the DNA that confers resistance to the aforementioned compound (Ia).

Example A9
Cloning of a Homologue of the Resistant Gene to the Aforementioned Compound (Ia) from the Clinical Isolate of C. albicans

[0249] PCR amplification was carried out using as template a genomic DNA that was purified from a clinical isolate of C.
albicans that is stored by the inventors, and SEQ ID NO:21 and SEQ ID NO:22 as primers. A DNA fragment of approximately 1.6 kb was amplified from all three of the independent PCR samples, the amplified fragments were purified, subcloned into a pT7-Blue vector (Novagen), and the nucleotide sequence was determined, and thereby, the DNA sequence of SEQ ID NO:5 was discovered. The sequence was different at three positions as compared to the DNA of Example A7 (SEQ ID NO:3).

Furthermore, in the nucleotide sequence of the C. albicans gene determined at Stanford University Sequence Center (on the worldwide web at sequence-www.stanford.edu), a homologue of the DNA of Example A7 was found (SEQ ID NO:7), and the sequence was different at four positions as compared to the DNA of Example A7 (SEQ ID NO:3).

Example A10
Construction of S. cerevisiae Overexpressing the GWT1 Gene Product

[0251] PCR amplification was carried out using a plasmid purified from the resistant clone to the aforementioned compound (la) obtained in Example A6 as a template, and SEQ ID NO:23 and SEQ ID NO:24 as primers. A PCR product cleaved with PvuII was inserted into the SalI-HindIII cleavage site of pRLW631 produced in Example A1. The entire insert was excised with BamHI-KpnI, and was inserted into the MCS (multi-cloning site) of PRS304 (Sikorski R S et al, Genetics, 122(1): 19-27, 1989) to produce a vector for integration.

[0252] S. cerevisiae CW63 strain having a cephalosporinase gene as the reporter gene was cultured by the method according to Example A1, TRP1 of the integration vector was cleaved with EcoRV, and then transformation was carried out by the method of Example A1. GWT1-overexpressed strain (S. cerevisiae CW63/GWT1 strain) was obtained by cultivating in SD(Trp) medium at 30°C for 3 days.

[0253] Other than showing resistance to the aforementioned compound (la), GWT1-overexpressed strain is not different from the wild type strain, and was sensitive towards other antifungal agents, cycloheximide, benomyl, and amphotericin B.

Example A11
Construction of S. cerevisiae Mutant Lacking the GWT1 Gene

[0254] His5 cassette containing the GWT1 sequence on both ends was amplified by PCR using the his5 gene of S. pombe (Longtime M S et al, Yeast, 14: 953-961, 1998) as template and SEQ ID NO:25 and SEQ ID NO:26 as primers. S. cerevisiae G2-10 was cultured and the cells were collected by the method according to Example A1, and the abovementioned PCR product was transformed by the method according to Example A1. A GWT1-deficient strain was obtained by cultivation in SD(His-) medium at 30°C for 5 to 7 days.

[0255] Although the GWT1-deficient strain shows very slow growth, it was suggested that the growth is not influenced by the aforementioned compound (la), and the GWT1 gene product is the target of the compound. Furthermore, the GWT1-deficient strain indicated the following characteristics: it cannot grow at high temperatures; the cells are swollen; and in the observation by a transmission electron microscope, the flocculent fibrous structure of the outermost layer of the fungal cell having high electron density had disappeared.

Example A12
Activity of the Aforementioned Compound (Ia) in S. cerevisiae Overexpressing the GWT1 Gene Product

[0256] Using S. cerevisiae CW63 strain and GWT1 gene introduced S. cerevisiae CW63/GWT1, activity of the aforementioned compound (Ia) was examined by a method according to the method described in Example A2.

[0257] As a result, even at a concentration (0.39 to 1.56 μg/ml) of the aforementioned compound (la) at which cephalosporinase activity in the culture supernatant fraction is increased, and the activity in the cell wall fraction is decreased in S. cerevisiae CW63 strain, no influence was observed in the S. cerevisiae CW63/GWT1 strain, and even at a concentration (>3.13 μg/ml) of the aforementioned compound (la) at which growth is inhibited in S. cerevisiae CW63 strain, growth inhibition was not observed in the S. cerevisiae CW63/GWT1 strain (FIG. 6).

Example A13
Synthesis of (4-butylyphenyl)(1-isoquinoxylnyl)ketone

[0258] Under a nitrogen atmosphere, 1-bromo 4-butylobenzene (2.29 mL, 13.0 mmol) was added to a mixed solution of magnesium (338 mg, 13.9 mmol) and tetrahydrofuran (6.5 mL), and as an initiator, catalytic amount of 1,2-dibromoethane was added, and this was stirred under reflux for 10 minutes. The solution was cooled to 0°C, a tetrahydrofuran solution of 1-isoquinoxylnecarboutrile (1.0 g, 6.49 mmol) was added, and was stirred for another 1 hour at room temperature, and at 70°C for 3 hours. Subsequently, the solution was cooled again to 0°C, concentrated hydrochloric acid (2.56 ml) and methanol (11 ml) were added, and then refluxed for 2 hours. The concentrated residue was dissolved in 5 N sodium hydroxide and toluene, and was filtered through celite. The toluene layer of the filtrate was divided, washed with water, dried over magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography to give 1.72 g of the title compound.

[0259] 1H-NMR (CDCl3, δ (ppm)): 0.93 (3H, t), 1.32-1.43 (2H, m), 1.58-1.66 (2H, m), 2.68 (2H, t), 7.28 (2H, d), 7.61 (1H, td), 7.74 (1H, d), 7.80 (1H, d), 7.87 (2H, d), 7.92 (1H, d), 8.20 (1H, d), 8.60 (1H, d)

Example A14
Synthesis of [1-(4-butylyphenyl)isoquinoxylnyl], the Aforementioned Compound of the Formula (Ia)

[0260] The compound of Example A13 (1.72 g, 5.95 mmol), hydrazine monohydrate (836 mg, 16.7 mmol), and potassium hydroxide (769 mg, 13.7 mmol) were added to diethyl glycol (8.5 ml), and were stirred at 80°C for 1 hour, at 160°C for 3 and a half hours, and at 200°C for 1 hour. Upon cooling to room temperature, ice water was added and extracted with ethyl acetate. This was washed with water, then dried over magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography to give 914 mg of the aforementioned compound of the formula (Ia).
Another Method for Producing \{1-(4-butylbenzyl)isoquinoline\}, the Aforementioned Compound of the Formula (Ia).

To a dimethylformamide (1.8 ml) solution of 60% sodium hydride (16 mg, 0.40 mmol), a dimethylformamide (3.6 ml) solution of 1-cyano-2-benzoyl-1,2-dihydroisoquinoline (100 mg, 0.38 mmol) synthesized according to the literature of Org. Synth., V, 115 (1988), and 4-4-butylbenzyl chloride (70 mg, 0.38 mmol) was added dropwise under nitrogen atmosphere at -16°C., and was further stirred at room temperature for 30 minutes. Water was added, this was concentrated, and toluene and water were added to this residue. The toluene layer was washed with water, dried over potassium carbonate, and concentrated. To an ethanol (1.6 ml) solution of the residue, 50% aqueous sodium hydroxide solution (0.63 ml) was added, and this was refluxed for 2 hours. After concentration, toluene and water were added. The toluene layer was washed with water, then dried over calcium carbonate, and then concentrated. The residue was purified by silica gel column chromatography to give 18 mg of the aforementioned compound of the formula (Ia).

Cloning of the C. albicans Homologue of the S. cerevisiae GWT1 Gene

The C. albicans genomic DNA (25 µg) treated with HindIII (TaKaRa) for 16 hours was separated by 0.75% agarose gel electrophoresis method, and the DNA fragments ranging in size from approximately 3.5 to 4.5 kb were recovered from the gel. The recovered DNA fragments were inserted into the HindIII site of the pKF3 vector (TaKaRa), and a Candida genomic library was produced.

Using the produced library, approximately 10,000 colonies were displayed on an LB/Ampicillin plate, colony lifting was performed using a Colony/Plaque Screen (NEB) membrane, and this was subjected to hybridization. A probe was produced by labeling 20 ng of the approximately 1.5 kb DNA fragment of SEQ ID NO:1 with alpha 32P-dCTP by the random primer method, and purifying using a GeneQuant column (Amersham-Pharmacia). Hybridization was carried out by pre-incubating the membrane in a PERFECTHYB™ (TOYOBO) solution at 65°C. for 1 hour, then adding the labeled probe mentioned above, and incubating further at 65°C. for 2 hours. Washing was carried out with (i) 2xSSC, 0.05% SDS solution at 25°C. for 5 minutes, (ii) 2xSSC, 0.05% SDS solution at 25°C. for 15 minutes, and (iii) 0.1xSSC, 0.1% SDS solution at 50°C. for 20 minutes. The washed membrane was wrapped with Saran Wrap, contacted with an X-RAY FILM (KONICA) for 24 hours at room temperature, and then developed. The E. coli colonies corresponding to the exposed spots were isolated, and were subjected to secondary screening. Approximately 200 of the isolated colonies were displayed on each LB/Ampicillin plate, colony lifting was performed in a similar manner to primary screening, which was followed by hybridization. The conditions for hybridization were the same as the conditions for primary screening.

As a result, a single colony of E. coli that reacts strongly with the probe was isolated. Plasmids were collected from this colony, and when the contained sequence was determined, a novel sequence having the same sequence as that revealed in Example A9 (SEQ ID NO:5) was found (the sequence of Candida GWT1), and was presumed to be a C. albicans homologue.

The S. Pombe Homologue of the S. cerevisiae GWT1 Gene

S. Pombe genes that show homology to the S. cerevisiae GWT1 gene (SEQ ID NO:27, and the amino acid sequence of the gene product thereof: SEQ ID NO:28) were found from a database search, and were considered to be the S. Pombe homologues of GWT1.

Cloning of the Aspergillus fumigatus Homologue of the S. cerevisiae GWT1 Gene

By genetic sequence analysis, the inventors discovered two highly conserved regions in the protein encoded by the GWT1 genes of S. cerevisiae, S. pombe, and C. albicans (FIG. 7, SEQ ID NOs:64-69). Based on the presumed DNA that encodes the amino acid sequence of this conserved region, primers of SEQ ID NO:29, SEQ ID NO:30, and SEQ ID NO:31 were designed. PCR amplification was carried out using 1 µl of the library purchased from STRAUengeance (Aspergillus fumigatus cDNA library: #937053) as a template, and using primers of SEQ ID NO:29 and SEQ ID NO:31. Furthermore, as a result of carrying out nested-PCR using 1 µg of this amplified sample as a template, and using primers of SEQ ID NO:29 and SEQ ID NO:30, amplification of a single fragment of approximately 250 bp was confirmed. When the sequence of this fragment was determined, a novel sequence having homology to the GWT1 gene of S. cerevisiae, shown in SEQ ID NO:32, was obtained, and this was presumed to be the homologue of A. fumigatus.

To obtain a full length cDNA, primers of SEQ ID NO:33 and SEQ ID NO:34 were designed based on the sequence of the amplified fragment. Furthermore, primers outside the gene insertions site of the library, SEQ ID NO:35 and SEQ ID NO:36, were designed. As a result of performing PCR using the A. fumigatus cDNA library as a template, and the primer set of SEQ ID NO:33 and SEQ ID NO:35, or the primer set of SEQ ID NO:34 and SEQ ID NO:36, amplification of a DNA fragment of approximately 1 kb was confirmed (by both primer sets). As a result of determining the nucleotide sequences of these fragments, a novel sequence that is highly homologous to the GWT1 genes of S. cerevisiae shown in SEQ ID NO:1 was obtained. Since the sequence is highly homologous to the GWT1 genes of S. cerevisiae, S. pombe, and C. albicans throughout the entire gene, this sequence was strongly suggested to be a homologue of A. fumigatus.

To clone the entire homologue of A. fumigatus, the primer shown in SEQ ID NO:37 that corresponds to the sequence upstream of the initiation codon, and the primer of SEQ ID NO:38 that corresponds to the sequence downstream of the stop codon were newly designed based on the obtained
sequence. As a result of performing 35 cycles of PCR using the A. fumigatus cDNA library (STRATAGENE) and the A. fumigatus genomic library (STRATAGENE) as templates, and primers of SEQ ID NO:37 and SEQ ID NO:38, a single amplified fragment of approximately 1.6 kb was detected from both templates. As a result of determining the nucleotide sequence of this fragment by Direct-Sequencing, the nucleotide sequence shown in SEQ ID NO:39 was found from the cDNA library, and was suggested to encode a protein comprising 501 amino acids shown in SEQ ID NO:40. Furthermore, the nucleotide sequence of SEQ ID NO:41 was found from the genomic library, and was found to have an intron comprising 77 base pairs in one position.

Example A19
Cloning of the Cryptococcus Homologue of the S. cerevisiae GWT1 Gene

1). Database Search

[0271] As a result of database searching for homology to the S. cerevisiae GWT1 gene, the sequence of 502042C05.x1 was found from the server of the Genome Center at Stanford University (on the worldwide web at baggage.stanford.edu/cgi-misc/cneoformans/). Furthermore, the sequence of b6c06cn.f1 was found from the server at Oklahoma University, U.S.A. (on the worldwide web at www.genome.ou.edu/cneo_blast.html).

2). PCR Using Genomic DNA as Template

[0272] The primer of SEQ ID NO:42 was constructed based on the sequence of 502042C05.x1, and the primer of SEQ ID NO:43 was constructed based on the sequence of b6c06cn.f1. When PCR amplification was carried out using the genomic DNA of Cryptococcus (Cryptococcus neoformans) as a template, and using the primer of SEQ ID NO:42, and the primer of SEQ ID NO:43, an amplified fragment of approximately 2 kb was detected. When the nucleotide sequence of this fragment was determined, a novel sequence showing homology to the GWT1 gene of S. cerevisiae, shown in SEQ ID NO:44, was obtained.

[0273] In order to obtain the sequence upstream of the initiation codon of the Cryptococcus GWT1 gene, the primer of SEQ ID NO:45 was designed based on the sequence of 502042C05.x1, and the primer of SEQ ID NO:46 was designed based on the sequence of SEQ ID NO:44. When PCR amplification was carried out using the genomic DNA of Cryptococcus as a template, and using the primer of SEQ ID NO:45, and the primer of SEQ ID NO:46, an amplified fragment of approximately 500 bp was detected. When the nucleotide sequence of this fragment was determined, the sequence of SEQ ID NO:47 was obtained, and this was found to overlap with SEQ ID NO:44.

3). 3'-RACE

[0274] To obtain the 3'-terminal sequence of the Cryptococcus GWT1 gene, 3'-RACE was carried out. Reverse transcription was carried out by priming with the adaptor-primer of SEQ ID NO:48, which is based on 16 μg of total RNA extracted from Cryptococcus, and by using SuperScript II Reverse Transcriptase (GIBCO/BRL), and a single stranded cDNA, which is to become the template for the RT-PCR that follows, was produced. As a result of performing 35 cycles of PCR using the single stranded cDNA as a template, and the primers of SEQ ID NO:49 and SEQ ID NO:50, an amplified fragment of approximately 1.2 kb was detected. When the nucleotide sequence of this fragment was analyzed by the Direct-Sequencing method, the novel sequence shown in SEQ ID NO:51 showing homology to the S. cerevisiae GWT1 gene was obtained.

4). PCR of a Full Length Genomic DNA

[0275] Using the primer of SEQ ID NO:52 that was designed based on SEQ ID NO:47, and the primer of SEQ ID NO:53 that was designed based on SEQ ID NO:51, 35 cycles of PCR was carried out on three independent preparations with the genomic DNA of Cryptococcus as template. As a result, an amplified fragment of approximately 2 kb was detected from all three of the independent tubes, and therefore, each of them were individually subjected to Direct-Sequencing, and their entire nucleotide sequences were determined. As a result, three independent sequences completely matched, and a sequence comprising the full length GWT1 gene homologue of Cryptococcus shown in SEQ ID NO:54 was obtained.

5). Determination of the cDNA Sequence

[0276] Comparison of the sequence of the Cryptococcus GWT1 gene derived from the genome shown in SEQ ID NO:54 with the cDNA sequence (SEQ ID NO:51) obtained by 3'-RACE suggested the presence of introns at two positions. Furthermore, since the open reading frame following the ATG initiation codon is not continuous, the presence of another intron was suggested. Therefore, the cDNA structure was predicted from the presumed amino acid sequence and the splicing donor/acceptor sequence, and the primers of SEQ ID NO:55 and SEQ ID NO:56 were designed at the position predicted to be the junction between exons. As a result of performing 35 cycles of PCR using the single stranded cDNA derived from Cryptococcus as template with the above-mentioned primers, an amplified fragment of approximately 1.4 kb was confirmed. As a result of determining the nucleotide sequence by subjecting the fragment to Direct-Sequencing, the sequence of SEQ ID NO:57 was obtained, and by comparing with SEQ ID NO:54, the cDNA sequence of the GWT1 gene of Cryptococcus was suggested to have the structure of SEQ ID NO:58 and encode the protein shown in SEQ ID NO:59. Since the sequence shows high homology at certain regions with the GWT1 genes of S. cerevisiae, S. pombe, C. albicans, and A. fumigatus, this sequence was strongly suggested to be a homologue of Cryptococcus.

Example A20
Genetic Mutation that Confers Resistance to the Aforementioned Compound of the Formula (Ia)

[0277] S. cerevisiae LW63 strain having a lysozyme gene as the reporter gene due to introduction of rKLW63T was treated with ethyl methanesulphonate, then by culturing in a SD medium containing the aforementioned compound of the formula (Ia) at concentrations of 1.56, 3.13, and 6.25 μg/ml at 37°C for 3 days, five resistant mutant strains (R1 to R5) were obtained. Among them, the R1 mutant strain and the R5 mutant strain were found to have acquired a specific resistant characteristic to the aforementioned compound of the formula (Ia) due to a mutation of a single gene. To confirm whether or not these two mutant strains have mutations on the GWT1 gene, genomic DNAs were extracted from both mutant strains, and the nucleotide sequence of the GWT1
the gene portion was determined. As a result, in the R1 mutant strain, guanine at position 1213 had been mutated to adenine. Furthermore, in the R5 mutant strain, guanine at position 418 had been mutated to adenine. Therefore, it was elucidated that in the R1 mutant strain, the 405th amino acid, isoleucine, had been changed to valine, and in the R5 mutant strain, the 140th amino acid, glycine, had been changed to arginine.

Next, to confirm whether or not these mutations are the cause of the acquisition of the specific resistant characteristic to the aforementioned compound of the formula (Ia), the mutant GWT1 gene (R1 or R5) was isolated using the genomic DNAs derived from both mutant strains as templates and the primers of SEQ ID NOs: 60 and 61. Simultaneously, the GWT1 promoter region (SEQ ID NO:62) and the terminator region (SEQ ID NO:63) were isolated, the GWT1 gene promoter, mutant GWT1 gene ORF, and the GWT1 gene terminator were inserted into the pRS316 vector, and plasmids that express a single copy of the mutant GWT1 gene were constructed (pRS316GWT1-R1, pRS316GWT1-R5). This was introduced to a diploid strain (WDG1) in which only a single copy of the GWT1 gene is disrupted. Spores were formed by culturing the colonies on a sporulation medium, and a clone in which the GWT1 gene on the chromosome is disrupted and also harbors the above-mentioned plasmid was obtained by performing a tetrad analysis. When this was cultured in a medium containing the aforementioned compound of the formula (Ia), resistance to the aforementioned compound of the formula (Ia) was seen, similarly to the original R1 mutant strain and R5 mutant strain. From the above, it was elucidated that the specific resistant characteristic to the aforementioned compound of the formula (Ia) is conferred by a point mutation accompanying an amino acid mutation, that occurred on the GWT1 gene, and this compound was strongly suggested to inhibit the function of the GWT1 protein by directly binding to the protein.

Example B

The compounds of this invention can be produced, for example, by the method of the Examples below. However, the Examples are for illustration purpose only and the compounds of this invention are not to be construed as being limited to those prepared in the following specific examples under any circumstances.

Example B1
1-(Chloromethyl)-4-n-butylbenzene

Thionyl chloride (2.5 ml, 34 mmol) was added to a solution of 4-n-butylbenzyl alcohol (2.0 g, 12 mmol) in ether (25 ml), and this mixture was stirred at room temperature for 3 hours. After concentration of the mixture, excess thionyl chloride was removed by azeotropic distillation with benzene to give the title compound (2.3 g). This compound was used in the following reaction without purification.

Example B2
1-(4-Butylbenzyl)isoquinoline

A solution of 1-cyano-2-benzoyl-1,2-dihydroisoquinoline (100 mg, 0.38 mmol), which was synthesized according to Org. Synth., VI, 115 (1988), and 4-n-butylbenzyl chloride (70 mg, 0.38 mmol) in dimethylformamide (3.6 ml) was added dropwise to a solution of 60% sodium hydride (16 mg, 0.40 mmol) in dimethylformamide (1.8 ml) under nitrogen atmosphere at −16°C, and this mixture was stirred at room temperature for 30 minutes. Water was added, the mixture was concentrated under reduced pressure, and toluene and water were added to the residue. The toluene layer was washed with water, dried over potassium carbonate, then concentrated under reduced pressure. A 50% aqueous sodium hydroxide solution (0.63 ml) was added to a solution of the residue in ethanol (1.6 ml). This mixture was heated under reflux for 2 hours and concentrated, and then toluene and water were added. The toluene layer was washed with water, dried over calcium carbonate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (18 mg).

Example B3
(4-Butylphenyl)(1-isoquinolyl)ketone

1-Bromo-4-butylbenzene (2.29 ml, 13 mmol) and a catalytic amount of 1,2-dibromoethane as an initiator were added to a mixed solution of magnesium (338 mg, 14 mmol) and tetrahydrofuran (6.5 ml) under nitrogen atmosphere, and this mixture was stirred under reflux for 10 minutes. The mixture was cooled to 0°C, a solution of 1-isoquinolinemerc-
bonitrile (1.0 g, 6.5 mmol) in tetrahydrofuran was added, and this mixture was stirred at room temperature for 1 hour, then at 70°C for 3 hours. Thereafter, the mixture was cooled again to 0°C, concentrated hydrochloric acid (2.6 ml) and methanol (11 ml) were added, and this mixture was heated under reflux for 2 hours. After the mixture was concentrated, the residue was dissolved in 5 N sodium hydroxide and toluene, and was filtered through celite. The toluene layer of the filtrate was separated, washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (1.7 g).

Example B4

Alternative Method for the Production of 1-(4-Butylbenzyl)isoquinoline

The compound of Example B3 (1.7 g, 6.0 mmol), hydrazine monohydrate (836 mg, 17 mmol), and potassium hydroxide (769 mg, 14 mmol) were added to diethylene glycol (8.5 ml), and this mixture was stirred at 80°C for 1 hour, at 160°C for 3.5 hours, then at 200°C for 1 hour. The mixture was cooled to room temperature, ice water was added, and this was extracted with ethyl acetate. The extract was washed with water, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (914 mg).

Example B5

1-(4-Ethylbenzyl)isoquinoline

Using p-ethylbenzyl chloride, the title compound was obtained in the same manner as in Example B2.

Example B6

(4-Propylphenyl)methanol

Example B7

1-(Chloromethyl)-4-propylbenzene

Example B8

1-(4-Propylbenzyl)isoquinoline

The title compound was obtained by treating the compound of Example B7 in the same manner as in Example B1. This compound was used in the following reaction without further purification.

Example B9

The title compound was obtained by treating the compound of Example B8 in the same manner as in Example B2.
Example B9

(4-Pentylphenyl)methanol

The title compound was obtained by reducing 4-n-amylbenzoic acid in the same manner as in Example B6.

Example B10

1-(Chloromethyl)-4-pentylbenzene

The title compound was obtained by treating the compound of Example B9 in the same manner as in Example B1. This compound was used in the following reaction without further purification.

Example B11

1-(4-Pentylbenzyl)isoquinoline

The title compound was obtained by treating the compound of Example B10 in the same manner as in Example B1. This compound was used in the following reaction without further purification.

Example B12

(4-Hexylphenyl)methanol

The title compound was obtained by reducing 4-n-hexylbenzoic acid in the same manner as in Example B6. This compound was used in the following reaction without further purification.

Example B13

1-(Chloromethyl)-4-hexylbenzene

The title compound was obtained by treating the compound of Example B12 in the same manner as in Example B1. This compound was used in the following reaction without further purification.

Example B14

1-(4-Hexylbenzyl)isoquinoline

The title compound was obtained by treating the compound of Example B13 in the same manner as in Example B1. This compound was used in the following reaction without further purification.
Example B15

1-(4-Isopropylbenzyl)isoquinoline

[0314] The title compound was obtained by treating p-isopropylbenzyl chloride in the same manner as in Example B2.

[0315] ¹H-NMR (CDCl₃) δ (ppm): 1.19 (6H, d), 2.80-2.87 (1H, m), 4.64 (2H, s), 7.11 (2H, d), 7.21 (2H, d), 7.51-7.56 (2H, m), 7.61-7.65 (1H, m), 7.81 (1H, d), 8.19 (1H, dd), 8.50 (1H, d)

Example B16

1-(4-(tert-Butyl)benzyl)isoquinoline

[0317] The title compound was obtained by treating 4-tert-butylbenzyl chloride in the same manner as in Example B2.

[0318] ¹H-NMR (CDCl₃) δ (ppm): 1.26 (9H, s), 4.64 (2H, s), 7.22 (2H, d), 7.27 (2H, d), 7.52-7.56 (2H, m), 7.62-7.66 (1H, m), 7.81 (1H, d), 8.19 (1H, dd), 8.50 (1H, d)

Example B17

(4-Isobutylphenyl)methanol

[0320] The title compound was obtained by reducing 4-isobutylbenzoic acid in the same manner as in Example B6. This was used in the following reaction without further purification.

Example B18

1-(Chloromethyl)-4-isobutylbenzene

[0322] The title compound was obtained by treating the compound of Example B17 in the same manner as in Example B1. This was used in the following reaction without further purification.

Example B19

1-(4-Isobutylbenzyl)isoquinoline

[0324] The title compound was obtained by treating the compound of Example B18 in the same manner as in Example B2.

[0325] ¹H-NMR (CDCl₃) δ (ppm): 0.86 (6H, d), 1.75-1.83 (1H, m), 2.39 (2H, d), 4.66 (2H, s), 7.02 (2H, d), 7.18 (2H, d), 7.52-7.58 (2H, m), 7.63-7.67 (1H, m), 7.82 (1H, d), 8.18 (1H, d), 8.50 (1H, d)

Example B20

1-(Chloromethyl)-4-(trifluoromethyl)benzene

[0327] The title compound was obtained by treating 4-trifluoromethylbenzyl alcohol in the same manner as in Example B1. This was used in the following reaction without further purification.
Example B21
1-4-(Trifluoromethyl)benzylisoquinoline

The title compound was obtained by treating the compound of Example B20 in the same manner as in Example B2.

1H-NMR (CDCl₃) δ (ppm): 4.67 (2H, s), 7.10 (2H, d), 7.27 (2H, d), 7.54-7.59 (2H, m), 7.64-7.68 (1H, m), 7.84 (1H, d), 8.11 (1H, dd), 8.50 (1H, d)

Example B22
1-(Chloromethyl)-4-(trifluoromethoxy)benzene

The title compound was obtained by treating 4-trifluoromethoxybenzyl alcohol in the same manner as in Example B1. This was used in the following reaction without further purification.

Example B23
1-4-(Trifluoromethoxy)benzylisoquinoline

The title compound was obtained by treating the compound of Example B22 in the same manner as in Example B2.

1H-NMR (CDCl₃) δ (ppm): 4.67 (2H, s), 7.10 (2H, d), 7.27 (2H, d), 7.54-7.59 (2H, m), 7.64-7.68 (1H, m), 7.84 (1H, d), 8.11 (1H, dd), 8.50 (1H, d)

Example B24
1-(Chloromethyl)-2-iodobenzene

The title compound was obtained by treating 4-iodobenzyl alcohol in the same manner as in Example B1. This was used in the following reaction without further purification.

Example B25
1-(2-Iodobenzyl)isoquinoline

The title compound was obtained by treating the compound of Example B24 in the same manner as in Example B2. This was used in the following reaction without further purification.

1H-NMR (CDCl₃) δ (ppm): 4.74 (2H, s), 6.81-6.84 (1H, m), 6.87-6.92 (1H, m), 7.11-7.15 (1H, m), 7.55-7.57 (1H, m), 7.60 (1H, d), 7.64-7.68 (1H, m), 7.83-7.86 (1H, m), 7.89-7.91 (1H, m), 8.00-8.02 (1H, m), 8.50 (1H, d)
Example B26
1-[2-(2-Phenyl-1-ethynyl)benzyl]isoquinoline

A solution of tetrakis(triphenylphosphine)palladium (58 mg, 0.05 mmol) and ethynylbenzene (204 mg, 2.0 mmol) in pyrroolidine (1.5 ml) was added to a solution of the compound of Example B25 (345 mg, 1.07 mmol) in pyrroolidine (1.5 ml) under nitrogen atmosphere, and the mixture was stirred at 80°C for 3 hours. The mixture was cooled to room temperature, diluted with ethyl acetate, washed with a saturated aqueous ammonium chloride solution, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (280 mg).

H-NMR (CDCl3) δ (ppm): 4.95 (2H, s), 6.98-7.06 (2H, m), 7.10-7.21 (2H, m), 7.31-7.35 (3H, m), 7.48-7.51 (3H, m), 7.57-7.65 (2H, m), 7.82 (1H, d), 8.25 (1H, d), 8.52 (1H, d)

Example B27
1-(2-Phenylethylbenzyl)isoquinoline

Palladium-carbon (10%, 230 mg) was added to a solution of the compound of Example B26 (280 mg, 0.88 mmol) in tetrahydrofuran (30 ml), and this mixture was stirred at room temperature under hydrogen atmosphere (1 atm) for 3 hours. The catalyst was removed by filtration and the obtained filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (162 mg).

H-NMR (CDCl3) δ (ppm): 2.90-2.94 (2H, m), 3.07-3.10 (2H, m), 4.67 (2H, s), 6.80 (1H, d), 7.02-7.06 (1H, m), 7.15-7.30 (7H, m), 7.49-7.53 (1H, m), 7.58 (1H, d), 7.64-7.68 (1H, m), 7.84 (1H, d), 7.95 (1H, d), 8.50 (1H, d)

Example B28
1-[2-[2-(2-Phenylethylbenzyl)isoquinoline]

A solution of tetrakis(triphenylphosphine)palladium (58 mg, 0.05 mmol) and 2-(3-butynyloxy)-tetrahydro-2H-pyran (208 mg, 2.0 mmol) in pyrroolidine (1.5 ml) was added to a solution of the compound of Example B25 (345 mg, 1.07 mmol) in pyrroolidine (1.5 ml) under nitrogen atmosphere, and this mixture was stirred for four days at room temperature, and for another 30 minutes at 80°C. The mixture was cooled to room temperature, diluted with ethyl acetate, washed with a saturated aqueous ammonium chloride solution, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (277 mg).

H-NMR (CDCl3) δ (ppm): 1.42-1.60 (4H, m), 1.64-1.68 (1H, m), 1.75-1.81 (1H, m), 2.76-2.80 (2H, m), 3.46-3.51 (1H, m), 3.60-3.66 (1H, m), 3.85-3.95 (2H, m), 4.64-4.66 (1H, m), 4.85 (2H, s), 6.95-6.98 (1H, m), 7.05-7.13 (2H, m), 7.44-7.46 (1H, m), 7.49-7.53 (1H, m), 7.56 (1H, d), 7.60-7.65 (1H, m), 7.80-7.82 (1H, m), 8.15-8.18 (1H, m), 8.49-8.51 (1H, m)

Example B29
4-[2-(1-Isoquinolylmethyl)phenyl]-3-butyn-1-ol

After the compound of Example B28 (200 mg, 0.54 mmol) was cooled to 0°C, a hydrochloric acid-methanol solution (10%, 5 ml) was added, and this mixture was stirred for 15 minutes. A saturated aqueous sodium hydrogenecarbon-
ate solution was added, and this mixture was extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (86 mg).

[0353] \[^1^H\text{-NMR (CDCl}_3\text{)} \delta \text{ (ppm): 2.72 (2H, t), 3.53-3.60 (1H, brs), 3.85 (2H, t), 4.85 (2H, s), 7.12-7.15 (2H, m), 7.22-7.24 (1H, m), 7.42-7.44 (1H, m), 7.55-7.59 (2H, m), 7.63-7.67 (1H, m), 7.81 (1H, d), 8.30 (1H, m), 8.46 (1H, m)\]

Example B30

4-[(1-Isoquinolylmethyl)phenyl]-1-butanol

[0354]

Palladium-carbon (10%, 10 mg) was added to a solution of the compound of Example B29 (44 mg, 0.15 mmol) in tetrahydrofuran (5 ml), and this mixture was stirred at room temperature under hydrogen atmosphere (1 atm) for 1 hour. After the catalyst was removed by filtration, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (18 mg).

[0355] \[^1^H\text{-NMR (CDCl}_3\text{)} \delta \text{ (ppm): 1.61-1.75 (4H, m), 2.33 (1H, brs), 2.77 (2H, t), 3.67 (2H, t), 4.70 (2H, s), 6.91 (1H, d), 7.02-7.06 (1H, m), 7.12-7.16 (1H, m), 7.19-7.21 (1H, m), 7.50-7.55 (1H, m), 7.57 (1H, d), 7.63-7.67 (1H, d), 7.83 (1H, d), 8.09 (1H, d), 8.47 (1H, d)\]

Example B31

1-Bromo-2-(chloromethyl)benzene

[0356]

The title compound was obtained by treating the compound of Example B31 in the same manner as in Example B2.

[0357] \[^1^H\text{-NMR (CDCl}_3\text{)} \delta \text{ (ppm): 2.71-2.74 (1H, t), 4.85 (2H, s), 7.12-7.15 (2H, s), 7.55-7.59 (2H, s), 8.46 (1H, d)\]

Example B32

1-(4-Bromobenzyl)isoquinoline

[0359]

N

Br

Example B33

Ethyl(E)-3-[4-(isoquinolylmethyl)phenyl]-2-propenoate

[0362]

Tris(2-methylphenyl)phosphine (20 mg, 0.067 mmol), palladium(II) acetate (7.5 mg, 0.034 mmol), and triethylamine (70 µL, 0.50 mmol) were added to a solution of the compound of Example B32 (100 mg, 0.34 mmol) and vinyl propionate (73 µL, 0.67 mmol) in dimethylformamide (1.0 ml) under nitrogen atmosphere, and this mixture was stirred at 100°C for 4 hours. After the mixture was cooled to room temperature, water was added, and this mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (74 mg).

[0363] \[^1^H\text{-NMR (CDCl}_3\text{)} \delta \text{ (ppm): 1.32 (3H, t), 4.24 (2H, q), 4.69 (2H, s), 6.36 (1H, d), 7.29 (2H, d), 7.42 (2H, d), 7.53-7.67 (4H, m), 7.83 (1H, d), 8.11-8.13 (1H, m), 8.50 (1H, d)\]
Example B34
Ethyl 3-[4-(1-isoquinolylmethyl)phenyl]propanoate

Palladium-carbon (10%, 20 mg) was added to a solution of the compound of Example B33 (71 mg, 0.22 mmol) in methanol (5.0 ml), and this reaction mixture was stirred at room temperature under hydrogen atmosphere at atmospheric pressure for 5.5 hours. After the catalyst was removed from the reaction mixture by filtration, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (52 mg).

Example B35
3-[4-(1-isoquinolylmethyl)phenyl]-1-propanol

Lithium aluminum hydride (6 mg, 0.16 mmol) was added to tetrahydrofuran (1.0 ml) cooled to 0°C. under nitrogen atmosphere. A solution of the compound of Example B34 (46 mg, 0.14 mmol) in tetrahydrofuran (1.0 ml) was further added, and this reaction mixture was stirred at that temperature for 3 hours. A mixed solution of methanol and water (9:1, 1.0 ml) was added to the reaction mixture, a saturated aqueous ammonium chloride solution was further added, then this mixture was extracted with chloroform. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (22 mg).

Example B36
1-isoquinolyl(4-methoxyphenyl)ketone

4-Bromoanisol (15.3 ml, 122 mmol) and a catalytic amount of 1,2-dibromoethane as an initiator were added to a mixed solution of magnesium (3059 mg, 125.8 mmol) and tetrahydrofuran (20 ml) under nitrogen atmosphere, and this reaction mixture was stirred while heating under reflux for 45 minutes. The mixture was cooled to 0°C., a solution of 1-isoquinolinecarboxitrite (10.78 g, 69.9 mmol) in tetrahydrofuran (30 ml) was added dropwise thereto, and this reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was cooled on ice, concentrated hydrochloric acid (24 ml) and methanol (120 ml) were added, and this mixture was heated under reflux for 1.5 hours. After cooling on ice, the mixture was adjusted to pH 8 by adding aqueous sodium hydroxide, extracted with ether, washed with saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (15.87 g).

Example B37
1-isoquinolyl(4-methoxyphenyl)methanol

Sodium borohydride (1855 mg) was added to an ice-cooled solution of the compound of Example B36 (8608 mg) in ethanol (170 ml), and this mixture was stirred at room temperature for 35 minutes. Sodium borohydride (957 mg) was further added, and this reaction mixture was stirred at 40°C. for 40 minutes. The reaction mixture was concentrated under reduced pressure, water was added, and this mixture
was extracted with ether. The organic layer was washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The obtained title compound (7881 mg) was used in the following reaction without further purification.

[0376] 1H-NMR (DMSO-d6) δ (ppm): 3.66 (3H, s), 6.30-6.52 (1H, brs), 6.81 (2H, d), 7.28 (2H, d), 7.54 (1H, dd), 7.68 (1H, dd), 7.76 (1H, d), 7.94 (1H, d), 8.37 (1H, d), 8.47 (1H, d).

[0377] The proton of the hydroxyl group was not observed in the NMR spectrum.

Example B38
1-Isoquinoly(4-methoxyphenyl)methyl acetate

[0378]

Acetic anhydride (20 ml) was added to a solution of the compound of Example B37 (7881 mg) in pyridine (100 ml), and this reaction mixture was stirred at 50°C for 4 hours. The reaction mixture was concentrated under reduced pressure and subjected to azotropic distillation with toluene. The residue was purified by silica gel column chromatography to give the title compound (8.79 g).

[0379] 1H-NMR (CDCl3) δ (ppm): 2.22 (3H, s), 3.76 (3H, s), 6.84 (2H, d), 7.39 (2H, d), 7.54 (1H, dd), 7.56 (1H, s), 7.60 (1H, d), 7.64 (1H, dd), 7.82 (1H, d), 8.19 (1H, d), 8.57 (1H, d).

Example B39
1-(4-Methoxybenzyl)isoquinoline

[0380] 1H-NMR (CDCl3) δ (ppm): 3.74 (3H, s), 4.61 (2H, s), 6.79 (2H, d), 7.21 (2H, d), 7.53 (1H, dd), 7.63 (1H, d), 7.80 (1H, d), 8.16 (1H, d), 8.49 (1H, d).

Example B38
4-(1-Isoquinolylmethyl)phenol

[0381] An aqueous hydrobromic acid solution (47%, 40 ml) was added to the compound of Example B39 (2185 mg), and this reaction mixture was heated under reflux for 14 hours. The reaction mixture was cooled to room temperature, further cooled on ice, neutralized with a 50% aqueous sodium hydroxide solution, and extracted with ethyl acetate. The ethyl acetate layer was washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The obtained powder was washed with petroleum ether to give the title compound (1822 mg).

[0382] 1H-NMR (DMSO-d6) δ (ppm): 4.48 (2H, s), 6.61 (2H, d), 7.07 (2H, d), 7.60 (1H, dd), 7.68 (1H, d), 7.71 (1H, dd), 7.92 (1H, d), 8.27 (1H, d), 8.41 (1H, d), 9.19 (1H, brs).

Example B41
4-(1-Isoquinolylmethyl)phenyl trifluoromethanesulfonate

[0383] Trifluoromethanesulfonic anhydride (0.55 ml) was added dropwise to an ice-cold solution of the compound of Example B40 (513 mg) in pyridine (10 ml), and this reaction mixture was stirred at that temperature for 45 minutes. After ice was added, the reaction mixture was extracted with ether. The organic layer was washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (546 mg).
Example B42
1-[4-(2-Phenyl-1-ethynyl)benzyl]isoquinoline

Phenylacetylene (53 µl), palladium acetate (9 mg), 1,1’-bis(diphenylphosphino)ferrocene (67 mg), copper(I) iodide (3 mg), lithium chloride (20 mg), and triethylamine (50 µl) were added to a solution of the compound of Example B41 (88 mg) in N,N-dimethylformamide (2.0 ml) that had been degassed and placed under nitrogen, and this mixture was stirred at 80°C for 8 hours. After cooling the mixture to room temperature, water was added, and this mixture was extracted with ethyl acetate. The organic layer was washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (53 mg).

Example B43
1-(4-Phenethylbenzyl)isoquinoline

Palladium-carbon catalyst (10%, 20 mg) was added to a solution of the compound of Example B42 (45 mg) in tetrahydrofuran (2 ml), and this mixture was stirred at room temperature under hydrogen atmosphere at atmospheric pressure for 2 hours. The catalyst was removed by filtration through celite, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (23 mg).

Example B44
1-[4-(4-Phenyl-1-butynyl)benzyl]isoquinoline

Example B45
1-[4-(4-Phenyl-1-butyl)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B44 and 4-phenyl-1-butynyl in the same manner as in Example B42.

Example B46
1-[4-(4-Phenyl-1-butynyl)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B44 in the same manner as in Example B43.
Example B46
1-4-{4-[tetrahydro-2H-2-pyranyloxy]-1-butynyl} benzyl]-isoquinoline

Example B48
4-4-{1-isoquinolylmethyl}phenyl]-1-butanol

[0402] [0409]

Example B47
4-{4-[(1-Isoquinolylmethyl)phenyl]-3-butyn-1-ol

Example B49
1-{4-[(3-Cyclopentyl-1-propynyl)benzyl]-isoquinoline

[0403] [0410]

[0404] ¹H-NMR (CDCl₃) δ (ppm): 1.48-1.90 (6H, m), 2.67 (2H, t), 3.49-3.55 (1H, m), 3.60 (1H, dd), 3.65-3.94 (2H, m), 4.66 (2H, s), 4.65-4.70 (1H, m), 7.14-7.20 (2H, m), 7.23-7.30 (2H, m), 7.53 (1H, dd), 7.58 (1H, d), 7.65 (1H, dd), 7.82 (1H, d), 8.10 (1H, d), 8.49 (1H, d).

Example B41 and 2-(3-butynyloxy)tetrahydro-2H-pyran in the same manner as in Example B42.

[0411] ¹H-NMR (CDCl₃) δ (ppm): 1.50-1.70 (4H, m), 2.57 (2H, t), 3.62 (2H, t), 4.64 (2H, s), 7.06 (2H, d), 7.18 (2H, d), 7.53 (1H, dd), 7.55 (1H, d), 7.63 (1H, dd), 7.80 (1H, d), 8.16 (1H, d), 8.49 (1H, d).

[0412] The title compound was obtained by treating the compound of Example B47 in the same manner as in Example B43.

Example B48
4-{4-{1-isoquinolylmethyl}phenyl]-1-butanol

[0413] [0417]

Example B49
1-{4-{3-Cyclopentyl-1-propynyl}benzyl]-isoquinoline

[0414] The title compound was obtained by treating the compound of Example B41 and 3-cyclopentyl-1-propyne in the same manner as in Example B42.

[0415] ¹H-NMR (CDCl₃) δ (ppm): 1.25-1.35 (2H, m), 1.45-1.70 (6H, m), 1.75-1.85 (2H, m), 2.05-2.13 (1H, m), 4.65 (2H, s), 7.17 (2H, d), 7.27 (2H, d), 7.51 (1H, dd), 7.56 (1H, d), 7.64 (1H, dd).

Example B50
1-{4-{3-Cyclopentylpropyl}benzyl]-isoquinoline

[0416] 7.81 (1H, d), 8.08 (1H, d), 8.49 (1H, d).

[0417] The proton of the hydroxyl group was not observed in the NMR spectrum.

[0406] The compound of Example B46 (1048 mg) was dissolved in a 10% hydrochloric acid-methanol solution (50 ml), and this reaction mixture was stirred at room temperature for 1.5 hours. The reaction mixture was cooled on ice, a saturated aqueous sodium hydrogencarbonate solution was added, and the resulting mixture was extracted with ethyl acetate. The organic layer was washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (666 mg).

[0407] ¹H-NMR (CDCl₃) δ (ppm): 2.65 (2H, t), 3.77 (2H, t), 4.65 (2H, s), 7.18 (2H, d), 7.29 (2H, d), 7.52 (1H, dd), 7.57 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.07 (1H, d), 8.49 (1H, d).

[0408] The proton of the hydroxyl group was not observed in the NMR spectrum.
The title compound was obtained by treating the compound of Example B49 in the same manner as in Example B43.

**Example B51**

4-(4-(1-Isoquinolylmethyl)phenyl)-2-methyl-3-butyne-2-ol

The title compound was obtained by treating the compound of Example B41 and 2-methyl-3-butyne-2-ol in the same manner as in Example B42.

**Example B52**

4-(4-(1-Isoquinolylmethyl)phenyl)-2-methyl-2-butanol

The title compound was obtained by treating the compound of Example B51 in the same manner as in Example B43.

**Example B53**

1-[4-(3-Methoxy-1-propynyl)benzyl]isoquinoline

The proton of the hydroxyl group was not observed in the NMR spectrum.

**Example B54**

1-[4-(3-Methoxypropyl)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B41 and methylpropargyl ether in the same manner as in Example B42.

**Example B55**

1-[4-(3-Methoxy-1-propynyl)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B51 in the same manner as in Example B43.

**Example B56**

1-[4-(3-Methoxypropyl)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B53 in the same manner as in Example B43.
Example B55

1-{4-[2-(2-Pyridyl)-1-ethynyl]benzyl}isoquinoline

[0433]

Example B57

1-{4-[2-(3-pyridyl)-1-ethynyl]benzyl}isoquinoline

[0439]

[0440] The title compound was obtained by treating the compound of Example B41 and 2-ethynylpyridine in the same manner as in Example B42.

[0441] 1H-NMR (CDCl₃) δ (ppm): 4.69 (2H, s), 7.27 (2H, d), 7.31 (1H, dd), 7.43 (2H, d), 7.55 (1H, dd), 7.59 (1H, d), 7.66 (1H, dd), 7.82 (1H, dd), 7.83 (1H, d), 8.10 (1H, d), 8.51 (1H, d), 8.60 (1H, dd), 8.77 (1H, d).

Example B56

1-{4-[2-(2-Pyridyl)ethyl]benzyl}isoquinoline

[0436]

Example B58

1-{4-[2-(3-Pyridyl)ethyl]benzyl}isoquinoline

[0442]

[0443] The title compound was obtained by treating the compound of Example B57 in the same manner as in Example B43.

[0444] 1H-NMR (CDCl₃) δ (ppm): 2.80-2.90 (4H, m), 4.65 (2H, s), 7.04 (2H, d), 7.15 (1H, dd), 7.19 (2H, d), 7.39 (1H, dd), 7.54 (1H, dd), 7.56 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.15 (1H, d), 8.40 (1H, d), 8.42 (1H, d), 8.49 (1H, d).

Example B59

N-(2-propynyl)acetamide

[0445]

[0446] Pyridine (16.3 ml) and acetic anhydride (10.4 ml) were added to an ice-cooled solution of propargylamine (3023 mg) in methylene chloride (30 ml), and this reaction
mixture was stirred at room temperature for 1 hour. The reaction mixture was poured on ice, extracted with ethyl acetate, washed successively with 1 N hydrochloric acid, a saturated aqueous sodium hydrogencarbonate solution, and saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (743 mg). The obtained compound was used in the following reaction without further purification.

Example B60
N-\{3-[4-(1-Isoquinolylmethyl)phenyl]-2-propynyl\}acetamide

Example B61
N-\{3-[4-(1-Isoquinolylmethyl)phenyl]propyl\}acetamide

Example B62
N-(2-Propynyl)phenylacetamide

Example B63
N-\{3-[4-(1-Isoquinolylmethyl)phenyl]-2-propynyl\}-methanesulfonamide

Example B64
Triethylamine (9.77 ml) was added to an ice-cooled solution of propargylamine (3023 mg) in methylene chloride (30 ml). After dropwise addition of methanesulfonyl chloride (5.19 ml), the reaction mixture was stirred for 3 hours at that temperature, warmed to room temperature, and further stirred for 2 hours. Ice was added to the reaction mixture, the resulting mixture was extracted with ethyl acetate, washed with saturated brine, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The residue was dissolved in methanol (120 ml), potassium carbonate (11.7 g) was added, and this reaction mixture was stirred at room temperature for 3 hours. The reaction mixture was concentrated under reduced pressure, neutralized with dilute hydrochloric acid while cooling on ice, and then extracted with ethyl acetate. The extract was washed with saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (6.67 g).

Example B65
\[^1^H\-NMR (CDCl\_3) \delta (ppm): 1.79 (3H, s), 4.04 (2H, s), 4.61 (2H, s), 7.45-7.68 (4H, m), 7.68-7.75 (2H, m), 7.90-8.00 (1H, m), 8.25-8.38 (2H, m), 8.40-8.45 (1H, m).\]

Example B66
\[^1^H\-NMR (CDCl\_3) \delta (ppm): 1.74-1.84 (2H, m), 2.55 (2H, t), 3.25 (2H, dt), 4.68 (2H, s), 7.10 (2H, d),\]

Example B67
\[^1^H\-NMR (CDCl\_3) \delta (ppm): 1.79 (3H, s), 4.04 (2H, s), 4.61 (2H, s), 7.45-7.68 (4H, m), 7.68-7.75 (2H, m), 7.90-8.00 (1H, m), 8.25-8.38 (2H, m), 8.40-8.45 (1H, m).\]
Example B64

3-[4-(1-isquinolylmethyl)phenyl]methanesulfonylamide

Example B65

1-[4-[3-(Ethylsulfanyl)-1-propynyl]benzyl]isoquinoline

Example B66

t-Butyl N-(2-propynyl)carbamate

Example B67

tert-Butyl N-[3-[4-(1-isquinolylmethyl)phenyl]-2-propynyl]-carbamate

Example B68

1H-NMR (DMSO-d6) δ (ppm): 1.36 (9H, s), 3.04 (1H, t), 3.62-3.70 (2H, m), 7.20-7.30 (1H, m)

Example B69

Example B70

1H-NMR (CDCl3) δ (ppm): 1.30 (3H, t), 2.73 (2H, q), 3.47 (2H, s), 4.67 (2H, s), 7.20-7.32 (4H, m), 7.52 (1H, dd), 7.57 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.08 (1H, d), 8.49 (1H, d).

Example B71

1H-NMR (CDCl3) δ (ppm): 1.45 (9H, s), 4.06-4.13 (2H, m), 4.66 (2H, s), 7.19 (2H, d), 7.20-7.28 (1H, m), 7.29 (2H, d), 7.52 (1H, dd), 7.57 (1H, d), 7.65 (1H, dd), 7.82 (1H, d), 8.08 (1H, d), 8.49 (1H, d).

The title compound was obtained by treating the compound of Example B41 and propargyl ethyl sulfide in the same manner as in Example B42.

The title compound was obtained by treating the compound of Example B41 and the compound of Example B66 in the same manner as in Example B42.
Example B68

tert-Butyl N-3-[4-(1-isooquinolylmethyl)phenyl] propylcarbamate

The title compound was obtained by treating the compound of Example B67 in the same manner as in Example B43.

Example B69

3-[4-(1-Isooquinolylmethyl)phenyl]-2-propyn-1-amine

Example B70

3-[4-(1-Isooquinolylmethyl)phenyl]-1-propanamine

The title compound was obtained by treating the compound of Example B68 in the same manner as in Example B69.

Example B71

N-methyl-N-(2-propynyl)acetamide

Example B72

N-3-[4-(1-Isooquinolylmethyl)phenyl]-2-propynyl)-N-methyl-acetamide

The title compound was obtained by treating the compound of Example B41 and the compound of Example B71 in the same manner as in Example B42.

Trifluoroacetic acid (0.3 ml) was added to an ice-cooled solution of the compound of Example B67 (4 mg) in methylene chloride (0.6 ml), and the reaction mixture was stirred at that temperature for 1 hour. After a saturated aqueous sodium hydrogencarbonate solution was added, the reaction mixture was extracted with ethyl acetate, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (4 mg).

Example B73

The title compound was obtained by treating N-methyl-N-(2-propynyl)amine in the same manner as in Example B59.

Example B74

1H-NMR (CDCl3) δ (ppm): 2.11 (2.1H, s), 2.17 (0.9H, s), 2.21 (0.7H, t), 2.31 (0.3H, t), 3.00 (0.9H, s), 3.08 (2.1H, s), 4.04 (0.6H, d), 4.23 (1.4H, d).

Example B75

The obtained compound contained a 7:3 mixture of geometrical isomers of the amide.

Example B76

N-3-[4-(1-Isooquinolylmethyl)phenyl]-2-propynyl)-N-methyl-acetamide

Example B77

The amine proton was not observed in the NMR spectrum.
The obtained compound contained a 3:2 mixture of geometrical isomers of the amide.

Example B73

N-[3-[4-(1-isoquinolylmethyl)phenyl]propyl]-N1-methylacetamide

The title compound was obtained by treating the compound of Example B72 in the same manner as in Example B43.

Example B74

N-methyl-N-(2-propynyl)methanesulfonamide

Triethylamine (6.55 ml) was added to an ice-cooled solution of N-methyl-N-(2-propynyl)amine (2603 mg) in methylene chloride (25 ml). Methanesulfonyl chloride (3.50 ml) was further added dropwise, the reaction mixture was stirred at that temperature for 1 hour, and then stirred further at room temperature for 2 hours. After ice was added, the reaction mixture was extracted with ethyl acetate, washed successively with 1 N hydrochloric acid, a saturated aqueous sodium hydrogencarbonate solution, and saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (4522 mg). The obtained compound was used in the following reaction without further purification.

The title compound was obtained by treating the compound of Example B41 and the compound of Example B74 in the same manner as in Example B42.

Example B76

N-[3-[4-(1-isoquinolylmethyl)phenyl]propyl]-N-methyl methanesulfonamide

Treating the compound of Example B75 in the same manner as in Example B43, the obtained residue was separated and purified by LC-MS [elucent: an acetonitrile solution containing 0.1% trifluoroacetic acid: an aqueous solution containing 0.1% trifluoroacetic acid=1:99 to 100:0/20-minute cycle, flow rate: 20 ml/minute, column: YMC CombiPrep ODS-AM, 20 mm Φx50 mm (long)] to give the title compound.

MS m/z (ESI: MH+): 369.2
Example B77
5-[4-(1-Isoquinolylmethyl)phenyl]-4-pentyn-2-ol

[0503]

The title compound was obtained by treating the compound of Example B41 and 4-pentyn-2-ol in the same manner as in Example B42.

[0504]  

[0505] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 1.27 (3H, t), 2.38-2.62 (2H, m), 3.95-4.03 (1H, m), 4.65 (2H, s), 7.19 (2H, d), 7.29 (2H, d), 7.52 (1H, dd), 7.57 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.08 (1H, d), 8.48 (1H, d).

[0506] The proton of the hydroxyl group was not observed in the NMR spectrum.

Example B78
5-[4-(1-Isoquinolylmethyl)phenyl]-2-pentanol

[0507]

[0508] Treating the compound of Example B77 in the same manner as in Example B43, the obtained residue was separated and purified by LC-MS [elucent: an acetonitrile solution containing 0.1% trifluoroacetic acid; an aqueous solution containing 0.1% trifluoroacetic acid: 1:99 to 100:0:20-minute cycle, flow rate: 20 ml/minute, column: YMC CombiPrep ODS-AM, 20 mm φ×50 mm (long)] to give the title compound.

[0509] MS m/z (ESI: MHI$^+$): 306.2

Example B79
3-Butylphenol

[0510]

[0511] The title compound was obtained by treating 1-butyl-3-methoxybenzene in the same manner as in Example B40.

[0512] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.94 (3H, t), 1.30-1.55 (2H, m), 1.55-1.62 (2H, m), 2.56 (2H, t), 4.76 (1H, brs), 6.63 (1H, dd), 6.66 (1H, d), 6.75 (1H, d), 7.12 (1H, dd).

Example B80
1-Butyl-3-(methoxymethoxy)benzene

[0513]

[0514] A 60% suspension of sodium hydride dispersed in mineral oil (102 mg) was added to an ice-cooled solution of the compound of Example B79 (318 mg) in dimethylformamide (5 ml), and the reaction mixture was stirred at room temperature for 30 minutes. The mixture was cooled again on ice, chloromethyl methyl ether (0.18 ml) was added, and this reaction mixture was stirred at room temperature for 12 hours. After water was added, the reaction mixture was extracted with ethyl acetate, washed with a saturated aqueous sodium hydrogen carbonate solution and saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (341 mg). The obtained compound was used in the following reaction without further purification.

[0515] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.94 (3H, t), 1.30-1.42 (2H, m), 1.55-2.04 (2H, m), 2.58 (2H, t), 3.49 (3H, s), 5.17 (2H, s), 6.80-6.87 (3H, m), 7.18 (1H, dd).

Example B81
4-Butyl-2-(methoxymethoxy)benzaldehyde

[0516]

[0517] A solution of t-butyl lithium in pentane (1.51 M, 10.6 ml) was added dropwise to a solution of the compound of Example B80 (2396 mg) in petroleum ether cooled to ~20°C., and this reaction mixture was stirred at a temperature in the range of ~10°C. to 0°C. for 1.5 hours. The reaction mixture was cooled to ~70°C., anhydrous ether (17 ml) and dimethylformamide (1.91 ml) were added, and the resulting mixture was stirred at that temperature for 3 hours, then stirred for another 1 hour at room temperature. The reaction mixture was cooled on ice, a saturated aqueous ammonium chloride solution was added, and the mixture was extracted
with ethyl acetate. The extract was washed with saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (1821 mg).

Example B82

[4-Butyl-2-(methoxymethoxy)phenyl][1-isoquinolyl] methanol

\[ \text{Example B82} \]

\[ \text{[4-Butyl-2-(methoxymethoxy)phenyl][1-isoquinolyl] methanol} \]

An aqueous sodium hydroxide solution (50%, 1.4 ml) was added to a solution of 1-cyano-benzoyl-1,2-dihydroisoquinoline (815 mg), which was synthesized according to Org. Synth., IV, 155 (1988), the compound of Example B81 (869 mg), and triethylbenzylammonium chloride (7 mg) in methylene chloride (1.6 ml), and the reaction mixture was subjected to ultrasonication in a water bath for 10 minutes. After methylene chloride (8.3 ml) and ethanol (4.4 ml) were added, the reaction mixture was further subjected to ultrasonication in a water bath for 85 minutes. Water was added and the resulting reaction mixture was extracted with methylene chloride. The extract was dried over anhydrous magnesium sulfate, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (1144 mg).

Example B83

[4-Butyl-2-(methoxymethoxy)phenyl][1-isoquinolyl] methyl acetate

The title compound was obtained by treating the compound of Example B82 in the same manner as in Example B83.

Example B84

1-[4-Butyl-2-(methoxymethoxy)benzyl]isoquinoline

The title compound was obtained by treating the compound of Example B83 in the same manner as in Example B84.

Example B85

5-Butyl-2-(1-isoquinolylmethyl)phenol

5 N hydrochloric acid (1.0 ml) was added to a solution of the compound of Example B84 (88 mg) in methanol (1.5 ml), and this reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was neutralized with a 5 N aqueous sodium hydroxide solution, adjusted to pH 6.8 with phosphate buffer, and extracted with ethyl acetate. The extract was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to give the title compound (44 mg).

Example B86

[4-Butyl-2-(methoxymethoxy)phenyl] (1-isoquinolyl)methyl acetate
The proton of the hydroxyl group was not observed in the NMR spectrum.

**Example B86**

\[
\text{N-\{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl\}-N,N-dimethyl-amine}
\]

**Example B87**

1-[4-3-(Tetrahydro-2H-2-pyranyloxy)-1-propynyl benzyl]iso-quinoline

**Example B88**

3-[4-(1-isoquinolylmethyl)phenyl]-2-propyn-1-ol

The title compound was obtained by treating the compound of Example B41 and 1-dimethylamino-2-propyne in the same manner as in Example B42.

**Example B89**

N,N-dimethyl-4-pentynamide

**Example B41**

\[
\text{\textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) (ppm): 2.04 (3H, s), 2.34 (3H, s), 3.47 (2H, s), 4.66 (2H, s), 6.70 (2H, d), 7.20 (2H, d), 7.32 (2H, d), 7.53 (1H, dd), 7.56 (1H, d), 7.65 (1H, dd), 7.82 (1H, d), 8.10 (1H, d), 8.50 (1H, d).}
\]

**Example B47**

\[
\text{\textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) (ppm): 1.20-1.30 (1H, m), 4.46 (2H, s), 4.67 (2H, s), 7.23 (2H, d), 7.31 (2H, d), 7.53 (1H, dd), 7.58 (1H, d), 7.65 (1H, dd), 7.83 (1H, d), 8.09 (1H, d), 8.49 (1H, d).}
\]

**Example B42**

\[
\text{\textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) (ppm): 1.96-1.99 (1H, m), 2.50-2.60 (4H, m), 2.96 (3H, s), 3.02 (3H, s).}
\]

Dimethylamine (2 M solution in tetrahydrofuran, 8.53 ml), triethylamine (2.59 ml), and 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (3221 mg), were added to a solution of 4-pentynoic acid (552 mg) in methylene chloride (150 ml) and this reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was washed successively with 1 N hydrochloric acid, a saturated aqueous sodium hydroxide solution, water, and saturated brine, dried over anhydrous magnesium sulfate, then concentrated under reduced pressure to give the title compound (129 mg). The obtained compound was used in the following reaction without further purification.

**Example B43**

\[
\text{\textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) (ppm): 1.45-1.85 (6H, m), 3.50-3.60 (1H, m), 3.84-3.90 (1H, m), 4.42 (1H, d), 4.48 (1H, d), 4.66 (2H, s), 4.87 (1H, dd), 7.15-7.21 (2H, m), 7.33-7.36 (2H, m), 7.50-7.70 (3H, m), 7.81-7.86 (1H, m), 8.07-8.10 (1H, m), 8.48-8.51 (1H, m).}
\]
Example B90

N,N-dimethyl-5-[4-(1-isquinolylmethyl)phenyl]-4-pentynamide

Example B92

1-[4-[3-(Tetrahydro-2H-2-pyranoxy)-1-butyryl]benzyl]-isoquinoline

The title compound was obtained by treating the compound of Example B41 and the compound of Example B89 in the same manner as in Example B42.

**Example B91**

1-Methyl-2-propynyltetrahydro-2H-2-pyran ether

The title compound was obtained by treating the compound of Example B41 and the compound of Example B91 in the same manner as in Example B42.

**Example B93**

4-[4-(1-Isquinolylmethyl)phenyl]-3-butyn-2-ol

The title compound was obtained by treating the compound of Example B92 in the same manner as in Example B47.

3,4-Dihydro-2H-pyran (7.15 ml) and pyridinium p-toluenesulfonate (2187 mg) were added to a solution of 3-butyn-2-ol (3051 mg) in dichloromethane (150 ml), and this reaction mixture was stirred at room temperature for 29 hours.

The reaction mixture was washed successively with a saturated aqueous sodium hydrogen carbonate solution, water, and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (4698 mg).

**Example B94**

1H-NMR (CDCl3, δ (ppm): 1.45 (1.05H, d), 1.48 (1.95H, d), 1.50-1.90 (6H, m), 2.37 (0.65H, d), 2.43 (0.35H, d), 3.50-3.60 (1.3H, m), 3.80-3.86 (0.7H, m), 4.4-4.50 (0.35H, m), 4.52-4.60 (0.65H, m), 4.77 (0.35H, t), 4.94 (0.65H, t).
Example B94
4-[4-(1-Isoquinolylmethyl)phenyl]-2-butanol

Treating the compound of Example B93 in the same manner as in Example B43, the obtained residue was separated and purified by LC-MS [eluent: an acetonitrile solution containing 0.1% trifluoroacetic acid; an aqueous solution containing 0.1% trifluoroacetic acid=1:99 to 100:0/20-minute cycle, flow rate: 20 ml/minute, column: YMC CombiPrep ODS-AM, 20 mm Φ×50 mm (long)] to give the title compound.

MS m/z (ESI: MH⁺): 292.2

Example B95
2-Methyl-4-pentyn-2-ol

Lithium acetylide-ethylendiamine complex was added gradually to a mixed solution of isobutylene oxide (1889 mg) in tetrahydrofuran (13 ml) and dimethyl sulfoxide (20 ml) cooled to 0°C, and this reaction mixture was stirred at 0°C for 5 hours. After water was added, the reaction mixture was extracted with ethyl acetate, washed with saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (3316 mg). This was used in the following reaction without further purification.

1H-NMR (CDCl₃) δ (ppm): 1.33 (6H, s), 2.09 (1H, t), 2.38 (2H, t).

The proton of the hydroxyl group was not observed in the NMR spectrum.

Example B96
5-[4-(1-Isoquinolylmethyl)phenyl]-2-methyl-4-pentyn-2-ol

The title compound was obtained by treating the compound of Example B41 and the compound of Example B95 in the same manner as in Example B42.

1H-NMR (DMSO-d6) δ (ppm): 1.18 (6H, s), 2.28 (1H, s), 2.42 (2H, s), 4.62 (2H, s), 7.10-7.30 (4H, m), 7.62 (1H, dd), 7.71 (1H, d), 7.72 (1H, dd), 7.94 (1H, d), 8.27 (1H, d), 8.42 (1H, d).

Example B97
5-[4-(1-Isoquinolylmethyl)phenyl]-2-methyl-2-pentanol

Treating the compound of Example B96 in the same manner as in Example B43, the obtained residue was separated and purified by LC-MS [eluent: an acetonitrile solution containing 0.1% trifluoroacetic acid; an aqueous solution containing 0.1% trifluoroacetic acid=1:99 to 100:0/20-minute cycle, flow rate: 20 ml/minute, column: YMC CombiPrep ODS-AM, 20 mm Φ×50 mm (long)] to give the title compound.

MS m/z (ESI: MH⁺): 320.2

Example B98
4-Benzylxoy-2-(methoxymethoxy)benzaldehyde

4-Benzylxoy-2-((methoxymethoxy)benzaldehyde
N,N-diisopropylethylamine (1.98 ml) and chloromethyl methyl ether (0.76 ml) were added to a solution of 4-benzyloxy-2-hydroxybenzaldehyde (2071 mg) in tetrahydrofuran (30 ml), and this reaction mixture was stirred and heated under reflux for 19 hours. N,N-diisopropylethylamine (2.7 ml) and chloromethyl methyl ether (1.04 ml) were further added, and the resulting mixture was stirred and heated under reflux for another 10 hours. After water was added, the reaction mixture was extracted with ethyl acetate, washed with a saturated aqueous ammonium chloride solution and saturated brine, dried over anhydrous magnesium sulfate, then filtered through silica gel and alumina. The filtrate was concentrated under reduced pressure to give the title compound (2470 mg). This compound was used in the following reaction without further purification.

**Example B99**

4-(Benzyloxy)-2-(methoxymethoxy)phenyl(1-isooquinolyl)methanol

**[0574]**

4-(Benzyloxy)-2-(methoxymethoxy)phenyl(1-isooquinolyl)methanol

**[0575]** The title compound was obtained by treating the compound of Example B98 in the same manner as in Example B82.

**[0576]** 1H-NMR (DMSO-d6) δ (ppm): 3.16 (3H, s), 5.01 (2H, s), 5.11 (1H, d), 5.14 (1H, d), 6.59 (1H, dd), 6.66-6.70 (2H, m), 7.18 (1H, d), 7.31 (1H, d), 7.34-7.42 (4H, m), 7.61 (1H, dd), 7.71 (1H, d), 7.75 (1H, d), 7.95 (1H, d), 8.28 (1H, d), 8.43 (1H, d).

**[0577]** The proton of the hydroxyl group was not observed in the NMR spectrum.

**Example B100**

4-(Benzyloxy)-2-(methoxymethoxy)phenyl(1-isooquinolyl)methyl acetate

**[0578]** The title compound was obtained by treating the compound of Example B99 in the same manner as in Example B38.

**[0579]** 1H-NMR (CDCl3) δ (ppm): 2.21 (3H, s), 3.42 (3H, s), 4.98 (1H, d), 5.00 (1H, d), 5.21-5.27 (2H, m), 6.54 (1H, dd), 6.81 (1H, d), 7.25 (1H, d), 7.30-7.41 (5H, m), 7.53 (1H, dd), 7.57 (1H, d), 7.63 (1H, dd), 7.80 (1H, d), 8.00 (1H, s), 8.29 (1H, d), 8.55 (1H, d).

**Example B101**

4-(1-Isooquinolylmethyl)-3-(methoxymethoxy)phenol

**[0580]** The title compound was obtained by treating the compound of Example B99 in the same manner as in Example B39.

**[0581]**

**Example B102**

4-(1-Isooquinolylmethyl)-3-(methoxymethoxy)phenyl trifluoro-methanesulfonate

**[0584]**

**Example B103**

4-(1-Isooquinolylmethyl)-3-(methoxymethoxy)phenyl trifluoro-methanesulfonate

**[0585]** The title compound was obtained by treating the compound of Example B101 in the same manner as in Example B41.

**[0586]** 1H-NMR (CDCl3) δ (ppm): 3.43 (3H, s), 4.65 (2H, s), 5.24 (2H, s), 6.77 (1H, dd), 7.04 (1H, d), 7.07 (1H, d), 7.54-7.61 (2H, d), 7.67 (1H, dd), 7.84 (1H, d), 8.16 (1H, d), 8.47 (1H, d).
Example B103

1-[2-(Methoxymethoxy)-4-(tetrahydro-2H-2-pyran-2yloxy)-1-butynyl]benzylisoquinoline

The title compound was obtained by treating the compound of Example B102 and 2-(3-butynyloxy)tetrahydro-2H-pyrin in the same manner as in Example B42.

\[ \text{H-NMR (CDCl}_3\text{)} \delta (ppm): 1.51-1.90 (6H, m), 2.68 (2H, t), 3.50 (3H, s), 3.49-3.55 (1H, m), 3.58-3.65 (1H, m), 3.84-3.94 (2H, m), 4.63-4.68 (1H, m), 4.65 (2H, s), 5.23 (2H, s), 6.76 (1H, dd), 7.04 (1H, d), 7.08 (1H, d), 7.49-7.69 (3H, m), 7.81 (1H, d), 8.14 (1H, d), 8.47 (1H, d). \]

Example B104

5-(4-Hydroxy-1-butynyl)-2-(1-isoquinolylmethyl)phenol

The title compound was obtained by treating the compound of Example B103 in the same manner as in Example B85.

\[ \text{H-NMR (CDCl}_3\text{)} \delta (ppm): 1.80 (1H, brs), 2.66 (2H, t), 3.73-3.82 (2H, m), 4.58 (2H, s), 6.87 (1H, d), 7.04 (1H, s), 7.23 (1H, d), 7.60 (1H, d), 7.69-7.78 (2H, m), 7.86 (1H, d), 8.37 (1H, d), 8.42 (1H, d). \]

Example B105

1-(t-Butyl)-1,1-dimethylsilyl[4-[4-(1-isoquinolylmethy]-2-methyl-3-butynyl]ether

Triphenylphosphine (18.37 g) was added to an ice-cooled solution of carbon tetrabromide (11.19 g) in methylene chloride (60 ml), and this reaction mixture was stirred at that temperature for 1 hour. A solution of 3-[1-(t-butyl)-1,1-dimethylsilyl]oxy]-2-methylpropanal, which was synthesized according to Tetrahedron Lett., 4347 (1979), in methylene chloride (14 ml) was added dropwise, and the resulting reaction mixture was further stirred for 1 hour. The reaction mixture was diluted with methylene chloride, washed successively with saturated aqueous sodium hydroxycarbonate solution, saturated an aqueous ammonium chloride solution and saturated brine, dried over magnesium sulfate, and then concentrated under reduced pressure. Ether was added to this residue, insoluble material was separated by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give t-butyl[4,4-dibromo-2-methyl-3-butenyloxy]-dimethylsilane (2385 mg).

Next, a 2.47 M n-butyl lithium solution in hexane (3.15 ml) was added dropwise to a solution of t-butyl[4,4-dibromo-2-methyl-3-butenyloxy]dimethylsilane (1326 mg) in tetrahydrofuran (10 ml) cooled to \(-70^\circ C\), and this mixture was stirred at that temperature for 1 hour. A saturated aqueous ammonium chloride solution was further added, and the resulting mixture was warmed to room temperature. After water was added, the reaction mixture was extracted with ether. The ether layer was washed with saturated brine, dried over anhydrous magnesium sulfate, then filtered through silica gel. The filtrate was concentrated under reduced pressure. The obtained residue and the compound of Example B41 were treated in the same manner as in Example B42 to obtain the title compound.

\[ \text{H-NMR (CDCl}_3\text{)} \delta (ppm): 0.07 (6H, s), 0.90 (9H, s), 1.18 (3H, d), 2.70-2.80 (1H, m), 3.47 (1H, dd), 3.70 (1H, dd), 4.65 (2H, s), 7.16 (2H, d), 7.27 (2H, d), 7.51 (1H, dd), 7.56 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.07 (1H, d), 8.49 (1H, d). \]
Example B106
4-{4-(1-Isoquinolymethyl)phenyl}-2-methyl-3-butyln-1-ol

Example B107
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B108
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B109
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B110
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Ethynyl magnesium bromide in tetrahydrofuran (0.5 M, 90 ml) was added to anhydrous tetrahydrofuran (20 ml) cooled to –78°C under nitrogen atmosphere. A solution of t-butyldimethylsilyloxycetaldehyde (6000 mg) in tetrahydrofuran (30 ml) was added dropwise, and the resulting mixture was stirred at –78°C for 45 minutes, warmed to room temperature, stirred for 1 hour 40 minutes, then cooled on ice. After a saturated aqueous ammonium chloride solution was added, the reaction mixture was extracted with ether, washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (8.55 g). This compound was used in the following reaction without further purification.

Ethynyl magnesium bromide in tetrahydrofuran (0.5 M, 90 ml) was added to anhydrous tetrahydrofuran (20 ml) cooled to –78°C, under nitrogen atmosphere. A solution of t-butylmethylsilyloxyacetaldheyde (6000 mg) in tetrahydrofuran (30 ml) was added dropwise, and the resulting mixture was stirred at –78°C for 45 minutes, warmed to room temperature, stirred for 1 hour 40 minutes, then cooled on ice. After a saturated aqueous ammonium chloride solution was added, the reaction mixture was extracted with ether, washed with water and saturated brine, dried over anhydrous magnesium sulfate, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (8.55 g). This compound was used in the following reaction without further purification.

Example B105
The title compound was obtained by treating the compound of Example B107 with the compound of Example B107 in the same manner as in Example B47.

Example B106
The title compound was obtained by treating the compound of Example B105 in the same manner as in Example B47.

Example B107
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B108
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B109
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B110
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B111
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B112
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B113
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B114
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B115
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B116
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B117
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B118
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B119
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B120
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B121
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B122
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B123
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B124
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B125
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B126
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B127
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B128
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B129
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B130
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B131
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B132
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B133
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B134
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B135
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B136
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B137
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B138
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B139
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B140
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B141
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B142
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B143
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}-3-butyln-2-ol

Example B144
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B145
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B146
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B147
1-(1-t-Butyl)-1,1-dimethylsilyloxy)-3-butyn-2-ol

Example B148
1-{1-(t-Butyl)-1,1-dimethylsilyloxy}methyl)-2-propynyl acetate

Example B149
4-{4-(1-Isoquinolylmethyl)phenyl}-3-butyln-1,2-diol

Example B150
1-{4-[2-(2,2-Dimethyl-1,3-dioxolan-4-yl)-1-ethynyl benzyl]-isoquinoline

Example B151
1-(1-t-Butyl)-1,1-dimethylsilyloxy)-3-butyn-2-ol
2,2-Dimethoxypropane (0.36 ml), 10-camphorsulfonic acid (43 mg), and molecular sieves (4 Å) were added to a solution of the compound of Example B109 (34 mg) in dimethylformamide (2 ml), and this reaction mixture was stirred at 75°C for 9 hours. After an saturated aqueous sodium carbonate solution was added, the reaction mixture was extracted with ethyl acetate, washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (14 mg).

**Example B111**
1-Butyl [[2-(1-ethoxyethoxy)-3-butylnyl]oxy]dimethylsilane

Ethyl vinyl ether (1.21 ml) and pyridinium p-toluene sulfonate (317 mg) were added to a solution of 1-[[1-(t-butyl)-1,1-dimethylsilyloxy]-3-butyln-2-ol (1687 mg) in methylene chloride (90 ml), and this mixture was stirred at room temperature for 1 hour. The methylene chloride layer was washed with a saturated aqueous sodium hydrogen carbonate solution and saturated brine, dried over anhydrous magnesium sulfate, then concentrated under reduced pressure to give the title compound (1962 mg). This compound was used in the following reaction without further purification.

**Example B112**
1-[[[1-(t-butyl)-1,1-dimethylsilyloxy]-3-butyln-2-ol (1687 mg) in methylene chloride (90 ml), and this mixture was stirred at room temperature for 1 hour. The methylene chloride layer was washed with a saturated aqueous sodium hydrogen carbonate solution and saturated brine, dried over anhydrous magnesium sulfate, then concentrated under reduced pressure to give the title compound (1962 mg). This compound was used in the following reaction without further purification.

**Example B113**
1-[(1-isoquinolyl-methyl)]phenyl]-3-butyln-2-ol

Pyridinium p-toluenesulfonate (486 mg) was added to a solution of the compound of Example B112 (474 mg) in methanol (15 ml), and this reaction mixture was stirred at room temperature for 24 hours. After ethyl acetate was added, the reaction mixture was washed with a saturated aqueous sodium hydrogen carbonate solution and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (265 mg).

**Example B114**
1-[(1-isoquinolyl-methyl)]phenyl]-3-butyln-2-ol

A solution of the compound of Example B113 (116 mg) in methylene chloride (2 ml) was added dropwise to a solution of (diethylamino)sulfur trifluoride (44 μl) in methylene chloride (2 ml) cooled to -78°C under nitrogen atmosphere. Upon stirring for 15 minutes, the reaction mixture was stirred at room temperature for another 8 hours. A saturated
aqueous sodium hydrogen carbonate solution was added, the resulting reaction mixture was extracted with methylene chloride. The methylene chloride layer was washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (42 mg).

**Example B115**

2-Fluoro-4-[4-(1-isoquinolylmethyl)phenyl]-3-butyryl-1-ol

**Example B116**

1-(t-Butyl)-1,1-dimethylsilyl [6-[4-(1-isoquinolylmethyl)phenyl]-5-hexynyl]ether

**Example B117**

6-[4-(1-isoquinolylmethyl)phenyl]-5-hexyn-1-ol

**Example B118**

6-[4-(1-isoquinolylmethyl)phenyl]-1-hexanol

**Example B119**

2-(4-Pentynyloxy)tetrahydro-2H-pyran
The title compound was obtained by treating 4-pentyn-1-ol in the same manner as in Example B91.

**Example B120**

1-(4-{5-(Tetrahydro-2H-2-pyranloxy)-1-pentynyl}benzyl)-isoquinoline

The title compound was obtained by treating the compound of Example B41 and the compound of Example B119 in the same manner as in Example B42.

**Example B121**

5-(4-{1-Isoquinolyimethyl}phenyl)-4-pentynyl cyanide

The title compound was obtained by treating the compound of Example B41 and 5-cyano-1-pentyne in the same manner as in Example B42.

**Example B122**

5-(4-{1-Isoquinolyimethyl}phenyl)-4-pentynyl cyanide

The title compound was obtained by treating the compound of Example B41 and the compound of Example B119 in the same manner as in Example B42.

**Example B123**

1-(4-(3-Methyl-1-butynyl)benzyl)isoquinoline

The title compound was obtained by treating the compound of Example B41 and 3-methyl-1-butyne in the same manner as in Example B42.

**Example B124**

1-(4-{5-Methyl-1-hexynyl}benzyl)isoquinoline
The title compound was obtained by treating the compound of Example B41 and 5-methyl-1-hexyne in the same manner as in Example B42.

Example B127

4-Pentynamide

1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (6775 mg) and ammonium hydrogen carbonate (5905 mg) were added to a solution of 4-pentynoic acid (2446 mg) in chloroform (75 ml), and this reaction mixture was stirred at room temperature for 17.5 hours. The reaction mixture was filtered through celite and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (249 mg).

Example B128

5-[4-(1-Isoquinolylmethyl)phenyl]-4-pentynoate

Benzyltriethylammonium chloride (5.92 g), potassium carbonate (93.4 g), and t-butyl bromide (143 ml) were added to a solution of 4-pentynoic acid (2550 mg) in N,N-dimethylacetamide (230 ml), and this reaction mixture was stirred at 55°C for 24 hours. After water was added, the reaction mixture was extracted with ethyl acetate, washed with water, dried over anhydrous magnesium chloride, and then filtered through silica gel. The filtrate was concentrated under reduced pressure to give the title compound (2.10 g). This compound was used in the following reaction without further purification.

Example B127

t-Butyl 4-pentynoate

1-H-NMR (CDCl₃) δ (ppm): 0.91 (6H, d), 1.47 (2H, dt), 1.68-1.77 (1H, m), 2.37 (2H, t), 4.65 (2H, s), 7.17 (1H, d), 7.28 (2H, d), 7.52 (1H, dd), 7.57 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.09 (1H, d), 8.49 (1H, d).

Example B125

4-Pentynamide

1H-NMR (DMSO-d₆) δ (ppm): 2.21 (2H, t), 2.29-2.33 (2H, m), 2.73 (1H, t), 6.78-6.88 (1H, m), 7.28-7.38 (1H, m).

Example B126

5-[4-(1-Isoquinolylmethyl)phenyl]-4-pentynoate

1-H-NMR (CDCl₃) δ (ppm): 1.46 (9H, s), 1.96-1.97 (1H, m), 2.45-2.47 (4H, m).

Example B127

t-Butyl 4-pentynoate

1-H-NMR (CDCl₃) δ (ppm): 0.91 (6H, d), 1.47 (2H, dt), 1.68-1.77 (1H, m), 2.37 (2H, t), 4.65 (2H, s), 7.17 (1H, d), 7.28 (2H, d), 7.52 (1H, dd), 7.57 (1H, d), 7.64 (1H, dd), 7.81 (1H, d), 8.09 (1H, d), 8.49 (1H, d).
Treating the compound of Example B128 in the same manner as in Example B69, the obtained residue was separated and purified by LC-MS [elu: an acetonitrile solution containing 0.1% trifluoroacetic acid: an aqueous solution containing 0.1% trifluoroacetic acid=1:99 to 100:0/20-minute cycle, flow rate: 20 ml/minute, column: YMC Combiprep ODS-AM, 20 mm Φ×50 mm (long)] to give the title compound.

MS m/z (ESI: MH+): 316.1

The following compounds were synthesized as follows. That is, the title compound was obtained by reacting the compound of Example B41 with various reactants described below, according to Example B33. The various reactants are acrylamide, N,N-dimethylacrylamide, t-buty1 acrylate, and methyl vinyl sulfone. Furthermore, the coupling product obtained in this manner was subjected to either the reduction according to Example B39 or the deprotection of t-buty1 ester according to Example B40, or both. The resulting product was purified by silica gel column chromatography or by LC-MS [elu: an acetonitrile solution containing 0.1% trifluoroacetic acid: an aqueous solution containing 0.1% trifluoroacetic acid=1:99 to 100:0/20-minute cycle, flow rate: 20 ml/minute, column: YMC Combiprep ODS-AM, 20 mm Φ×50 mm (long)].

MS m/z (ESI: MH+): 317.3

MS m/z (ESI: MH+): 289.3

MS m/z (ESI: MH+): 319.1
Example B134
t-Butyl (E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenoate

[0681]

Example B135
(E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenoic acid

[0683]

Example B136
t-Butyl 3-[4-(1-isoquinolylmethyl)phenyl]propanoate

[0685]

Example B137
3-[4-(1-isoquinolylmethyl)phenyl]propanoic acid

[0687]

Example B138
(E)-2-[4-(1-isoquinolylmethyl)phenyl]-1-ethenyl methylsulfone

[0689]

Example B139
1-[4-[2-(Methylsulfonylethyl)benzyl]isoquinoline

[0691]

Example B134

[0682] \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) (ppm): 1.51 (9H, s), 4.68 (2H, s), 6.28 (1H, d), 7.27 (2H, d), 7.39 (2H, d), 7.49-7.60 (3H, m), 7.65 (1H, dd), 7.82 (1H, d), 8.11 (1H, d), 8.50 (1H, d).

Example B136

[0684] MS m/z (ESI: MH\(^+\)): 290.2

Example B137

[0688] MS m/z (ESI: MH\(^+\)): 292.1

Example B138

[0689] MS m/z (ESI: MH\(^+\)): 324.1

Example B139

[0691] MS m/z (ESI: MH\(^+\)): 326.1
Example B140

2-Benzoyl-6,7-dimethoxy-1,2-dihydro-1-isquinolinecarbonitrile

Example B141

1-(4-Butylbenzyl)-6,7-dimethoxyisoquinoline

Example B142

1-(3-Methoxyphenyl)-2-nitro-1-ethanol

An aqueous potassium cyanide (1.0 g, 16 mmol) solution (2.3 ml) and benzoyl chloride (1.1 ml, 9.5 mmol) were added to a solution of 6,7-dimethoxyisoquinoline (1.0 g, 5.3 mmol), which was synthesized according to Tetrahedron, 37 (23), 3977 (1981), in methylene chloride (6.0 ml), and this reaction mixture was stirred while heating under reflux for 2 hours. The reaction mixture was cooled to room temperature, filtered through celite, and washed with methylene chloride and water. After the obtained filtrate was separated, the methylene chloride layer was washed successively with water, 2 N hydrochloric acid, water, and 2 N sodium hydroxide, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (573 mg).

Example B143

2-Amino-1-(3-methoxyphenyl)-1-ethanol

Example B144

2-(4-Butylphenyl)acetic acid

An aqueous sodium hydroxide solution (1.5 g of sodium hydroxide (37 mmol) was dissolved in 15 ml of water) was added dropwise to a solution of m-anisaldehyde (5.0 g, 37 mmol) and nitromethane (4.0 ml, 73 mmol) in methanol (50 ml) keeping the temperature of the solution at not higher than 30°C. The reaction mixture was then stirred at room temperature for 4 hours. Upon cooling on ice, an aqueous acetic acid solution (glacial acetic acid (37 mmol) was dissolved in 250 ml of water) was added, the resulting reaction mixture was extracted with ethyl acetate. The ethyl acetate layer was washed successively with water and a 5% aqueous sodium hydrogen carbonate solution, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (6.09 g).

Example B145

1H-NMR (DCl3) δ (ppm): 3.92 (3H, s), 3.94 (3H, s), 5.99 (1H, d), 6.51-6.55 (2H, m), 6.73 (1H, s), 6.85 (1H, s), 7.45-7.49 (2H, m), 7.53-7.56 (1H, m), 7.58-7.61 (2H, m)

Example B146

Palladium-carbon (10%, 0.64 g) and ammonium formate (4.8 g) were added to a mixed solution of the compound of Example B142 (3.0 g, 15 mmol) in tetrahydrofuran (43 ml) and methanol (43 ml), and this mixture was stirred at room temperature for 18 hours. The catalyst was removed by filtration, the filtrate was diluted with ether, and the mixture was stirred at room temperature for 18 hours. The catalyst was removed by filtration, and the obtained filtrate was concentrated under reduced pressure to give the title compound (1.82 g). This compound was used in the following reaction without further purification.

Example B147

2-(4-Butylphenyl)acetic acid

The title compound was obtained by treating the compound of Example B140 and the compound of Example B1 in the same manner as in Example B2.

Example B148

1H-NMR (DCl3) δ (ppm): 0.90 (3H, t), 1.27-1.36 (2H, m), 1.51-1.58 (2H, m), 2.54 (2H, t), 3.88 (3H, s), 4.01 (3H, s), 4.57 (2H, s), 7.05 (1H, s), 7.07 (2H, d), 7.19 (2H, d), 7.32 (1H, d), 7.43 (1H, d), 8.37 (1H, d)
Thionyl chloride (4.7 ml, 66 mmol) was added dropwise to a solution of 4-n-butylbenzyl alcohol (9.6 g, 59 mmol) in ether (120 ml), and this mixture was stirred at room temperature for 2 hours. The solvent was removed under reduced pressure, and excess thionyl chloride was removed by azotropic distillation with benzene. The residue was dissolved in dimethyl sulfoxide (50 ml), sodium cyanide (86 g, 1.8 mol) and n-tetrabutylammonium iodide (2.2 g, 5.9 mmol) were added to this solution, and the resulting mixture was stirred at room temperature for 16 hours. Water was added, and this mixture was extracted with ethyl acetate. The ethyl acetate layer was washed successively with water and saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give n-butylphenylacetanilide (8.2 g) as a yellow oil. Next, concentrated sulfuric acid (48 ml) was added dropwise to water (58 ml), this solution was cooled to 50°C, and n-Butylphenylacetanilide (8.2 g) obtained above was added dropwise to the solution. The resulting mixture was stirred while heating under reflux for 16 hours. Upon cooling to room temperature, the precipitated crystals were collected by filtration, washed with water, and dissolved in a 0.1 N aqueous sodium hydroxide solution (200 ml). Norit (5 g) was added, and this mixture was stirred and refluxed for 2 hours. After Norit was removed by filtration through celite, the filtrate was cooled to room temperature and acidified with 1 N hydrochloric acid to precipitate crystals. The precipitated crystals were collected by filtration, washed with water, and dried to give the title compound (3.5 g).

**Example B145**

N-[2-Hydroxy-2-(3-methoxyphenyl)ethyl]-2-(4-butylphenyl)-acetamide

Thionyl chloride (0.76 ml, 10 mmol) was added to a solution of the compound of Example B144 (1.0 g, 5.2 mmol) in benzene (10 ml), and the mixture was stirred under reflux for 2 hours. Upon concentration, excess thionyl chloride was removed by azotropic distillation with benzene. The obtained residue and the compound of Example B143 (0.87 g, 5.2 mmol) were dissolved in ether (5 ml), an aqueous sodium hydroxide solution (0.21 g of sodium hydroxide was dissolved in 4.2 ml of water) was added thereto, and the mixture was stirred vigorously at room temperature for 30 minutes. The ether layer was separated and concentrated under reduced pressure to give the title compound (600 mg).

**Example B146**

1-(4-Butylbenzyl)-6-methoxyisoquinoline

Phosphorus oxychloride (1.6 ml) was added to a solution of the compound of Example B145 (600 mg, 1.7 mmol) in acetonitrile (15 ml), and the mixture was stirred under reflux for 1 hour 30 minutes. The mixture was cooled on ice, made alkaline with a 5% aqueous sodium hydroxide solution, extracted with ethyl acetate, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (82 mg).

**Example B147**

1-(4-Butylbenzyl)-6-isoquinolinol

A 47% hydrobromic acid solution was added to the compound of Example B146 (82 mg), and the mixture was stirred under reflux for 19 hours. The mixture was concentrated under reduced pressure, water was added, and the resulting mixture was neutralized with sodium carbonate to precipitate crystals. The obtained crystals were collected by filtration, washed with water, and then dried to give the title compound (74 mg).
Example B148
1-(4-Butylbenzyl)-6-propanoylisoquinoline

Silver carbonate (40 mg, 0.14 mmol) was added to a solution of the compound of Example B147 (20 mg, 0.069 mmol) and 1-iodopropane (0.4 ml, 4.1 mmol) in toluene (1.0 ml), and the mixture was stirred in the dark at 50°C for 4 hours. Upon cooling to room temperature, the mixture was filtered through Celite and washed with a mixed solution of toluene and methanol (9:1). The obtained filtrate was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography to give the title compound (13 mg).

Example B149
1-(4-Butylbenzyl)-6-(2-piperidinoethoxy)isoquinoline

The title compound was obtained in the same manner as in Example 148.

Example B150
N-(1-(4-Butylbenzyl)-6-isquinolyl)oxy)-ethyl-N, N-dimethylamine

The title compound was obtained in the same manner as in Example 148.

Example B151
2-Benzoyl-7-methoxy-1,2-dihydro-1-isquinolinecarbonitrile

The title compound was obtained by treating the compound of Example B1 and the compound of Example B151 in the same manner as in Example B2.
Example B153
1-(4-Bromobenzyl)-7-methoxyisoquinoline

The title compound was obtained by treating the compound of Example B31 and the compound of Example B151 in the same manner as in Example B2.

Example B154
1-(4-Butylbenzyl)-7-isoquinolinol

The title compound was obtained by treating the compound of Example B152 in the same manner as in Example B147.

Example B155
1-(4-Butylbenzyl)-7-isoquinolinyl trifluoromethanesulfonate

4-Nitrophenol triflate (0.72g, 2.7 mmol), which was synthesized according to J. Org. Chem., 64, 7638 (1999), and potassium carbonate (1.1 g, 8.1 mmol) were added to a solution of the compound of Example B154 (1.0 g, 2.7 mmol) in dimethylformamide (30 ml), and the mixture was stirred at room temperature for 2 hours. After water was added, the resulting mixture was extracted with ethyl acetate. The ethyl acetate layer was washed with 1 N sodium hydroxide and saturated brine, dried over magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (1.0 g).

Example B156
1-(4-Butylbenzyl)-7-isoquinolinecarbonitrile

Zinc cyanide (215 mg, 1.8 mmol), tetrakis(triphenylphosphine)palladium (41 mg, 0.35 mmol), and lithium chloride (120 mg, 2.8 mmol) were added to a solution of the compound of Example B155 (400 mg, 0.95 mmol) in dimethylformamide (2 ml) under nitrogen atmosphere, and the mixture was stirred at 120° C. for 2 hours. After cooling to room temperature, saturated sodium hydrogencarbonate was added, and the resulting mixture was extracted with ethyl acetate. The ethyl acetate layer was washed with saturated brine, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (71 mg).
Example B157
1-(4-Butylbenzyl)-7-[2-(1,1,1-trimethylsilyl)-1-ethynyl]-isoquinoline

[0746] Palladium acetate (11 mg, 0.047 mmol), 1,1'-bis(diphenylphosphino)ferrocene (72 mg, 0.13 mmol), and lithium chloride (25 mg, 0.59 mmol) were added to a solution of the compound of Example B155 (100 mg, 0.24 mmol) and trimethylsilylethynylene (65 μL, 0.47 mmol) in dimethylformamide (3.0 mL), and the reaction system was purged with nitrogen. Triethylamine (59 μL, 0.43 mmol) and copper iodide (2 mg, 0.018 mmol) were added, and the resulting mixture was stirred at 80°C for 21 hours, then cooled to room temperature. After water and ethyl acetate were added for partition, the ethyl acetate layer was washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (7.0 mg).

[0747] 1H-NMR (CDCl₃) δ (ppm): 0.88-0.32 (9H, m), 0.92 (3H, t), 1.32-1.38 (2H, m), 1.54-1.57 (2H, m), 2.57 (2H, t), 4.63 (2H, s), 7.10 (2H, d), 7.20 (2H, d), 7.52 (1H, d), 7.67-7.69 (1H, m), 7.75 (1H, d), 8.34 (1H, d), 8.51 (1H, d)

Example B158
1-(4-Butylbenzyl)-7-(1-ethynyl)isoquinoline

[0750] Potassium carbonate (13 mg, 0.094 mmol) was added to a solution of the compound of Example B157 (6 mg, 0.016 mmol) in methanol (1.0 mL), and the mixture was stirred at room temperature for 1 hour. Upon concentration under reduced pressure, the obtained residue was purified by silica gel column chromatography to give the title compound (3.0 mg).

[0751] 1H-NMR (CDCl₃) δ (ppm): 0.91 (3H, t), 1.29-1.38 (2H, m), 1.52-1.57 (2H, m), 2.55 (2H, t), 3.19 (1H, s), 4.62 (2H, s), 7.09 (2H, d), 7.20 (2H, d), 7.53 (1H, d), 7.67-7.69 (1H, m), 7.77 (1H, d), 8.50 (1H, s), 8.52 (1H, d)

Example B159
1-(4-Butylbenzyl)-7-ethylisoquinoline

[0755] Palladium carbonate (10%, 5.0 mg) was added to a solution of the compound of Example B158 (2.0 mg) in tetrahydrofuran (2.0 mL), and the mixture was stirred at room temperature under nitrogen atmosphere (1 atm) for 1 hour. The catalyst was removed by filtration, and the filtrate was concentrated. The residue was purified by silica gel column chromatography to give the title compound (0.21 mg).

Example B160
1-(4-Butylbenzyl)-7-[4-(tetrahydro-2H-2-pyran-2-yl)-1-butylnyl]-isoquinoline

[0756] 1H-NMR (CDCl₃) δ (ppm): 0.90 (3H, t), 1.28-1.38 (2H, m), 1.52-1.67 (6H, m), 1.72-1.79 (1H, m), 1.79-1.88 (1H, m), 2.54 (2H, t), 2.78 (2H, t), 3.53-3.56 (1H, m), 3.66-3.72 (1H, m), 3.91-3.99 (2H, m), 4.60 (2H, s), 4.71-4.73 (1H, m), 7.08 (2H, d), 7.19 (2H, d), 7.50 (1H, d), 7.59-7.62 (1H, m), 7.72 (1H, d), 8.24 (1H, s), 8.48 (1H, d)
Example B161
4-[1-(4-Butylbenzyl)-7-isoquinoly]-3-butyn-1-ol

The title compound was obtained by treating the compound of Example B160 in the same manner as in Example B29.

\[\text{H-NMR (CDCl}_3\text{) } \delta \text{ (ppm): } 0.89 \text{ (3H, t), 1.05 \text{ (3H, } t), 1.27-1.39 \text{ (2H, m), 1.51-1.57 \text{ (2H, m), 1.84 \text{ (1H, brs), 2.05 \text{ (2H, t), 2.75 \text{ (2H, d), 3.84-3.89 \text{ (2H, m), 4.08 \text{ (2H, d), 7.18 \text{ (2H, d), 7.50 \text{ (1H, d), 6.60-6.62 \text{ (1H, m), 7.73 \text{ (1H, d), 8.25 \text{ (1H, d), 8.46 \text{ (1H, d) }}}})}}}}}}\]

Example B162
4-[1-(4-Butylbenzyl)-7-isoquinoly]-1-butanol

The title compound was obtained by treating the compound of Example B161 in the same manner as in Example B30.

\[\text{H-NMR (CDCl}_3\text{) } \delta \text{ (ppm): } 0.89 \text{ (3H, t), 1.28-1.36 \text{ (2H, m), 1.50-1.59 \text{ (4H, m), 1.67-1.77 \text{ (3H, m), 2.53 \text{ (2H, t), 2.79 \text{ (2H, t), 3.63 \text{ (2H, t), 4.62 \text{ (2H, s), 7.06 \text{ (2H, d), 7.18 \text{ (2H, d), 7.47-7.52 \text{ (2H, m), 7.73 \text{ (1H, d), 7.92 \text{ (1H, s), 8.43 \text{ (1H, d) }}}})}}}}\]

Example B163
1-(4-Butylbenzyl)-7-proxolyisoquinoline

Example B164
1-(4-Butylbenzyl)-7-(2-piperidinoethoxy)isoquinoline

The title compound was obtained by treating the compound of Example B154 in the same manner as in Example B148.

\[\text{H-NMR (CDCl}_3\text{) } \delta \text{ (ppm): } 0.90 \text{ (3H, t), 1.05 \text{ (3H, t), 1.27-1.36 \text{ (2H, m), 1.50-1.56 \text{ (2H, m), 1.76-1.84 \text{ (2H, m), 2.53 \text{ (2H, t), 3.92 \text{ (2H, t), 4.58 \text{ (2H, s), 7.06 \text{ (2H, d), 7.19 \text{ (2H, d), 7.26-7.29 \text{ (1H, m), 7.34 \text{ (1H, d), 7.48 \text{ (1H, d), 7.70 \text{ (1H, d), 8.38 \text{ (1H, d) }}}})}}}}\]

Example B165
N-(2-[[1-(4-Butylbenzyl)-7-isoquinoly]oxy]ethyl)-N,N-dimethyl-amine

The title compound was obtained in the same manner as in Example B148.

\[\text{H-NMR (CDCl}_3\text{) } \delta \text{ (ppm): } 0.89 \text{ (3H, t), 1.27-1.36 \text{ (2H, m), 1.50-1.57 \text{ (2H, m), 2.35 \text{ (6H, s), 2.53 \text{ (2H, t), 2.75 \text{ (2H, t), 4.06 \text{ (2H, t), 4.58 \text{ (2H, s), 7.06 \text{ (2H, d), 7.18 \text{ (2H, d), 7.30-7.33 \text{ (1H, m), 7.36 \text{ (1H, d), 7.48 \text{ (1H, d), 7.70 \text{ (1H, d), 8.39 \text{ (1H, d) }}}})}}}}\]

Example B166
The title compound was obtained in the same manner as in Example B148.

\[\text{H-NMR (CDCl}_3\text{) } \delta \text{ (ppm): } 0.89 \text{ (3H, t), 1.27-1.36 \text{ (2H, m), 1.43-1.58 \text{ (4H, m), 1.61-1.69 \text{ (4H, m), 2.51-2.55 \text{ (6H, m), 2.79 \text{ (2H, t), 4.11 \text{ (2H, t), 4.57 \text{ (2H, s), 7.06 \text{ (2H, d), 7.18 \text{ (2H, d), 7.28-7.30 \text{ (1H, m), 7.36 \text{ (1H, d), 7.48 \text{ (1H, d), 7.70 \text{ (1H, d), 8.38 \text{ (1H, d) }}}})}}}}\]

Example B167
N-(2-[[1-(4-Butylbenzyl)-7-isoquinoly]oxy]ethyl)-N,N-dimethyl-amine
Example B166
1-(4-Butylbenzyl)-7-isooquinolyl-(2-morpholinoethyl)ether

[0771]

[0772] The title compound was obtained in the same manner as in Example B148.

[0773] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.50-1.58 (2H, m), 2.51-2.58 (6H, m), 2.81 (2H, t), 3.75 (4H, t), 4.11 (2H, t), 4.58 (2H, s), 7.06 (2H, d), 7.17 (2H, d), 7.28-7.31 (1H, m), 7.35 (1H, d), 7.49 (1H, d), 7.71 (1H, d), 8.39 (1H, d)

Example B167
7-(Benzzyloxy)-1-(4-butylbenzyl)isoquinoline

[0774]

[0775] The title compound was obtained in the same manner as in Example B148.

[0776] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.50-1.54 (2H, m), 2.54 (2H, t), 4.54 (2H, s), 5.06 (2H, s), 7.05 (2H, d), 7.14 (2H, d), 7.34-7.43 (7H, m), 7.49 (1H, d), 7.72 (1H, d), 8.39 (1H, d)

Example B168
1-(4-Butylbenzyl)-7-(2-pyridylmethoxy)isoquinoline

[0777]

[0778] The title compound was obtained in the same manner as in Example B148.

[0779] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.49-1.57 (2H, m), 2.52 (2H, t), 4.51 (2H, s), 5.25 (2H, s), 7.02 (2H, d), 7.14 (2H, d), 7.24-7.27 (1H, m), 7.40 (1H, dd), 7.47-7.50 (3H, m), 7.68-7.72 (1H, d), 7.74 (1H, d), 8.39 (1H, d), 8.64-8.66 (1H, m)

Example B169
1-(4-Butylbenzyl)-7-(3-pyridylmethoxy)isoquinoline

[0780]

[0781] The title compound was obtained in the same manner as in Example B148.

[0782] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.50-1.58 (2H, m), 2.54 (2H, t), 4.57 (2H, s), 5.06 (2H, s), 7.07 (2H, d), 7.15 (2H, d), 7.31-7.36 (2H, m), 7.42 (1H, d), 7.51 (1H, d), 7.74-7.76 (2H, m), 8.42 (1H, d), 8.61-8.62 (1H, m), 8.69-8.70 (1H, m)

Example B170
1-(4-Butylbenzyl)-7-(4-pyridylmethoxy)isoquinoline

[0783]

[0784] The title compound was obtained in the same manner as in Example B148.

[0785] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.50-1.56 (2H, m), 2.54 (2H, t), 4.53 (2H, s), 5.09 (2H, s), 7.04 (2H, d), 7.09 (2H, d), 7.33-7.39 (4H, m), 7.51 (1H, d), 7.76 (1H, d), 8.41 (1H, d), 8.63-8.64 (2H, m)
Example B171
1-(4-Butylbenzyl)-7-[(2-methoxybenzyl)oxy]isoquinoline

Example B172
1-(4-Butylbenzyl)-7-[(3-methoxybenzyl)oxy]isoquinoline

Example B173
1-(4-Butylbenzyl)-7-[(4-methoxybenzyl)oxy]isoquinoline

Example B174
7-[(1,3-Benzodioxol-5-ylmethoxy)-1-(4-butylbenzyl)isoquinoline

Example B175
1-(4-Butylbenzyl)-7-[(2-nitrobenzyl)oxy]isoquinoline

Example B176
The title compound was obtained in the same manner as in Example B148.

Example B177
The title compound was obtained in the same manner as in Example B148.

Example B178
The title compound was obtained in the same manner as in Example B148.

Example B179
The title compound was obtained in the same manner as in Example B148.

Example B180
The title compound was obtained in the same manner as in Example B148.
Example B176
1-(4-Butylbenzyl)-7-{(3-nitrobenzyl)oxy}isoquinoline

Example B179
1-(4-Butylbenzyl)-7-(2-cyclohexylethoxy)isoquinoline

[0801] The title compound was obtained in the same manner as in Example B148.

[0802] 1H-NMR (CDCl₃) δ (ppm): 0.89 (3H, t), 1.26-1.36 (2H, m), 1.49-1.57 (2H, m), 2.52 (2H, t), 4.18 (2H, t), 4.56 (2H, s), 7.04 (2H, d), 7.16 (2H, d), 7.26-7.28 (4H, m), 7.33-7.35 (3H, m), 7.48 (1H, d), 7.70 (1H, d), 8.38-8.39 (1H, m)

Example B177
1-(4-Butylbenzyl)-7-(phenethoxy)isoquinoline

Example B180
6-Benzoyl-5,6-dihydro[1,3]dioxolo[4,5-g]isoquinoline-5-carbonitrile

[0803] 1H-NMR (CDCl₃) δ (ppm): 0.89 (3H, t), 1.26-1.36 (2H, m), 1.49-1.57 (2H, m), 2.52 (2H, t), 3.10 (2H, t), 4.18 (2H, t), 4.56 (2H, s), 7.04 (2H, d), 7.16 (2H, d), 7.26-7.28 (4H, m), 7.33-7.35 (3H, m), 7.48 (1H, d), 7.70 (1H, d), 8.38-8.39 (1H, m)

Example B178
1-(4-Butylbenzyl)-7-(3-phenylpropoxy)isoquinoline

Example B181
5-(4-Butylbenzyl)[1,3]dioxolo[4,5-g]isoquinoline

[0804] The title compound was obtained in the same manner as in Example B148.

[0805] The title compound was obtained in the same manner as in Example B148.

[0806] 1H-NMR (CDCl₃) δ (ppm): 0.89 (3H, t), 1.26-1.36 (2H, m), 1.49-1.57 (2H, m), 2.52 (2H, t), 3.10 (2H, t), 4.18 (2H, t), 4.56 (2H, s), 7.04 (2H, d), 7.16 (2H, d), 7.26-7.28 (4H, m), 7.33-7.35 (3H, m), 7.48 (1H, d), 7.70 (1H, d), 8.38-8.39 (1H, m)

Example B179
1-(4-Butylbenzyl)-7-(2-cyclohexylethoxy)isoquinoline

Example B180
6-Benzoyl-5,6-dihydro[1,3]dioxolo[4,5-g]isoquinoline-5-carbonitrile

[0810] The title compound was obtained in the same manner as in Example B148.

[0811] The title compound was obtained in the same manner as in Example B148.

[0812] 1H-NMR (CDCl₃) δ (ppm): 0.89 (3H, t), 0.94-1.02 (2H, m), 1.17-1.36 (4H, m), 1.36-1.57 (4H, m), 1.65-1.76 (7H, m), 2.53 (2H, t), 3.98 (2H, t), 4.58 (2H, s), 7.06 (2H, d), 7.19 (2H, d), 7.25-7.28 (1H, m), 7.33 (1H, d), 7.47 (1H, d), 7.69 (1H, d), 8.37 (1H, d)

Example B180
6-Benzoyl-5,6-dihydro[1,3]dioxolo[4,5-g]isoquinoline-5-carbonitrile

[0813] The title compound was obtained by treating [1,3]dioxolo[4,5-g]isoquinoline in the same manner as in Example B140.

[0814] The title compound was obtained by treating [1,3]dioxolo[4,5-g]isoquinoline in the same manner as in Example B140.

[0815] 1H-NMR (CDCl₃) δ (ppm): 5.94-5.96 (1H, m), 6.03 (1H, d), 6.04 (1H, d), 6.47-6.54 (2H, m), 6.70 (1H, s), 6.83 (1H, s), 7.45-7.49 (2H, m), 7.54-7.62 (3H, m)

Example B181
5-(4-Butylbenzyl)[1,3]dioxolo[4,5-g]isoquinoline
[0817] The title compound was obtained by treating the compound of Example B180 and the compound of Example B1 in the same manner as in Example B2.

[0818] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.90 (3H, t), 1.28-1.37 (2H, m), 1.51-1.57 (2H, m), 2.54 (2H, t), 4.50 (2H, s), 6.05 (2H, s), 7.05-7.07 (3H, m), 7.16 (2H, d), 7.38 (7.40 (2H, m), 8.35 (1H, d)

Example B182
2-Benzoyl-6-bromo-1,2-dihydro-1-isoquinolinecarbonitrile

[0819]

[0820] The title compound was obtained by treating 6-bromoisoquinoline, which was synthesized according to J. Am. Chem. Soc., 183 (1942), in the same manner as in Example B140.

[0821] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 6.01 (1H, d), 6.53 (1H, brs), 6.70 (1H, brd), 7.24 (1H, d), 7.33 (1H, d), 7.47-7.51 (3H, m), 7.56 (3H, m)

Example B183
6-Bromo-1-(4-butylbenzyl)isoquinoline

[0822]

[0823] The title compound was obtained by treating the compound of Example B182 and the compound of Example B1 in the same manner as in Example B2.

[0824] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.89 (3H, t), 1.27-1.36 (2H, m), 1.50-1.58 (2H, m), 2.53 (2H, t), 4.60 (2H, s), 7.06 (2H, d), 7.15 (2H, d), 7.46 (1H, d), 7.59 (1H, q), 7.98 (1H, d), 8.02 (1H, d), 8.51 (1H, d)

Example B184
A mixture of 2-benzoyl-5-bromo-1,2-dihydro-1-isoquinoline-carbonitrile and 2-benzoyl-7-bromo-1,2-dihydro-1-isoquinoline-carbonitrile

[0825] A mixture of 2-benzoyl-5-bromo-1,2-dihydro-1-isoquinoline-carbonitrile and 2-benzoyl-7-bromo-1,2-dihydro-1-isoquinoline-carbonitrile

[0826] The title compounds were obtained by treating 5- or 7-bromoisoquinoline, which was synthesized according to J. Am. Chem. Soc., 61, 183 (1939), in the same manner as in Example B140. The obtained compounds were used in the following reaction without separation and purification.

Example B185
7-Bromo-1-(4-butylbenzyl)isoquinoline

[0827]

[0828] The title compound was obtained by treating the compound of Example B184 and the compound of Example B1 in the same manner as in Example B2.

[0829] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 0.90 (3H, t), 1.28-1.37 (2H, m), 1.51-1.58 (2H, m), 2.55 (2H, t), 4.58 (2H, s), 7.09 (2H, d), 7.18 (2H, d), 7.51-7.53 (1H, m), 7.69-7.70 (2H, m), 8.33-8.34 (1H, m), 8.52 (1H, d)

Example B186
5-Benzoyl-4,5-dihydrothieno[3,2-c]pyridine-4-carbonitrile

[0830]

[0831] The title compound was obtained by treating thieno[3,2-c]pyridine, synthesized according to J. Heterocycl. Chem., 30, 183 (1993), in the same manner as in Example B140.

[0832] $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 6.05 (1H, d), 6.57 (1H, brd), 6.66 (1H, s), 7.07 (1H, d), 7.32 (1H, d), 7.46-7.50 (2H, m), 7.54-7.62 (3H, m)
Example B187
4-(4-Butylbenzyl)thieno[3,2-c]pyridine

[0833] The title compound was obtained by treating the compound of Example B186 and the compound of Example B1 in the same manner as in Example B2.

[0834] $^1$H-NMR (CDCl$_3$) δ (ppm): 0.90 (3H, t), 1.27-1.37 (2H, m), 1.51-1.59 (2H, m), 2.54 (2H, t), 4.47 (2H, s), 7.07 (2H, d), 7.19 (2H, d), 7.42 (1H, d), 7.47 (1H, d), 7.68 (1H, d), 8.41 (1H, d)

Example B188
4-(4-Methoxybenzyl)thieno[3,2-c]pyridine

[0836] The title compound was obtained by treating the compound of Example B186 and 4-methoxybenzyl chloride in the same manner as in Example B2.

[0837] $^1$H-NMR (CDCl$_3$) δ (ppm): 3.75 (3H, s), 4.44 (2H, s), 6.79-6.82 (2H, m), 7.19-7.22 (2H, m), 7.43 (1H, d), 7.46 (1H, d), 7.68 (1H, d), 8.41 (1H, d)

Example B189
4-(Thieno[3,2-c]pyridin-4-ylmethyl)phenyl trifluoromethane-sulfonate

[0839]

Example B190
4-(4-Bromobenzyl)thieno[3,2-c]pyridine

[0840] A solution of boron tribromide in methylene chloride (1.0 M, 10 ml, 10 mmol) was added dropwise to a solution of the compound of Example B188 (510 mg, 2.0 mmol) in methylene chloride (10 ml) cooled to 0°C, and this reaction mixture was stirred at that temperature for 1.5 hours. The reaction mixture was made weakly alkaline by addition of a saturated aqueous sodium hydrogencarbonate solution, extracted with ethyl acetate, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The obtained residue was dissolved in pyridine, and the resulting solution was cooled to 0°C. After trifluoromethanesulfonic anhydride (0.34 ml, 2.1 mmol) was added dropwise thereto, the mixture was stirred at that temperature for 2 hours, poured on ice, extracted with ethyl acetate, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel chromatography to give the title compound (312 mg).

Example B191
4-(4-Bromo-2-fluorobenzyl)thieno[3,2-c]pyridine

[0843] The title compound was obtained by treating the compound of Example B186 and the compound of Example B31 in the same manner as in Example B2.

Example B192
4-(4-Bromo-2-fluorobenzyl)thieno[3,2-c]pyridine

[0845] The title compound was obtained by treating the compound of Example B186 and 4-bromo-2-fluorobenzyl bromide in the same manner as in Example B2.
Example B192

4-{4-[4-(Tetrahydro-2H-2-pyran)-1-butynyl]-benzyl}thieno[3,2-c]pyridine

Example B193

4-{4-[Thieno[3,2-c]pyridin-4-ylmethyl]phenyl}-3-butyn-1-ol

Example B194

6-Benzoyl-6,7-dihydrothieno[2,3-c]pyridine-7-carbonitrile

Example B195

7-(4-Butylbenzyl)thieno[2,3-c]pyridine

Example B196

7-(4-Methoxybenzyl)thieno[2,3-c]pyridine

Example B197

7-(4-Methoxybenzyl)thieno[2,3-c]pyridine

Example B198

7-(4-Methoxybenzyl)thieno[2,3-c]pyridine
Example B197
4-(Thieno[2,3-c]pyridin-7-ylmethyl)phenyl trifluoromethane-sulfonate

The title compound was obtained by treating the compound of Example B196 in the same manner as in Example B189.

Example B198
7-(4-Bromobenzyl)thieno[2,3-c]pyridine

Similarly, the title compound was obtained by treating the compound of Example B194 and 4-bromo-2-fluorobenzyl bromide in the same manner as in Example B2.

Example B200
7-{4-[4-(Tetrahydro-2H-2-pyranoxy)-1-butynyl]benzyl}thieno[2,3-c]pyridine

The title compound was obtained by treating the compound of Example B197 and 2-(3-butyloxy)tetrahydro-2H-pyran in the same manner as in Example B42.

Example B201
4-[4-(Thieno[2,3-c]pyridin-7-ylmethyl)phenyl]-3-butyn-1-ol

The title compound was obtained by treating the compound of Example B200 in the same manner as in Example B47.
Example B202
2-Chloro-3-(methoxymethoxy)pyridine

Sodium hydride (66%, 633 mg, 17.4 mmol) was added to an ice-cooled solution of 2-chloro-3-hydroxy pyridine (2.05 g, 15.8 mmol) in tetrahydrofuran (30 ml) under nitrogen atmosphere, and this reaction mixture was stirred at that temperature for 15 minutes. Chloromethyl methyl ether (1.32 ml, 17.4 mmol) was added, and the resulting reaction mixture was stirred at that temperature for 30 minutes, then at room temperature for another 2 hours. After water was added, the reaction mixture was extracted with ethyl acetate, washed with saturated brine, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (2.44 g).

Example B203
2-Chloro-4-iodo-3-(methoxymethoxy)pyridine

A solution of the compound of Example B202 (1.40 g, 8.06 mmol) in diethyl ether (8 ml) was added dropwise to a solution of 1.51 M t-butyllithium-p-pentane solution (8.01 ml, 12.1 mmol) in diethyl ether (15 ml) cooled to -78° C. under nitrogen atmosphere, and the reaction mixture was stirred at that temperature for 15 minutes. After iodine (3.07 g, 12.1 mmol) was added, the reaction mixture was gradually warmed to room temperature. An aqueous sodium thiosulfate solution was further added, and the diethyl ether layer was separated, washed with saturated brine, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (356 mg).

Example B204
7-Chlorofuro[2,3-c]pyridine

Trimethylsilylacetylene (28.3 ml, 0.201 mmol) and triethylamine (59.8 ml, 0.429 mmol) were added to a solution of the compound of Example B203 (36.6 mg, 0.143 mmol), tetraakis(triphenylphosphine)palladium (16.5 mg, 0.0143 mmol), and copper(I) iodide (2.7 mg, 0.0144 mmol) in dimethylformamide (1.5 ml), and this mixture was stirred at 50° C. for 4 hours. After allowing the mixture to cool to room temperature, water was added thereto, and the resulting mixture was extracted with ethyl acetate, washed with saturated brine, and then concentrated under reduced pressure. The residue was dissolved in methanol (5 ml), potassium carbonate (100 mg, 0.724 mmol) was added thereto, and the resulting mixture was stirred at room temperature for 1 hour. After water was added, the mixture was extracted with diethyl ether, washed with saturated brine, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (5.5 mg).

Example B205
4-Butylbenzylmagnesium chloride

A mixed solution of the compound of Example B1 (1.04 g, 5.69 mmol), magnesium (761 mg, 31.3 mmol), and a catalytic amount of 1,2-dibromoethane in diethyl ether (11 ml) was initiated by heating under reflux. After the heat source was removed, a solution of the compound of Example B1 (4.16 g, 22.8 mmol) in diethyl ether (60 ml) was added dropwise to the reaction mixture at a rate that maintains gentle reflux, and the mixture was heated under reflux for 30 minutes. The mixture was then allowed to cool to room temperature to give the title compound as a 0.4 M solution in diethyl ether. This solution was used in the following reaction as it is.

Example B206
7-(4-Butylbenzyl)furo[2,3-c]pyridine

The compound of Example B205 (300 ml, 0.1 mmol) was added to a solution of the compound of Example B204 (5.0 mg, 0.033 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichloronickel(II) (4.5 mg, 0.0005 mmol) in tetrahydrofuran (1 ml), and the mixture was stirred at 50° C. for 1 hour. After allowing the mixture to cool to room temperature, ethyl acetate was added thereto. The resulting mixture was washed with a saturated aqueous ammonium chloride solu-
The compound of Example B205 (800 µl, 0.3 mmol) was added to a solution of 1-chloropyrrolopyridine (19.4 mg, 0.127 mmol), which was synthesized from 2-chloro-3-amino pyridine according to the method described in J. Heterocycl. Chem., 2, 196 (1965), and dichloro(diphenylphosphinopropane)nickel (6.9 mg, 0.013 mmol) in tetrahydrofuran (1 ml) under ice-cooling, and the mixture was stirred while heating under reflux for 4 hours. After allowing the mixture to cool to room temperature, ethyl acetate was added thereto. The resulting mixture was washed with a saturated aqueous ammonium chloride solution and saturated brine, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (7.1 mg).

The compound of Example B205 (3.45 ml, 1.38 mmol) was added to a solution of 1-chloroimidazopyridine (88.6 mg, 0.577 mmol), which was synthesized from 4-amino-2-chloropyridine according to the method described in J. Heterocycl. Chem., 2, 196 (1965), and dichloro(diphenylphosphinopropane)nickel (31.3 mg, 0.0577 mmol) in tetrahydrofuran (2 ml), and the mixture was stirred while heating under reflux for 2 hours. After allowing the mixture to cool to room temperature, ethyl acetate was added thereto. The resulting mixture was filtered through silica gel and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (64.2 mg).

Example B208
4-(4-Butylbenzyl)-1-imidazo[4,5-c]pyridine

Example B209
4-Bromo-1-isoquinolinol

Example B210
1,4-Dibromoisoquinoline

A mixed solution of the compound of Example B209 (1.40 g, 8.06 mmol) and phosphorus tribromide (6 ml) was stirred at 150°C for 1 hour, and then heated under reflux for another 1 hour. The reaction mixture was allowed to cool to room temperature, poured on ice, then warmed to room temperature. Ethyl acetate was added, and the resulting mix-
ture was washed with saturated brine and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (845 mg).

Example B211
4-Bromo-1-(4-butylbenzyl)isoquinoline

Example B212
1-(4-Butylbenzyl)-5,6,7,8-tetrahydroisoquinoline

Example B213
1-[2-(Phenyl)benzyl]isoquinoline

The compound of Example B205 (2.5 ml, 1 mmol) was added to a solution of the compound of Example B210 (200 mg, 0.697 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichlororonicl(II) (75.6 mg, 0.139 mmol) in tetrahydrofuran (2 ml), and the mixture was stirred at room temperature for 30 minutes. After ethyl acetate was added, the resulting mixture was washed successively with a saturated aqueous ammonium chloride solution, a saturated aqueous sodium hydrogen carbonate solution, and saturated brine, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (98 mg).

Example B214
1-[4-Fluoro-2-(trifluoromethyl)benzyl]isoquinoline

The title compound was obtained by treating 2-phenylbenzyl bromide instead of n-butylbenzyl chloride in the same manner as in Example B2.

Example B215
1-[4-Fluoro-2-(trifluoromethyl)benzyl]isoquinoline

The title compound was obtained by treating 4-fluoro-2-(trifluoromethyl)benzyl methanesulfonate instead of n-butylbenzyl chloride in the same manner as in Example B2.

The compound of Example B211 (13.0 mg, 0.0367 mmol) was dissolved in a mixed solution of ethyl acetate and methanol (1:1, 1 ml), 10% palladium-carbon (containing 50% water, 13 mg) was added, and the mixture was stirred at room temperature under hydrogen atmosphere at atmospheric pressure for 12 hours. After purging the reaction system with nitrogen, the catalyst was removed by filtration through celite. The obtained filtrate was concentrated under reduced pressure to give the title compound (8.8 mg).

1H-NMR (CDCl3) δ (ppm): 7.76-7.80 (1H, m), 7.86-7.90 (1H, m), 8.19 (1H, d), 8.31-8.34 (1H, m), 8.48 (1H, s)

Example B216
1-[2-(Phenyl)benzyl]isoquinoline

Example B217
1-[2-(Phenyl)benzyl]isoquinoline
Example B215

1,3-Benzodioxoyl-4-yl-(1-isoquinolyl)methanol

The title compound was obtained by treating 2,3-methylenedioxybenzaldehyde in the same manner as in Example B82.

[0921] ¹H-NMR (CDCl₃) δ (ppm): 5.97-5.99 (1H, m), 6.09 (1H, brs), 6.20-6.40 (1H, m), 6.54-6.60 (2H, m), 6.65-6.70 (2H, m), 7.52 (1H, dd), 7.63 (1H, d), 7.64 (1H, dd), 7.84 (1H, d), 8.04 (1H, d), 8.53 (1H, d).

Example B216

1,3-Benzodioxoyl-4-yl-(1-isoquinolyl)methyl acetate

The title compound was obtained by treating the compound of Example B215 in the same manner as in Example B38.

[0924] ¹H-NMR (CDCl₃) δ (ppm): 2.23 (3H, s), 5.98-6.02 (2H, m), 6.74-6.79 (1H, m), 6.90-6.93 (1H, m), 7.15-7.19 (1H, m), 7.23-7.28 (1H, m), 7.58 (1H, dd), 7.60 (1H, d), 7.66 (1H, dd), 7.83 (1H, d), 8.28 (1H, d), 8.57 (1H, d).

Example B217

1-(1,3-Benzodioxoyl-4-ylmethyl)isoquinoline

The title compound was obtained by treating the compound of Example B215 in the same manner as in Example B39.

[0927] ¹H-NMR (CDCl₃) δ (ppm): 4.62 (2H, s), 6.02 (2H, s), 6.64-6.70 (3H, m), 7.57 (1H, dd), 7.58 (1H, d), 7.66 (1H, dd), 7.83 (1H, d), 8.23 (1H, d), 8.50 (1H, d).

Example B218

1-(1-Naphthylmethyl)isoquinoline

The title compound was obtained by treating 1-(chloromethyl)naphthalene instead of n-butylbenzyl chloride in the same manner as in Example B2.

[0930] ¹H-NMR (CDCl₃) δ (ppm): 5.13 (2H, s), 6.96 (1H, d), 7.29 (1H, d), 7.45-7.67 (5H, m), 7.72 (1H, d), 7.84-7.90 (2H, m), 8.08 (1H, d), 8.26 (1H, d), 8.52 (1H, d).

Example B219

3-Bromophenylbutyrate

n-Butyryl chloride (7.25 ml) was added to an ice-cooled solution of 3-bromophenol (10.0 g) in pyridine (50 ml), and this reaction mixture was stirred at that temperature for 3 hours, then at room temperature for another 3.5 hours. After ice was added, the reaction mixture was extracted with ethyl acetate, washed with 1 N hydrochloric acid and water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (12.77 g).

[0933] ¹H-NMR (CDCl₃) δ (ppm): 1.04 (3H, t), 1.72-1.82 (2H, m), 2.54 (2H, t), 7.04 (1H, dd), 7.22-7.29 (2H, m), 7.36 (1H, d).

Example B220

1-(4-Bromo-2-hydroxyphenyl)-1-butanone

[0934]
Aluminum chloride (10.51 g) was added to a solution of the compound of Example B219 (12.77 g) in chlorobenzene (70 ml) under nitrogen atmosphere, and this reaction mixture was stirred while heating under reflux for 9 hours. After the reaction mixture was cooled to room temperature, ice was added thereto. The resulting mixture was extracted with ethyl acetate, washed with water, dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The compound thus obtained was used in the following reaction without further purification.

**Example B221**

1-(4-Bromo-2-methoxyphenyl)-1-butanone

Potassium carbonate (9.07 g) and methyl iodide (3.92 ml) were added to a solution of the compound of Example B220 (13.30 g) in acetone (75 ml), and this reaction mixture was stirred while heating under reflux for 4 hours. The reaction mixture was filtered through celite, ether was added to remove insoluble material by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (9.52 g).

**Example B222**

4-Bromo-1-butyl-2-methoxybenzene

The title compound was obtained by treating the compound of Example B221 in the same manner as in Example B3.

**Example B223**

(4-Butyl-3-methoxyphenyl)(1-isoquinolyl)ketone

A mixture containing the title compound was obtained by treating the compound of Example B222 in the same manner as in Example B220.

**Example B224**

(4-Butyl-3-methoxyphenyl)(1-isoquinolyl)methanol

A mixture containing the title compound was obtained by treating the compound of Example B223 in the same manner as in Example B37.

**Example B225**

(4-Butyl-3-methoxyphenyl)(1-isoquinolyl)methyl acetate

The title compound was obtained by treating the compound of Example B224 in the same manner as in Example B38.

**Example B226**

(4-Butyl-3-methoxyphenyl)(1-isoquinolyl)ethyl acetate
Example B226

1-(4-Butyl-3-methoxybenzyl)isoquinoline

\[ \text{N} \quad \text{OMe} \]

**[0952]**

The title compound was obtained by treating the compound of Example B225 in the same manner as in Example B39.

**[0953]**

\[ ^1\text{H-NMR (CDCl}_3\text{)} \delta (ppm): 0.89 (3H, t), 1.27-1.38 (2H, t), 1.45-1.54 (2H, t), 2.52 (2H, t), 3.72 (3H, s), 4.63 (2H, s), 6.78 (1H, d), 6.79 (1H, s), 6.99 (1H, d), 7.53 (1H, dd), 7.55 (1H, d), 7.64 (1H, dd), 7.80 (1H, d), 8.19 (1H, d), 8.49 (1H, d). \]

Example B227

2-Butyl-5-(1-isoquinolylomethyl)phenol

**[0955]**

The title compound was obtained by treating the compound of Example B226 in the same manner as in Example B40.

**[0956]**

\[ ^1\text{H-NMR (CDCl}_3\text{)} \delta (ppm): 0.91 (3H, t), 1.30-1.40 (2H, m), 1.52-1.65 (2H, m), 2.55 (2H, t), 4.55 (2H, s), 6.46 (1H, brs), 6.85 (1H, d), 7.03 (1H, d), 7.32-7.40 (1H, m), 7.55 (1H, dd), 7.68 (1H, dd), 7.81 (1H, d), 7.94-8.05 (1H, m), 8.14 (1H, d). \]

**[0957]**

The proton of the phenolic hydroxyl group was not observed in the NMR spectrum.

Example B228

2-Bromo-3-(methoxymethoxy)pyridine

**[0959]**

**[0960]**

The title compound was synthesized in the same manner as in Example B202 by using 2-bromo-3-hydroxy-pyridine.

Example B229

2-(4-Butylbenzyl)-3-(methoxymethoxy)pyridine

**[0962]**

**[0963]**

The compound of Example B205 (7 ml, 3 mmol) was added to an ice-cooled mixed solution of the compound of Example B228 (524 mg, 2.40 mmol) and dichloro(di-phenylphosphinophosphate)nickel (65.0 mg, 0.120 mmol) in tetrahydrofuran (10 ml), and the mixture was stirred while heating under reflux for 5 hours. After allowing the mixture to cool to room temperature, ethyl acetate was added. The resulting mixture was washed successively with a saturated aqueous ammonium chloride solution, a saturated aqueous sodium hydrogencarbonate solution, and saturated brine, then concentrated under reduced pressure. The residue was filtered through NH-silica gel. After concentrating under reduced pressure, the residue was dissolved in methanol (15 ml), triethylamine (500 μl, 3.59 mmol) and 10% palladium-carbon (containing 50% water, 50 mg) were added, and the resulting mixture was stirred at room temperature under hydrogen atmosphere at atmospheric pressure for 3 hours. After purging the reaction system with nitrogen, the catalyst was removed by filtration through celite, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (280 mg).

Example B230

2-(4-Butylbenzyl)-3-pyridinol

**[0965]**

Trifluoroacetic acid (1 ml) was added to a solution of the compound of Example B229 (256 mg, 0.849 mmol) in methylene chloride (5 ml), and this reaction mixture was...
stirred at room temperature overnight. After a saturated aqueous sodium hydrogencarbonate solution and ethyl acetate were added, the reaction mixture was washed with saturated brine and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (187 mg).

**[0967]** $^1$H-NMR (CDCl$_3$) δ (ppm): 0.90 (3H, t), 1.28-1.37 (2H, m), 1.51-1.58 (2H, m), 2.54 (2H, t), 4.20 (2H, s), 7.02-7.08 (4H, m), 7.22 (2H, d), 8.08-8.09 (1H, m)

**[0968]** The proton of the phenolic hydroxyl group was not observed in the NMR spectrum.

### Example B231

2-(4-Butylbenzyl)-3-methoxypyridine

![Example B231](image)

**[0969]** Potassium carbonate (33.0 mg, 0.239 mmol) and methyl iodide (14.9 μl, 0.239 mmol) were added to a solution of the compound of Example B230 (19.2 mg, 0.0796 mmol) in acetone (1 mL), and this reaction mixture was stirred at room temperature for 3 hours. After ethyl acetate was added, the reaction mixture was washed with saturated brine and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (1.47 mg).

**[0970]** $^1$H-NMR (CDCl$_3$) δ (ppm): 0.90 (3H, t), 1.32-1.34 (2H, m), 1.53-1.57 (2H, m), 2.54 (2H, t), 3.82 (3H, s), 4.14 (2H, s), 7.06 (2H, d), 7.10-7.11 (2H, m), 7.21 (2H, d), 8.12-8.14 (1H, m)

### Example B232

2-(4-Butylbenzyl)-3-chloropyridine

![Example B232](image)

**[0972]** The compound of Example B205 (12 mL, 5 mmol) was added to an ice-cooled mixed solution of 2,3-dichloropyridine (525 mg, 3.55 mmol) and dichloro(diphenylphosphinophinopropane)nickel (96.2 mg, 0.178 mmol) in tetrahydrofuran (4 mL), and this reaction mixture was stirred at room temperature for 1 hour. After ethyl acetate was added, the reaction mixture was washed successively with a saturated aqueous ammonium chloride solution, a saturated aqueous hydrogencarbonate solution, and saturated brine, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (199 mg).

**[0974]** $^1$H-NMR (CDCl$_3$) δ (ppm): 0.91 (3H, t), 1.29-1.38 (2H, m), 1.52-1.60 (2H, m), 2.56 (2H, t), 4.28 (2H, s), 7.08-7.13 (3H, m), 7.21 (2H, d), 7.64 (1H, dd), 8.46 (1H, dd)

### Example B233

2-(4-Butylbenzyl)-3-ethylpyridine

![Example B233](image)

**[0976]** Ethylmagnesium chloride (0.97 M, 102 μl, 0.993 mmol) was added to a mixed solution of the compound of Example B232 (12.9 mg, 0.0496 mmol) and dichloro(diphenylphosphinoferrocene)nickel (3.4 mg, 0.0050 mmol) in tetrahydrofuran (1 mL). The reaction mixture was stirred at 50° C. for 1 hour, then heated under reflux for another 2 hours. After allowing the reaction mixture to reach room temperature, ethyl acetate was added thereto. The reaction mixture was washed with a saturated aqueous ammonium chloride solution and saturated brine, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (3.29 mg).

**[0977]** $^1$H-NMR (CDCl$_3$) δ (ppm): 0.90-0.93 (6H, m), 1.30-1.37 (2H, m), 1.54-1.59 (2H, m), 2.55-2.59 (4H, m), 4.12 (2H, s), 7.05-7.18 (5H, m), 7.55-7.59 (1H, m), 8.53-8.55 (1H, m)

### Example B234
tert-Butyl N-(2-bromo-3-pyridyl)carbamate

![Example B234](image)

**[0978]**

**[0979]** N-bromosuccinimide (7.51 g, 42.2 mmol) was added to an ice-cooled mixed solution of 3-aminopyridine (3.97 g, 42.2 mmol) in dimethylformamide (25 mL), and this reaction mixture was stirred at that temperature for 30 minutes. After ethyl acetate was added, the reaction mixture was washed with saturated brine and concentrated under reduced pressure. A solution of the residue in methylene chloride (20 ml) was cooled on ice, then triethylamine (3.74 ml, 26.8 mmol), a catalytic amount of dimethylaminopyridine, and dibutyl dicarbonate (3.08 ml, 15.4 mmol) were added to the
solution, and the mixture was stirred at room temperature overnight. After concentration under reduced pressure, the residue was purified by silica gel column chromatography to give the title compound (344 mg).

Example B235

2-Bromo-3-(N-t-butoxycarbonyl-N-methyl)aminopyridine

Methyl iodide (157 µl, 2.52 mmol) and 66% sodium hydride (91.6 mg, 2.52 mmol) were added to an ice-cooled solution of the compound of Example B234 (344 mg, 1.26 mmol) in dimethylformamide (5 ml), and this reaction mixture was stirred at that temperature for 40 minutes. After ethyl acetate was added, the reaction mixture was washed with saturated brine and filtered through silica gel. The organic layer was concentrated under reduced pressure to give the title compound (356 mg).

Example B236

N-[2-(4-Butylbenzyl)-3-pyridyl]-N-methylamine

To a methylene chloride solution (2 ml) of a compound, which was obtained by introduction of a 4-butylbenzyl group to the compound of Example B235 (62.8 mg, 0.219 mmol) in the same manner as in Example B211, trifluoroacetic acid (2 ml) was added at room temperature. The mixture was stirred at room temperature for 1 hour, and then added dropwise to an aqueous solution of sodium hydrogen carbonate. After ethyl acetate was added, the mixture was washed with saturated brine and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (29.7 mg).

Example B237

N-[2-(4-Butylbenzyl)-3-pyridyl]-N,N-dimethylamine

Acetic acid (12.1 µl, 0.211 mmol), 37% formalin (15.8 µl, 0.211 mmol), and sodium triacetoxyborohydride (44.7 mg, 0.211 mmol) were added to an ice-cooled solution of the compound of Example B236 (26.8 mg, 0.105 mmol) in methylene chloride (2 ml), and the mixture was stirred at room temperature for 30 minutes. After ethyl acetate was added, the mixture was washed with saturated aqueous sodium hydrogen carbonate solution and saturated brine and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (23.3 mg).

Example B238

2-(4-Butylbenzyl)-4-methoxypyridine

The title compound was obtained in the same manner as in Example B211 using 2-chloro-4-methoxypyridine.

Example B239

2-(4-Butylbenzyl)-4-methoxypyridine

The title compound was obtained in the same manner as in Example B211 using 2-chloro-4-methoxypyridine.

Example B240

2-(4-Butylbenzyl)-4-methoxypyridine

The title compound was obtained in the same manner as in Example B211 using 2-chloro-4-methoxypyridine.
Example B239
2-(4-Butylbenzyl)-4-chloropyridine

[0993]

Phosphorus oxychloride (57.0 μl, 0.612 mmol) was added to an ice-cooled solution of the compound of Example B238 (52.0 mg, 0.204 mmol) in dimethylformamide (1 ml), and this reaction mixture was stirred at 100° C. for 8 hours. The reaction mixture was allowed to cool, poured on ice, and warmed to room temperature. After ethyl acetate was added, the mixture was washed with a saturated aqueous sodium hydrogen carbonate solution and saturated brine, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give the title compound (2.29 mg).

[0995] 1H-NMR (CDCl₃, δ (ppm)): 0.92 (3H, t), 1.31-1.38 (2H, m), 1.53-1.61 (2H, m), 2.59 (2H, t), 4.10 (2H, s), 7.12-0.18 (6H, m), 8.44 (1H, d)

Example B240
2-Chloro-3-methoxypyridine

[0996]

The title compound was obtained in the same manner as in Example B231 using 2-chloro-3-methoxypyridine.

[0998] 1H-NMR (CDCl₃, δ (ppm)): 3.93 (3H, s), 7.21-7.22 (2H, m), 7.99-8.01 (1H, m)

Example B241
2-Chloro-3,4-dimethoxypyridine

[0999]

A solution of diisopropylamine (84.0 μl, 0.599 mmol) and the compound of Example B240 (860 mg, 5.99 mmol) in tetrahydrofuran (4 ml) was added to a solution of 1.06 M phenyllithium cyclopentane-diethyl ether solution in tetrahydrofuran (11 ml) cooled to −78° C. under nitrogen atmosphere. This reaction mixture was stirred at −40° C. for 1 hour, then at −18° C. for another 20 minutes. The reaction mixture was cooled again to −78° C., trimethoxyborate (2.04 ml, 18.0 mmol) was added dropwise thereto, and the resulting mixture was stirred at 0° C. for 20 minutes. At that temperature, aqueous ammonia (29%, 30 ml), ammonium chloric (4.5 g), and an aqueous hydrogen peroxide solution (30%, 12 ml) were added in this order, and the mixture was stirred at room temperature for 2 hours. Saturated sodium thiosulfate, acetic acid and ethyl acetate were added, and the mixture was washed with saturated brine. The ethyl acetate layer obtained upon filtration through silica gel was concentrated under reduced pressure. The resulting residue was treated in the same manner as in Example B231 to obtain the title compound (31.3 mg).

[1001] 1H-NMR (CDCl₃, δ (ppm)): 3.89 (3H, s), 3.94 (3H, s), 6.82 (1H, d), 8.05 (1H, d)

Example B242
2-(4-Butylbenzyl)-3,4-dimethoxypyridine

[1002]

The title compound was obtained in the same manner as in Example B206 using the compound of Example B241.

[1004] 1H-NMR (CDCl₃, δ (ppm)): 0.90 (3H, t), 1.26-1.35 (2H, m), 1.33-1.57 (2H, m), 2.54 (2H, t), 3.70 (3H, s), 3.89 (3H, s), 4.12 (2H, s), 6.72 (1H, d), 7.06 (2H, d), 7.21 (2H, d), 8.20 (1H, d)

Example B243
2,4-Di-(4-butylbenzyl)-3-methoxypyridine

[1005]

A solution of the compound of Example B240 (436 mg, 3.04 mmol) in diethyl ether (2 ml) was added to a solution of 1.43 M t-butyllithium n-pentane solution (2.76 ml, 3.95 mmol) in diethyl ether (5 ml) cooled to −78° C. under nitrogen atmosphere, and this reaction mixture was stirred at that temperature for 30 minutes. A solution of tetramethylethylendiamine (688 μl, 4.56 mmol) and hexachloroethane (719 mg, 3.04 mmol) in diethyl ether (5 ml) was further added and the reaction mixture was stirred at that temperature for 1 hour.

[1006] 1H-NMR (CDCl₃, δ (ppm)): 3.95 (3H, s), 7.21-7.22 (2H, m), 7.99-8.01 (1H, m)
After warming gradually to room temperature, ethyl acetate was added, and the mixture was washed with saturated brine. The ethyl acetate layer obtained upon filtration through silica gel was concentrated under reduced pressure. The resulting residue was treated in the same manner as in Example B206 to obtain the title compound (10.1 mg).

**Example B244**

2-(4-Bromo-2-fluorobenzyl)-3-(methoxymethoxy) pyridine

**Example B245**

2-(4-Bromo-2-fluorobenzyl)-3-pyridinol

**Example B246**

2-(4-Bromo-2-fluorobenzyl)-3-methoxypyridine

**Example B247**

2-(4-Bromo-2-fluorobenzyl)-3-ethoxypyridine
Example B248
2-(4-Bromo-2-fluorobenzyl)-3-propoxypyridine

Example B252
2-(4-Bromo-2-fluorobenzyl)-3-(2-fluoroethoxy)pyridine

MS m/z (ESI: MH⁺): 324.0

Example B249
2-(4-Bromo-2-fluorobenzyl)-3-butoxypyridine

Example B253
2-(4-Bromo-2-fluorobenzyl)-3-(3-fluoropropoxy)pyridine

MS m/z (ESI: MH⁺): 328.0

Example B250
2-(4-Bromo-2-fluorobenzyl)-3-(pentyloxy)pyridine

Example B254
2-(4-Bromo-2-fluorobenzyl)-3-isopropoxypyridine

MS m/z (ESI: MH⁺): 342.0

Example B251
2-(4-Bromo-2-fluorobenzyl)-3-(hexyloxy)pyridine

Example B255
2-(4-Bromo-2-fluorobenzyl)-3-(2,2,2-trifluoroethoxy)pyridine

MS m/z (ESI: MH⁺): 324.0

Example B252
2-(4-Bromo-2-fluorobenzyl)-3-propoxypyridine

MS m/z (ESI: MH⁺): 366.0

Example B256
2-(4-Bromo-2-fluorobenzyl)-3-propoxypyridine

MS m/z (ESI: MH⁺): 364.0
Example B256
2-(4-Bromo-2-fluorobenzyl)-3-(3,3,3-trifluoropropoxy)pyridine

Example B257

Compounds were evaluated using the S. cerevisiae reporter system of Example A2. The lowest concentration at which cephalosporinase activity in the cell wall fraction became 50% or less compared to that obtained where the compound was not treated, was defined to be the IC50 value. Effects of the representative compounds are shown in Table 1.

TABLE 1

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INDUSTRIAL APPLICABILITY

The present invention revealed genes encoding the proteins participating in the transport process of the GPI-anchored proteins to the cell wall. Furthermore, this invention discloses a method of screening for compounds that inhibit the activity of these proteins, and also discloses representative compounds having the inhibitory activity.

Using novel compounds, the present invention showed that antifungal agents having a novel mechanism of inhibiting the transport process of the GPI-anchored proteins to the cell wall can be provided.

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act ggt ggc aca att gas gaa att tat gct gta acc agg ata gca tta
Thr Gly Thr Ile Glu Glu Ile Tyr Ala Val Thr Ser Ile Ala Leu
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144
Arg Ile Ser Lys Lys Gln His Lys Lys Glu Leu Leu Leu Leu Phe Phe Ser
340 345 350

gtc gcc act act cag gga tta taa tgg gca tgt atc ttc tat cac tta
355 360 365

Val Ala Thr Thr Gly Leu Tyr Leu Ala Cys Ile Phe Tyr His Leu
1104

gct ttc agt tgt ttc atc agc taa tca ttc ttc tga cca cca att tca
370 375 380

Ala Phe Ser Leu Phe Ile Ser Arg Leu Ser Phe Leu Glu Pro Ile Ser
1152

aga cga tgg gcc aat ttc ccc tac gtc atg tgg gtc gtt tgg tac aat
385 390 395 400

Arg Arg Leu Ala Arg Phe Pro Tyr Val Met Trp Val Val Ser Tyr Ann
1200

gct acg ttt tta taa tgt tat gac tta att gaa aag aat att cgg ggg
405 410 415

Ala Thr Phe Leu Leu Cys Tyr Arg Leu Ile Glu Phe Ile Pro Gly
1248

aac ctt act tct act gta tgg gac tct att aat aac aat ggt tta ttt
420 425 430

Ann Leu Thr Ser Thr Val Leu Arg Ser Ile Arg Met Ser Ile
1296

atc ttc tgg gtc agc aat tta taa aca ggg tgt att aac atg tct atc
435 440 445

Ile Phe Leu Val Ser Leu Leu Thr Gly Phe Ile Arg Met Ser Ile
1344

aac act tgg gaa act aag act aag gca tgt att atc tgg att ggc
450 455 460

Ann Thr Leu Glu Thr Ser Ser Arg Met Ala Val Ile Ile Leu Ile Gly
1392

tat agt ctt act tgg aca tgg gct ttc tta tgt gat aag agg aag
465 470 475 480

Tyr Ser Leu Thr Thr Tyr Thr Leu Ala Leu Tyr Leu Arg Leu Arg Ile
1440

atc tac att aag ctt tag
485

Ile Tyr Ile Lys Leu
1458

<210> SEQ ID NO 4
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Candida albicans
<220> FEATURE: OTHER INFORMATION: CaGMT1
<400> SEQUENCE: 4

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20 25 30

Ser Ser Tyr Ser Leu Ser Phe Arg Leu Leu Lys Ser Leu Gly Asp Leu
35 40 45

Ala Leu Ile Tyr Asp Tyr Ile Leu Asn Val Leu Thr Ile Leu Ala Ser
50 55 60

Ile Thr Val Tyr Ser Asn Ser Pro Ser Tyr Leu His Tyr Phe Ile Val
65 70 75 80

Ile Pro Ser Leu Val Ile Tyr Leu Val Ann Tyr His Val Glu Lys Pro
85 90 95

Ser Ser Pro His Arg Glu Asn Arg Thr Lys Glu Asp Ser Asp Glu
100 105 110

Leu Leu Pro Arg Lys Glu Phe Ile Thr Ala Tyr Arg Ser Glu Met Leu
115 120 125

Ile Ile Thr Asn Leu Ala Ile Ile Ala Val Asp Phe Pro Ile Phe Pro
130 135 140
Arg Arg Phe Ala Lys Val Glu Thr Trp Gly Thr Ser Met Met Asp Leu
145 150 155 160
Gly Val Gly Ser Phe Val Phe Ser Met Gly Leu Ala Asn Ser Arg Gin
165 170 175
Leu Ile Lys Arg His Thr Asp Asn Tyr Lys Phe Ser Trp Lys Ser Tyr
180 185 190
Leu Lys Thr Ile Lys Gln Asn Phe Ile Lys Ser Val Pro Ile Leu Val
195 200 205
Leu Gly Ala Ile Arg Phe Val Ser Val Lys Gin Leu Asp Tyr Gin Glu
210 215 220
His Glu Thr Glu Tyr Gly Ile His Trp Asn Phe Phe Thr Leu Gly
225 230 235 240
Phe Leu Pro Ile Val Leu Gly Ile Leu Asp Pro Val Leu Asn Leu Val
245 250 255
Pro Arg Phe Ile Ile Gly Ile Gln Ser Ile Ala Tyr Gly Val Ala
260 265 270
Leu Asn Lys Thr Gly Leu Leu Lys Phe Ile Leu Ser Ser Glu Asn Arg
275 280 285
Leu Glu Ser Leu Ile Thr Met Asn Lys Gin Gly Ile Phe Ser Phe Ile
290 295 300
Gly Tyr Leu Cys Ile Phe Ile Ile Gly Gin Ser Phe Gly Ser Phe Val
305 310 315 320
Leu Thr Gly Tyr Lys Thr Lys Asn Asn Leu Ile Thr Ile Ser Lys Ile
325 330 335
Arg Ile Ser Lys Lys Thr Gly Leu Leu Lys Leu Leu Phe Phe Ser
340 345 350
Val Ala Thr Thr Gin Gly Leu Tyr Leu Ala Cys Ile Phe Tyr His Leu
355 360 365
Ala Phe Ser Leu Phe Ile Ser Asn Ser Leu Ser Ser Phe Leu Gin Pro Ile Ser
370 375 380
Arg Arg Leu Ala Asn Phe Pro Tyr Val Met Trp Val Val Ser Tyr Ann
385 390 395 400
Ala Thr Phe Leu Leu Cys Tyr Asp Leu Ile Glu Lys Phe Ile Pro Gly
405 410 415
Asn Leu Thr Ser Thr Val Leu Asp Ser Ile Asn Asn Asn Gly Leu Phe
420 425 430
Ile Phe Leu Val Ser Asn Leu Thr Gly Phe Ile Ann Met Ser Ile
435 440 445
Asn Thr Leu Glu Thr Ser Asn Lys Met Ala Val Ile Ile Leu Ile Gly
450 455 460
Tyr Ser Leu Thr Trp Thr Leu Leu Ala Leu Tyr Leu Asp Lys Arg Lys
465 470 475 480
Ile Tyr Ile Lys Leu
485
<210> SEQ ID NO 5
<211> LENGTH: 1458
<212> TYPE: DNA
<213> ORGANISM: Candida albicans
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1) ..(1458)
<223> OTHER INFORMATION: CaGWT1

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<490> SEQUENCE: 5

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acg ggt ggc aca att gaa gaa att tat gtt gta acc agt ata gca tta
Thr Gly Gly Thr Ile Glu Ile Tyr Ala Val Thr Ser Ile Ala Leu
     20     25     30
```

```
tca tct tat tgg tcc ttt aga ttg ttg aaa aag tct ctt ggt gat tta
Ser Ser Tyr Leu Ser Phe Arg Leu Lys Lys Ser Glu Asp Leu
     35     40     45
```

```
gct ttg att tac gac tac att ctt aat tgt ttg aca att cta gca tcc
Ala Leu Ile Tyr Asp Tyr Ile Leu Arg Val Leu Thr Ile Leu Ala Ser
     50     55     60
```

```
att act gtt tat agc aac aac cct tct tat tgg cat tat ttt att gtt
Ile Thr Val Tyr Ser Arg Ser Pro Ser Tyr Tyr Phe Ile Val
     65     70     75     80
```

```
att cca tca tta gtt ats ats cta gtt gat att taa tac cat gtt ggt aas aca
Ile Pro Ser Leu Val Ile Tyr Leu Val Asn Val Tyr His Tyr Glu Pro
     85     90     95
```

```
tct tca ccc cat aga caa aat gat aca caa gaa gat aag tca gcg gaa
Ser Ser Pro His Arg Glu Asn Arg Thr Lys Glu Asp Lys Ser Asp Glu
     100    105    110
```

```
cta ttg ccg aga aaa caa ctt att ats gga ccc tat gct tct cta asg tgg
Leu Leu Asp Arg Lys Ile Thr Ala Tyr Ser Arg Ser Met Leu
     115    120    125
```

```
ata att act ats cta gct ats gtt gat ttt cct att ttc cca
Ile Ile Thr Asp Ala Leu Val Asp Asp Tyr Phe Pro Ile Phe Pro
     130    135    140
```

```
aga aga ttt ggc aaa ctt gaa aca tgg ggc aac tca atg atg gat tta
Arg Arg Phe Ala Lys Val Glu Thr Trp Gly Thr Ser Met Met Asp Leu
     145    150    155    160
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```
gga gtt ggg tgg gtt ttc gcc tct gtt gcc tgg ttg gat att taa cca gca
Gly Gly Val Ser Phe Val Phe Ser Met Leu Ala Asn Arg Gin
     165    170    175
```

```
tgg atc aag cac acc gac aac ats ccc ttt aag tgg aag atg tat
Leu Ile Lys Asp Ser Tyr Tyr Phe Ser Phe Ser Tyr Tyr Lys
     180    185
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tgg aag aca atc aag ccg aac ccc gcc gac aac ttt atg tgg aag atg tat
Leu Lys Thr Ile Arg Phe Val Ser Val Val Lys Gin Leu Asp Tyr Tyr Glu
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```
tta gga got att cgt ttt gtt aag gtt caa ttg gac tat cag gaa
Leu Gly Ala Ile Arg Phe Val Ser Val Lys Gin Leu Asp Tyr Tyr Glu
     210    215    220
```

```
cac gaa aca ggg tgg aat ttt ctc ttc atc gta aat ttt gcc tgg
His Thr Gln Thr Glu Thr Lys His Thr Asp Phe Phe Thr Leu Gly
     225    230    235    240
```

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ttc ttg ccc atc gta tgg gaa ats cca gcc gtt ttg aat tgg ggt
Phe Leu Pro Ile Val Leu Gly Leu Asp Phe Pro Val Leu Leu Met Leu
     245    250    255
```

```
cca cgc tcc atc atc gcc att ggt atg atc cta att gct ttt gat gaa ggc
Pro Arg Phe Ile Gly Ile Gly Asp Ile Tyr Phe Thr Leu Ala Val
     260    265    270
```

```
ttt atc aag act gct ttt gtt aag ttt ccc gcc tgg ttg aat tgg ggt
Leu Asn Lys Thr Gly Leu Leu Leu Leu Asp Ser Gly Arg Gin
     275    280    285
```

```
cgg gaa gcc ttt atc gcc atg ctt ccc gcc gta cgg
Val Glu Gin Trr Glu Gin Ser Tyr Gln Leu
     290    295
```

```
cct gaa ttt gcc atg aat aca cgg att ttt tct gtt ttt att
Leu Glu Ser Leu Ala Met Arg Tyr Glu Gin Phe Ser Phe Ile
     300    305
```

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gga tat ctt tg att ttt ata att ggt cag tct ttt ggg tcg ttt gtt
Gly Tyr Leu Cys Ile Phe Ile Ile Gly Gin Ser Phe Gly Ser Phe Val 305 310 315 320

960

tta cca ggc tac aca aca aag aac tta ata acc att agc aaa att
Leu Thr Gly Tyr Lys Thr Lys Asn Leu Ile Thr Ile Ser Lys Ile 325 330 335

1008

cgt att cta aaa aca cac aag aac gag tgc tgc tgg ttt ttc tca
Arg Ile Ser Lys Gin His Lys Lyu Glu Leu Leu Phe Phe Ser 340 345 350

1056

gtc gcc act act cag gga tta tat tgg gca tgt atc ttc tat cac tta
Val Ala Thr Thr Gin Leu Tyr Leu Ala Cys Ile Phe Tyr His Leu 355 360 365

1104

gtc ttc agt ttc ttc atc aac tta tca ttc tgt caa cca att tca
Ala Phe Ser Leu Phe Ile Ser Am Leu Ser Phe Leu Gin Pro Ile Ser 370 375 380

1152

aga cga tgg gcc aat tcc ccc tac gtc atg tgg gtc gtt tgc tac aat
Arg Arg Leu Ala Am Phe Pro Tyr Val Met Trp Val Val Ser Tyr Am 395 395 400

1200

gct acg ctt tta tta tgt tat gac tta att gaa aaa ttt atc ccc ggg
Ala Thr Phe Leu Phe Gin Tyr Arg Leu Ile Glu Lys Phe Ile Pro Gly 405 410 415

1248

aac ctt act tct act gta tgt gac tct att aat aac aat ggt tta ttt
Asn Leu Thr Ser Thr Val Leu Am Ser Ile Am Asn Gin Leu Phe 420 425 430

1296

atc ttc tgt gtc agc aat tta tca aca ggg ttt att aac atg tcc atc
Ile Phe Leu Phe Ser Am Leu Leu Thr Gin Phe Ile Asn Gin Met Ser Ile 445 445

1344

aac act tgt gaa act agc aat aaa atg gca tgt att atc tgg att ggc
Ann Thr Leu Glu Thr Ser Asn Leu Met Ala Val Ile Gin Lys 450 450 460

1392

tgt tgt ctt act tgt gcc ttc tat tgt gat aag agg aag
Tyr Ser Leu Thr Thr Thr Leu Ala Leu Tyr Asp Lys Arg Lys 465 470 475 480

1440

atc tac atc aag ctt tag
Ile Tyr Ile Lys Leu 485

1458

<210> SEQ ID NO 6
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGASMIC: Candida albicans
<220> FEATURE: 
<223> OTHER INFORMATION: CaGNT1

<400> SEQUENCE: 6

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Ser Ser Tyr Leu Ser Phe Arg Leu Leu Lys Ser Leu Gin Asp Leu 35 40 45
Ala Leu Ile Tyr Asp Tyr Ile Leu Am Val Leu Thr Ile Leu Ala Ser 50 55 60
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<212> TYPE: DNA
<213> ORGANISM: Candida albicans
<220> FEATURE:
<223> OTHER INFORMATION: homologue of S. cerevisiae GNT1

<400> SEQUENCE: 7

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tttatagtctcc tttgtga tgttacat caccctttta ttttaattgg tttaatcc 180
atttctgat cctttcgctc ttatacaac acgtctcttt cttcatatttttctttg 240
attttcatct tagtattcatt tttgtgcatt attcatctttt gcacacacttc ttttcccttttcc 300
agaacatcag tatactcagact gcgaaagtaa agcagggac acatatattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

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<211> SEQ ID NO 10
<212> LENGTH: 33
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

<400> SEQUENCE: 10

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<211> SEQ ID NO 11
<212> LENGTH: 32
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

<400> SEQUENCE: 11

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<211> SEQ ID NO 12
<212> LENGTH: 49
<213> ORGANISM: Artificial Sequence
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

<400> SEQUENCE: 12

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<211> SEQ ID NO 13
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

<400> SEQUENCE: 13

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<211> SEQ ID NO 14
<212> LENGTH: 33
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer

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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer  
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<212> TYPE: DNA  
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<210> SEQ ID NO 19  
<211> LENGTH: 32  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer  
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<210> SEQ ID NO 20  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
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Cys Phe Thr Ala Gly Thr Asn Thr Val Thr Phe Asn Asp Gly Asp Lys
1  5  10  15
Amp Ile

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

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<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

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<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

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<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

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Met Ser Tyr Lys Leu Glu Lys Ala Phe Val Ser Arg Leu Thr Gly
1 5 10 15

tca aat tcc att gac aca tgt ggc ttg tta tta ata gga att gct tgc
Ser Ser Ser Ile Thr Cys Gly Leu Leu Leu Ile Gly Ile Ala Cys
20 25 30

aac gtt ttg tgg gta aac atg act ggc aga aac atc tta ccc aaa ggg
Amm Val Leu Thr Val Arg Thr Ala Arg Arg Ile Leu Pro Gly
35 40 45

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Phe Val Ile Ser Ser Val Ser Lys Val Phe Thr Leu Cys Ile
65 70 75 80

gcc tct ttt tgt ctt ctc ttc gcc tgt atc gtt ata aat cca att aat
Ala Ser Phe Leu Pro Pro Val Val His Val Ile Ser Pro Ile
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Trp Asp Val Leu Arg Arg Pro Arg Arg Ile Leu Phe Thr Lys Arg
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gaa aat act ttt gat cga cga att gtt gga gtc cca ttt tat ctt tct
Glu Thr Thr Asp Arg Ala Ala Gly Val Thr Phe Thr Arg Ser
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caa atg atg tgg gtt act gtt atc gtc act tgg gcc gtc gtt ggc
Gln Met Met Leu Val Thr Thr Thr Cys Ile Leu Ala Val Asp Phe Thr
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Leu Phe Pro Arg Tyr Ala Lys Val Glu Thr Thr Gly Thr Ser Leu
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Met Asp Leu Gly Val Gly Ser Phe Ser Ser Gly Thr Val Ala
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gga cgg aas eat gag atc att aaa aca eat gcy ttt aas eat gta ttg
Gly Arg Lys Asp Ile Lys Lys Pro Arg Ala Phe Lys Arg Val Leu
180 185 190

tgg gaa cag gtc aag aat ttc ctt ttg aat ttc gga ttg gcc gcc aat tta
Trp Asp Ser Val Leu Leu Ile Leu Gly Phe Ala Arg Met Phe Leu
195 200 205

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Thr Lys Ser Ile Asn Tyr Gin Glu His Val Ser Gly Tyr Gly Met His
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<223> OTHER INFORMATION: n = g, a, c or t
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<210> SEQ ID NO 31
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<213> ORGANISM: Aspergillus fumigatus
<220> FEATURE:
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Val His Arg Thr Thr Thr Glu Ser Glu Ser Glu Arg Val Asp Phe
420  425  430
Ala Thr Ser Arg Ile Met Ser Ala Phe Asn Lys Asn Ser Leu Ala Ile
435  440  445
Phe Leu Leu Ala Asn Leu Leu Thr Gly Ala Val Asn Leu Ser Ile Ser
450  455  460
Thr Ile Asp Ala Ann Thr Ala Glu Ala Ile Val Leu Ile Gly Tyr
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Ser Ser Ile Ile Thr Gly Val Ala Leu Ala Leu His His Ala Asn Ile
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Lys Val Leu Pro Phe
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<210> SEQ ID NO 41
<211> LENGTH: 1648
<212> TYPE: DNA
<213> ORGANISM: Aspergillus fumigatus
<220> FEATURE:
<222> OTHER INFORMATION: cloned homologue of S. cerevisiae GWT1 from genomic library
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: 26..121, 189..608)
<220> FEATURE:
<221> NAME/KEY: exon
<222> LOCATION: (26)..<(121)
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<221> NAME/KEY: intron
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<220> FEATURE:
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<222> LOCATION: (199)..<(608)

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          1   5

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       Glu Ala Phe Val Ser Gly Leu Ala Gly Gly Ser Ile Leu Glu Ile Ann
          10  15  20  25

gcc gtc acc tgt gtt gct tgt ctt gtctggtta ctatctatt gttgctactt 151
       Ala Thr Leu Val Ser Al A Ser
             30

cgctaaatt gtttctgagc taaacagtc ttcttgcat caactcg gta tcc gtt 207
       Val Ser Val
             35

ttt ctc tgg tca att cta cca aaa tct cgg cta tcc ttt ttc aca ccc tac 255
       Phe Leu Trp Ser Ile Leu Gln Ser Arg Leu Ser Phe Phe Thr Pro Tyr
             40  45  50

gac gcc got gcc ctt ctc gtt gat ttc ctt ctc aat gta cta gct atc 303
       Ser Ala Ala Leu Leu Val Asp Phe Leu Leu Asn Val Leu Ala Ile
             55  60  65

ttg tcc gca acc act tta tac tct ctc tgt ctc gct ctt ctc atc aat tct 351
       Leu Phe Ala Thr Thr Leu Tyr Ser Ser Ala Pro Leu Leu Leu Asn Leu
             70  75  80
-continued

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  aaa gag gac tct aac gac aac gcc ttc cta gag ttc 495
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cat ccc ccc gtc aac ggc aac aag cag ctc 543
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ttc ctc ttc ggc gga gta ttc gct ccc ctc ata aag aac 687
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  arg ggt gct ccc aag gtc ttc cct ctt cgc aag tgg ttc 735
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tgc agc cag ctc atc ctt cgg cgg ctc ctc cgc cgg 793
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tac gtc aca gtc tat gac ggc gac gac tac gcc 831
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gta cat tgg aac ttc ttc ttc aca ctt cgc ggt gct 879
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tcc gtt ggg atc gcc gtc tgt cgg ctc gca gtc 975
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ggc gtt cca gcc tca tgt gtt ggc gcc tcc act 1215
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tat ggc atg gga tac ggt atc ctt gtc tcc cgc cgc ctc gct 1263
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  385
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aac atg ccc tat gtc ctt tgg gtg gtc ggg ttc aac acc ggc gaa cta
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ttt gtt ttc tgc tgg atc gaa aca ttc tgc ttc ctc gca gtt cat gc
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Phe Val Phe Cys Leu Ile Glu Thr Leu Cys Phe Pro Ala Val His Arg

aca acg act cca gaa gac gaa tct gag cga gtc gat ttc gtt acg gc
420 425 430 435
Thr Thr Thr Gln Glu Ser Glu Ser Glu Arg Val Asp Phe Ala Thr Ser

cga ttc atg tgg gcc ttc aat aag aac act ctc ggg tgc atc ttt cttgg
440 445 450
Arg Ile Met Ser Ala Phe Asn Lys Asn Ser Leu Ala Ile Phe Leu Leu

gcc aat ctt ctc act gga ggt gtt gta atg aag atc tcc aca at gat
455 460 465
Ala Asn Leu Leu Thr Gly Ala Val Asn Leu Ser Ile Ser Thr Ile Asp

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470 475 480
Ala Asn Thr Gln Ala Ile Ala Val Leu Ile Gly Tyr Ser Ser Ile

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485 490 495
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Pro Phe

<210> SEQ ID NO 42
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

<400> SEQUENCE: 42
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<210> SEQ ID NO 43
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:PCR amplification primer

<400> SEQUENCE: 43
cattaaaccc ccattgaca accacg
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<210> SEQ ID NO 44
<211> LENGTH: 169
<212> TYPE: DNA
<213> ORGANISM: Cryptococcus neoformans
<220> FEATURE:
<223> OTHER INFORMATION: amplified fragment showing homology to S. cerevisiae GAT1

<400> SEQUENCE: 44
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cattggaga cgtttatata tssggtatgc aggccatctg tcgtctcttg agtcgcttat
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gatcaccatt tttcagctgt acgcagctgc acatgttgtc atgactctgt acgatcctct 780
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tgatcagcct gcagcaggtgc actgtttagt ggtggtgggc acctagggattg gcctagtgattt 1560
ggcggggagt ggcagttacgc agggggttag taaatccgaca ctctttgtata tatttaccc 1620
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aaacacacca atcagctgtaa tcgctctctc tgcgtggtggct tataaaaaa acgycttgttgc 1800
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atgccgaggg 1869

<210> SEQ ID NO 45
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: OTHER INFORMATION: Description of Artificial Sequence: PCR amplification primer

<400> SEQUENCE: 45
gtaaaggaag gcgctagaaaa agatat 27

<210> SEQ ID NO 46
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
FEATURE:
DESCRIPTION: Description of Artificial Sequence: PCR amplification primer

SEQUENCE: 46
ctcatcggag tcgtatggtctagcc 26

SEQ ID NO: 47
LENGTH: 470
TYPE: DNA
ORGANISM: Cryptococcus neoformans
FEATURE:
DESCRIPTION: amplified fragment over lapping SEQ ID NO: 44

SEQUENCE: 47
gaagggccta gaaagatag ggtcttggca tagcattaaa tccgccgcat aataagctac 60
tgaattgca tgggggatta caagtgccc aagaggcct ttgctcggga taacccaggt 120
gccttcatct gcagatgacaa cgytgccac tctgtcggac ctgatgtgtag ctggtctccc 180
gaggggttct gcattttgga gacggctatt aatggggct gcagggcaca tagcctctct 240
ggatggcct atgcggctac atcgcggcag gactctgga caaactacctg atctgtggttc 300
ttccctatt atggggctg acaatctcttc caaetttgcct ctcctgtattt aacctttttt 360
tggtcatttt ttcctctctg ttcatacgga aatccaaaa atcgcctcaa aatcgtcagtt 420
cgccagaaaa gccaaagggg cattggctag ccgaatcagca ctcgatgag 470

SEQ ID NO: 48
LENGTH: 37
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
DESCRIPTION: Description of Artificial Sequence: 3' - RACE adaptor-primer

SEQUENCE: 48
ggccacgctg cgactagtac ttttttttt tttttttt 37

SEQ ID NO: 49
LENGTH: 29
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
DESCRIPTION: Description of Artificial Sequence: PCR amplification primer

SEQUENCE: 49
catcttggcg ttagattttg aagttgctcc 29

SEQ ID NO: 50
LENGTH: 20
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
DESCRIPTION: Description of Artificial Sequence: PCR amplification primer

SEQUENCE: 50
ggccacgctg cgactagtac 20

SEQ ID NO: 51
LENGTH: 1136
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```<212> TYPE: DNA
<213> ORGANISM: Cryptococcus neoformans
<220> FEATURE:
<223> OTHER INFORMATION: amplified fragment showing homology to S. cerevisiae GW1

<400> SEQUENCE: 51

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etttctccct cacttcaccc tcttacgcce ttctegcccg ctctcaacct tcaatctatt 180
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tctgccctgg cgttataatc gttgattatg gctgaggagt ctgattatcc tctgacatgtg 300
agacagatcc cggctgacgt gaaatttttc tctacccctgc cattgctgctcc tctgtgtgcgc 360
gtgggacact caacagcggcg gtatgggact tggctgaggt tggcttgaggtaatc attatattct 420
tttggtcct acgtctgttggtt acatattatat ccccaacccca cgccttctttt attgggctgc 480
tcaggatct ttctagaaaa caaggaaggg tcttccccct tctgctggta tttccctat 540

<210> SEQ ID NO: 52
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: PCR amplification primer

<400> SEQUENCE: 52

gtctgtgcat aagattaaat ccocgcgc

<210> SEQ ID NO: 53
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: PCR amplification primer

<400> SEQUENCE: 53

gaacccagat atcacttacgc ctgctaggg

<210> SEQ ID NO: 54
<211> LENGTH: 2045
<212> TYPE: DNA
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ORGANISM: Cryptococcus neoformans
OTHER INFORMATION: full length homologue of S. cerevisiae GNT1
LOCATION: join(44..136,159..891,943..1635,1687..2004)
OTHER INFORMATION: homologue of S. cerevisiae GNT1
FEATURE: exon
LOCATION: (44) (136)
NAME: exon
LOCATION: (137) (198)
NAME: exon
LOCATION: (199) (891)
NAME: inton
LOCATION: (892) (942)
NAME: exon
LOCATION: (943) (1635)
NAME: inton
LOCATION: (1636) (1696)
NAME: exon
LOCATION: (1687) (2004)

SEQUENCE: 54

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gtaaatctgtagcgttgg gat tag gcc aac gag gct ttt gtc
gtaaatctgtagcgttgg gat tag gcc aac gag gct ttt gtc
gtaaatctgtagcgttgg gat tag gcc aac gag gct ttt gtc
gtaaatctgtagcgttgg gat tag gcc aac gag gct ttt gtc

Lyser Ala Lys Glu Ala Phe Val Ser Asp Arg His Gly Leu Leu Asn
Leu Thr Ile Ala Leu Ser Pro Tyr Ile Arg His Gly Leu Leu Asn
Leu Thr Ile Ala Leu Ser Pro Tyr Ile Arg His Gly Leu Leu Asn
Leu Thr Ile Ala Leu Ser Pro Tyr Ile Arg His Gly Leu Leu Asn
Leu Thr Ile Ala Leu Ser Pro Tyr Ile Arg His Gly Leu Leu Asn
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55
103
156
210
258
306
354
402
450
498
546
594
Asp Pro Thr Thr Ser Pro Met Ser Pro Ser Ser Ser Ser Ala Ser Gly 150 155 160

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His Glu Asp Pro Leu Gly Ile Met Gly Val Asn Arg Arg Arg Ser Leu
165 170 175

tta gaa gga gtt ctc gat gtt ccc tca cat atc gac tcc aag gtc 690
Leu Glu Gly Val Ser Leu Asp Pro Ser His Ile Asp Ser Lys Val
180 185 190 195

aga ata tct cct gct ccc tac tgg agg ctc aaa aag tct agy gca acg 738
Arg Ile Pro Val Pro Tyr Leu Arg Leu Lys Lys Ser Arg Ala Thr
200 205 210

aag gcc caa tgg ggg aaa gaa aag gta taa cca ttt tgg aca gta 786
Lys Ala Gln Thr Val Lys Glu Arg Leu Pro Phe Leu Thr Val
215 220 225

tac cga gcc cac atg atg ctc atg act gtt aat atc tgc atc tgg gcc gta 834
Tyr Arg Ala His Met Met Ser Thr Leu Thr Val Ile Cys Ile Leu Ala Val
230 235 240

gat ttt gaa gtc ctc cct aag tga cag gcc aag tgc gaa gat ttt ggt 882
Asp Phe Glu Val Phe Pro Arg Thr Glu Asp Lys Arg Phe Gly
245 250 255

act act gtt gtaagttc cttcagcct ggtccagtg ctcaccgct Thr Ser Leu 931
260

actggcctg a atg gac gtt ggt gtt gtt ctc ttc gtc gtt ccc tcc gct 981
Met Asp Val Val Val Gly Val Ser Pro Val Phe Ser Leu Gly
265 270 275

cct gto ctc cca aca cct ctt ctt ctt ctt ctc aat ctc aag ccc 1029
Leu Val Ser Thr Leu Ser Leu Ser Pro Pro Pro Pro Thr Pro
280 285 290

tcc gtc ccc gct ctc aac tct ctc att ctc atc aag ctc ccc 1077
Ser Ser Pro Ala Leu Asn Ser Ser His Ile Ile Pro Leu Thr Pro Ser Pro
295 300 305

ttc act ctc atc ctc ctc aat ctc ccc atc ctc ctc aat ctc aag ctc 1125
Phe Thr Ser Ile Leu Leu Leu Arg Ser Ser Ile Ile Pro Ile Leu Val
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Arg Thr Ser Val Leu Val Ile Ile Ser Leu His Glu Trp
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Leu Thr Thr Tyr Leu Glu Ser Ile Pro Phe Ser Phe Arg Arg Gly
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Ile Phe Leu Asn Lys Glu Gly Phe Ser Ser Leu Pro Gly Thr Leu
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**<213> ORGANISM:** Artificial Sequence

**<220> FEATURE:**
**<223> OTHER INFORMATION:** Description of Artificial Sequence: PCR amplification primer predicted to be junction between exons

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**<210> SEQ ID NO 56**
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**<212> TYPE:** DNA
**<213> ORGANISM:** Artificial Sequence

**<220> FEATURE:**
**<223> OTHER INFORMATION:** Description of Artificial Sequence: PCR amplification primer predicted to be junction between exons

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<213> ORGANISM: Cryptococcus neoformans
<220> FEATURE:
<223> OTHER INFORMATION: amplified fragment showing homology to S. cerevisiae GNT1

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tccactctcg cctctcgtgt taactccttt ttgtctccatt atttccctcg ctctccacc 180
gaaatccaa aacggtcttc aacggtcgtg tgaacctgca aagccaaaag gcacagctgtt 240
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<210> SEQ ID NO: 58
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<213> ORGANISM: Cryptococcus neoformans
<220> FEATURE:
<223> OTHER INFORMATION: homologue of S. cerevisiae GNT1

<400> SEQUENCE: 58

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<213> ORGANISM: Cryptococcus neoformans
<220> FEATURE:
OTHER INFORMATION: homologue of S. cerevisiae GWT1

SEQUENCE: 59

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Thr Tyr Ala Leu Trp Ile Ala Leu Ser Pro Tyr Ile Arg His Gly Leu
35 40 45

Leu Asn Asn Tyr Leu Ile Cys Val Leu Pro Leu Leu Phe Gly Val Thr
50 55 60

Ile Phe Ser Thr Ser Pro Leu Val Phe Thr Ser Phe Leu Ser Ile Ile
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Ser Leu Ala Phe Ile Thr Lys Ser Gln Lys Cys Phe Lys Ser Val Ser
85 90

Ser Pro Glu Lys Pro Lys Gly Gln Trp Leu Asp Glu Ser Asp Ser Asp
100 105 110

Glu Glu Pro Ala Glu Pro Ala Ser Ala Ala Gly Ser Ala Ala Val Ser
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Pro Val Lys Leu Leu Pro Ser Gln Val Ala Phe Ala Ser Gly Ser Leu
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145 150 155 160

Ala Ser Gly His Glu Asp Pro Leu Gly Ile Met Gly Val Asn Arg Arg
165 170 175

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180 185 190

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Pro Ser Pro Phe Thr Ser Ile Leu Ile Ser Arg Lys Ser Ile Pro
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Ile Leu Val Leu Gly Phe Ile Arg Leu Ile Met Val Lys Gly Ser Asp
325 330 335

Tyr Pro Glu His Val Thr Glu Tyr Gly Val His Trp Asn Phe Phe Phe
340 345 350

Thr Leu Ala Leu Val Pro Val Ala Val Gly Ile Arg Pro Leu Thr
355 360 365

Gln Trp Leu Arg Trp Ser Val Leu Gly Val Ile Ile Ser Leu Leu His
370 375 380
Gln Leu Trp Leu Thr Tyr Tyr Leu Gln Ser Ile Val Phe Ser Phe Gly
385 390 395 400
Arg Ser Gly Ile Phe Leu Ala Asn Lys Glu Gly Phe Ser Ser Leu Pro
405 410 415
Gly Tyr Leu Ser Ile Phe Leu Ile Gly Leu Ser Ile Gly Asp His Val
420 425 430
Leu Arg Leu Ser Leu Pro Pro Arg Glu Arg Val Val Ser Glu Thr
435 440 445
Asn Glu Glu His Glu Gln Ser His Phe Glu Arg Lys Leu Asp Leu
450 455 460
Ile Met Glu Leu Ile Gly Tyr Ser Leu Gly Trp Trp Ala Leu Gly
465 470 475 480
Gly Trp Ile Trp Ala Gly Gly Glu Val Ser Arg Arg Leu Ala Asn Ala
485 490 495
Pro Tyr Val Phe Trp Val Ala Ala Tyr Thr Thr Phe Leu Leu Gly
500 505 510
Tyr Leu Leu Leu Thr His Ile Ile Pro Ser Pro Thr Ser Ser Gln Thr
515 520 525
Ser Pro Ser Ile Leu Val Pro Pro Leu Leu Asp Ala Met Asn Lys Ann
530 535 540
Gly Leu Ala Ile Phe Leu Ala Asn Leu Leu Thr Gly Leu Val Ann
545 550 555 560
Val Ser Met Lys Thr Met Tyr Ala Pro Ala Trp Leu Ser Met Gly Val
565 570 575
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<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<222> OTHER INFORMATION: Description of Artificial Sequence: R1 and R5 mutant GNTI gene primer

<400> SEQUENCE: 60
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<220> FEATURE:
OTHER INFORMATION: highly conserved GWI1 P-domain

SEQ ID NO 64
LENGTH: 31
TYPE: PRT
ORGANISM: Candida albicans
FEATURE:
OTHER INFORMATION: highly conserved GWI1 P-domain

SEQ ID NO 65
LENGTH: 31
TYPE: PRT
ORGANISM: Candida albicans
FEATURE:
OTHER INFORMATION: highly conserved GWI1 P-domain

SEQ ID NO 66
LENGTH: 31
TYPE: PRT
ORGANISM: Schizosaccharomyces pombe
FEATURE:
OTHER INFORMATION: highly conserved GWI1 P-domain

SEQ ID NO 67
LENGTH: 17
TYPE: PRT
ORGANISM: Saccharomyces cerevisiae
FEATURE:
OTHER INFORMATION: highly conserved GWI1 R-domain

SEQ ID NO 68
LENGTH: 17
TYPE: PRT
ORGANISM: Candida albicans
FEATURE:
OTHER INFORMATION: highly conserved GWI1 R-domain
What is claimed is:

1. A fungus comprising a DNA that encodes a protein having an activity to confer resistance of the fungus against the compound shown in formula (la) when the gene product of the DNA is overexpressed in the fungus, wherein the DNA is selected from the group consisting of:
   (a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NOs: 2, 4, 6, 28, 40 or 59,
   (b) a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58,
   (c) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are (i) 4×SSC at 65°C, then washing in 0.1×SSC for 1 hour at 65°C,
   (d) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are 4×SSC at 42°C in 50% formamide,
   (e) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are PerfectHyb™ (TOYOBO) solution for 2.5 hours at 65°C, then washing in (i) 2×SSC, 0.05% SDS solution at 25°C for 5 minutes, (ii) 2×SSC, 0.05% SDS solution at 25°C for 15 minutes, and (iii) 0.1×SSC, 0.1% SDS solution at 50°C for 20 minutes, and,
   (f) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NOs: 2, 4, 6, 28, 40 or 59, wherein one or more amino acids have been added, deleted, substituted, and/or inserted

2. A fungus comprising a DNA that encodes a protein having an activity to decrease the amount of a glycosylphosphatidylinositol (GPI)-anchored protein in the cell wall of the fungus, wherein the DNA is selected from the group consisting of:
   (a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NOs: 2, 4, 6, 28, 40 or 59,
   (b) a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58,
   (c) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are (i) 4×SSC at 65°C, then washing in 0.1×SSC for 1 hour at 65°C,
   (d) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are 4×SSC at 42°C in 50% formamide,
   (e) a DNA that hybridizes under stringent conditions to a DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 27, 39, 41, 54 or 58, wherein said stringent conditions are PerfectHyb™ (TOYOBO) solution for 2.5 hours at 65°C, then washing in (i) 2×SSC, 0.05% SDS solution at 25°C for 5 minutes, (ii) 2×SSC, 0.05% SDS solution at 25°C for 15 minutes, and (iii) 0.1×SSC, 0.1% SDS solution at 50°C for 20 minutes, and,
   (f) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NOs: 2, 4, 6, 28, 40 or 59, wherein one or more amino acids have been added, deleted, substituted, and/or inserted.

* * * * *