DESCRIPTION

NOVEL STEVIOL GLYCOSIDE AND PRODUCTION METHOD THEREFOR, AND SWEETENER COMPOSITION CONTAINING SAME

TECHNICAL FIELD

[0001] The present invention relates to a novel steviol glycoside, a method for producing the same, and a sweetener composition containing the same. Furthermore, the present invention also relates to a food or beverage, a plant, an extract thereof and a flavor controlling agent containing the novel steviol glycoside.

BACKGROUND ART

[0002] Leaves of *Stevia rebaudiana* contain a secondary metabolite called Steviol which is one type of diterpenoids, where steviol glycoside provides sweetness that is nearly 300 times the sweetness of sugar and is therefore utilized as a calorieless sweetener in the food industry. The demand for calorieless sweeteners is growing day by day as obesity has become a serious social problem worldwide and also for the sake of health promotion and reduction in the medical expenditure. Currently, aspartame and acesulfame potassium, which are artificially synthesized amino acid derivatives, are utilized as artificial sweeteners, but natural calorieless sweeteners like the steviol glycosides are expected to be safer and more likely to gain public acceptance.

[0003] The major steviol glycosides from stevia are ultimately glycosylated to a glycoside named rebaudioside A (Reb.A) that has four sugar moieties (Figure 1). Stevioside, namely, a tri-glycosylated steviol glycoside and a precursor of Reb.A, is the most abundant glycoside. These two glycosides are the main substances responsible for the sweetness of stevia. Stevioside accounts for the largest content in stevia leaves and is known to provide sweetness that is about 250-300 times the sweetness of sugar. Reb.A is a tetra-glycosylated steviol glycoside that has strong sweetness (350-450 times sugar) with good taste quality. They have been drawing attention as calorieless sweeteners. Besides them, existence of glycosides that are considered to be reaction intermediates and analogs having different types of sugar moieties are known. For example, while all of the four glycoside sugar moieties of Reb.A are glucose, rebaudioside C (Reb.C) is known to have rhamnose instead of glucose attached to C-2 of glucose at C-13, and rebaudioside F (Reb.F) is known to have xylose attached at the same position.

[0004] To date, attempts have been made to obtain a stevia plant having a higher Reb.A

content than wild-type stevia plants by breeding or the like since taste quality of Reb.A, in which all of the four glycoside sugar moieties are glucose, is good (for example, Patent document 1).

PRIOR ART DOCUMENT

Patent document

[0005] Patent document 1: Japanese Patent No. 3436317

SUMMARY OF INVENTION

Problem to be Solved by the Invention

[0006] Meanwhile, some of the stevia cultivers resulting from breeding may contain a minute amount of a steviol glycoside whose structure is not yet identified, where the presence of such steviol glycoside present in minute quantity may potentially be contributing to the flavor characteristic of the stevia extract. Moreover, although researches have been made thus far on steviol glycosides obtained by further attaching glucose to Reb.A and on cultivers containing the same, not much research has been made at this point on a cultiver containing an abundant amount of a steviol glycoside having rhamnose like Reb.C and on such a steviol glycoside.

[0007] Accordingly, the objective of the present invention is to determine the structure of a novel steviol glycoside present in minute quantity that affects the taste quality, and to identify the characteristics of its taste quality. In addition, further objectives of the present invention are to provide a novel steviol glycoside, a method for producing the same, and a sweetener composition containing the same.

Means for Solving the Problem

[0008] The present inventors have gone through extensive investigation to solve the above-described problem, and as a result of which succeeded in determining the structure of the novel steviol glycoside present in minute quantity that affects the taste quality. The present invention was made based on the above-described finding.

EFFECT OF THE INVENTION

[0009] The present invention can provide a novel steviol glycoside present in minute quantity that affects the taste quality. Furthermore, the present invention can also provide a method for producing the novel steviol glycoside, and a sweetener composition, a food or beverage, a plant, an extract thereof and a flavor controlling agent containing the novel steviol glycoside.

BRIEF DESCRIPTION OF DRAWINGS

[0010] Figure 1 illustrates a diagram showing structures and names of steviol glycosides.

Figure 2 illustrates a diagram showing a selected ion chromatogram of Sample 1 at m/z of 1095.4.

Figure 3 illustrates a diagram showing a selected ion chromatogram of Sample 1 at m/z of 1257.5.

Figure 4 illustrates a diagram showing MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 1.

Figure 5 illustrates a diagram showing MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 2.

Figure 6 illustrates (a) a diagram showing a ^{1}H-NMR spectrum of Compound 15 (800 MHz, Pyr-d5); and (b) a diagram showing a ^{13}C-NMR spectrum of Compound 15 (200 MHz, Pyr-d5).

Figure 7 illustrates (a) a diagram showing a ^{1}H-^{1}H cosy spectrum of Compound 15 (800 MHz, Pyr-d5); and (b) a diagram showing a HSQC spectrum of Compound 15 (800 MHz, Pyr-d5).

Figure 8 illustrates (a) a diagram showing a HMBC spectrum of Compound 15 (800 MHz, Pyr-d5); and (b) a diagram showing a TOCSY spectrum of Compound 15 (800 MHz, Pyr-d5).

Figure 9 illustrates a diagram showing a NOESY spectrum of Compound 15 (800 MHz, Pyr-d5).

Figure 10 illustrates (a) a diagram showing a ^{1}H-NMR spectrum of Compound 17 (800 MHz, Pyr-d5); and (b) a diagram showing a ^{13}C-NMR spectrum of Compound 17 (200 MHz, Pyr-d5).

Figure 11 illustrates (a) a diagram showing a ^{1}H-^{1}H cosy spectrum of Compound 17 (800 MHz, Pyr-d5); and (b) a HSQC spectrum of Compound 17 (800 MHz, Pyr-d5).

Figure 12 illustrates (a) a diagram showing a HMBC spectrum of Compound 17 (800 MHz, Pyr-d5); and (b) a diagram showing a TOCSY spectrum of Compound 17 (800 MHz, Pyr-d5).

Figure 13 illustrates a diagram showing a NOESY spectrum of Compound 17 (800 MHz, Pyr-d5).

Figure 14 illustrates a diagram showing extracted ion chromatograms of Novel steviol glycoside 1 (stevia leaf extract) and a chemically synthesized product (the β-form of Compound 15).

Figure 15 illustrates Diagrams showing MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 1 (stevia leaf extract) and a chemically synthesized product (the β-form of Compound 15).

Figure 16 illustrates a diagram showing extracted ion chromatograms of Novel steviol glycoside 2 (stevia leaf extract) and a chemically synthesized product (the β-form of Compound 17).

Figure 17 illustrates Diagrams showing MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 2 (stevia leaf extract) and a chemically synthesized product (the β-form of Compound 17).

Figure 18 illustrates a diagram showing a selected ion chromatogram of a sample obtained by biosynthesis at m/z of 1095.4.

Figure 19 illustrates a diagram showing a selected ion chromatogram of a sample obtained by biosynthesis at m/z of 1257.5.

Figure 20 illustrates Diagrams showing results of sensory evaluations for comparison of the novel steviol glycoside with rebaudioside A and rebaudioside D.

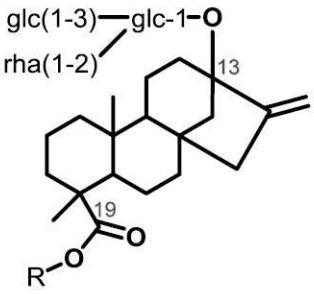
MODES FOR CARRYING OUT THE INVENTION

[0011] Hereinafter, the present invention will be described in detail. The following embodiment is provided for illustrating the present invention and not with the intention of limiting the present invention solely to this embodiment. The present invention may be carried out in various modes without departing from the scope thereof. All of the documents, publications, patent publications and other patent documents cited herein are incorporated herein by reference.

[0012] The terms "rebaudioside" and "Reb." as used herein have the same meaning and both refer to "rebaudioside". Similarly, the terms "dulcoside" and "dulcoside" as used herein have the same meaning and both refer to "dulcoside".

[0013] 1. Novel steviol glycoside

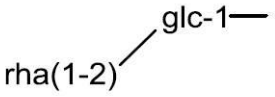
For the first time, the present inventors identified the structure of a minute amount of a novel steviol glycoside that affects taste quality. The novel steviol glycoside of the present invention (hereinafter, also referred to as the "glycoside of the present invention") is a compound represented by Formula (1):



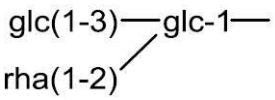
(1)

or a derivative, a salt or a hydrate thereof,

wherein R represents a sugar chain of Formula (2) or (3); and



(2)



(3)

glc represents glucose, and rha represents rhamnose.

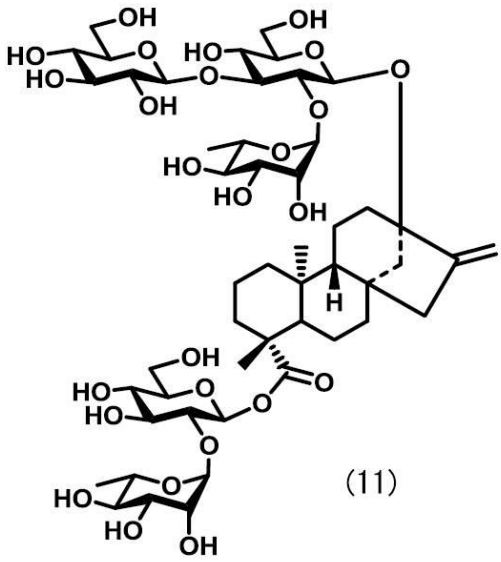
[0014] As represented above, the glycoside of the present invention has a sugar chain containing two glucose moieties and one rhamnose moiety at C-13 of steviol, and a sugar chain containing one glucose moiety and one rhamnose moiety, or two glucose moieties and one rhamnose moiety at C-19.

[0015] Furthermore, as represented above, glc represents glucose and rha represents rhamnose. "Glc" as used herein may be α- or β-glucose while rha may be α- or β-rhamnose. Alternatively, "glc" as used herein may be α- and β-glucose while rha may be α- and β-rhamnose. Moreover, "glc-1-" indicates that the carbon atom at C-1 of glucose is joined to steviol via a glycosidic bond, and "glc(1-3)-glc-1-" indicates that the carbon atom at C-3 of glucose represented by "glc-1-" is joined to a carbon atom at C-1 of another glucose via a glycosidic bond. Furthermore, "rha(1-2)-glc-1-" indicates that the carbon atom at C-2 of glucose represented by "glc-1-" is joined to a carbon atom at C-1 of rhamnose via a glycosidic bond.

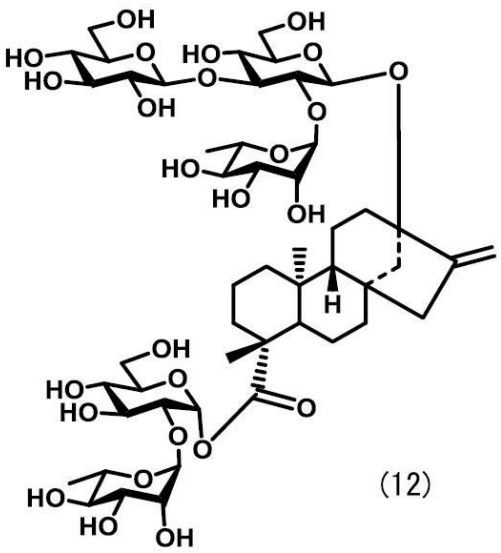
[0016] Herein, among the compounds represented by Formula (1), those in which R has a sugar chain of Formula (2) are referred to as "Glycoside A" while those in which R has a sugar chain of Formula (3) are referred to as "Glycoside B".

[0017] Examples of Glycoside A include glycosides having the structures represented by

Formulae (11) and (12).



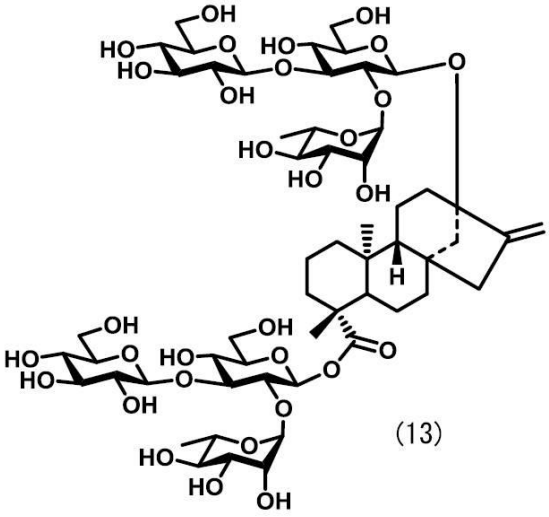
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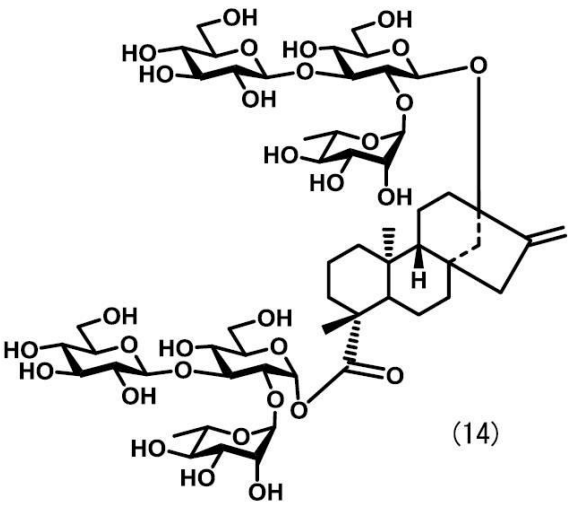
(12)

In Glycoside A represented by Formula (11), glucose is joined to the carboxylic group at C-19 of steviol via a β-glycosidic bond, whereas in Glycoside A represented by Formula (12), glucose is joined to the carboxylic group at C-19 of steviol via an α-glycosidic bond.

[0018] Examples of Glycoside B include glycosides having the structures represented by Formulae (13) and (14).



(13)



(14)

In Glycoside B represented by Formula (13), glucose is joined to the carboxylic group at C-19 of steviol via a β-glycosidic bond, whereas in Glycoside B represented by Formula (14), glucose is joined to the carboxylic group at C-19 of steviol via an α-glycosidic bond.

[0019] The glycoside of the present invention also comprises isomers such as the α- and β-forms as described above. Therefore, the glycoside of the present invention may comprise only those of the α-form, only those of the β-form or a mixture of the α- and β-forms. The glycoside of the present invention has the β-form in a proportion of preferably 80% or more, more preferably 90% or more, still more preferably 95% or more, and particularly preferably 99% or more. The α- and β-forms can be isolated/purified by a known method such as high-performance liquid chromatography (HPLC), ultra (high) performance liquid chromatography (UPLC), or the like.

[0020] The glycoside of the present invention may not only be the compound represented by Formula (1) but may also be a derivative, a salt or a hydrate thereof. The term "derivative" as used herein refers to a compound resulting from a structural change of a minor moiety of the compound, for example, a compound in which some of the hydroxyl groups are substituted with other substituents. Therefore, derivatives of the compound represented by Formula (1) include compounds in which some of the hydroxyl groups contained in the compound have been substituted with a substituent selected from hydrogen, a halogen, an alkyl group, an alkenyl group, an alkynyl group, an aryl group, an amino group, a cyano group or the like. As used herein, a "salt of the compound represented by Formula (1)" refers to a physiologically acceptable salt, for example, a sodium salt, of the compound represented by Formula (1). Furthermore, a "hydrate of the compound represented by Formula (1)" as used herein refers to a compound resulting from addition of a water molecule to the compound represented by Formula (1).

[0021] While the glycoside of the present invention is not particularly limited, it may be a plant-derived product, a chemically synthesized product or a biosynthetic product. For example, it may be isolated and purified from a plant with abundant Reb.C, or it may be obtained by chemical synthesis or biosynthesis. Details of a method for producing a glycoside of the present invention will be described later herein.

[0022] The glycoside of the present invention is sweeter than sugar (sucrose), has taste quality such as early sweetness onset and lingering aftertaste as good as sugar, and can affect the taste quality of foods/beverages in a small amount. Thus, the glycoside of the present invention can be used as a novel sweetener.

[0023] A glycoside in a preferable aspect of the present invention is selected from Glycoside A or Glycoside B. Glycoside B is sweeter than sugar (sucrose), has early sweetness onset, has sweet lingering as good as sugar, and is highly water soluble. Accordingly, it can favorably be used as a sweetener in various applications as will be described later. Glycoside A, although not much sweet as Glycoside B, is sweeter than

sugar (sucrose), has early sweetness onset, and has sweet lingering as good as sugar. Similar to Glycoside B, Glycoside A can also favorably be used as a sweetener in various applications. Since it has lower water solubility than Glycoside B, it can particularly favorably be used in a lacto-fermented beverage, a suspended fruit juice drink, and a turbid beverage. It can also favorably be used for adjusting sweetness of a pharmaceutical product or the like. While not wishing to be bound by any theory, a low water solubility can suppress bitterness sensed by tongue while enhancing the stimulating feeling going down the throat, and thus advantageous for enhancing the body of the beverage.

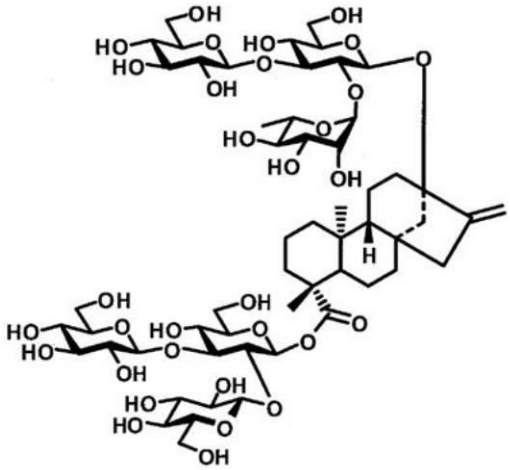
[0024] 2. Sweetener composition containing novel steviol glycoside

In one aspect of the present invention, a sweetener composition containing the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof (hereinafter, also referred to as the "sweetener composition of the present invention") is provided. The sweetener composition of the present invention is not particularly limited as long as it contains the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof, and it may be a composition containing an extract containing the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof.

[0025] The amount of the glycoside of the present invention contained in the sweetener composition of the present invention is not particularly limited.

[0026] Alternatively, the sweetener composition of the present invention is preferably a composition containing the glycoside of the present invention in a larger amount than the amount in a wild-type stevia or stevia extract by at least 0.01%. As mentioned above, the glycoside of the present invention was detected for the first time in a cultiver containing abundant Reb.C, and it is not contained in a wild-type stevia or an extract thereof at all or, if any, contained in an amount of the detection limit or less.

[0027] The sweetener composition of the present invention may further contain other steviol glycosides. For example, the sweetener composition of the present invention may contain, in addition to the glycoside of the present invention, one or more types of steviol glycosides selected from the group consisting of rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside N, rebaudioside M, rebaudioside O, rebaudioside Q, rebaudioside R, dulcoside A, dulcoside C, rubusoside, steviol, steviol monoside, steviol bioside and stevioside. Herein, "dulcoside C" refers to a compound having the following structure.



[0028] In a case where other steviol glycoside is contained, the composition ratio of the glycoside of the present invention and other steviol glycoside is preferably 0.01:9.99-6:4 in a mass ratio.

[0029] The sweetener composition of the present invention may further contain a general sweetener. Examples of such a general sweetener include natural sweeteners such as fructose, sugar, fructose-glucose syrup, glucose, maltose, sucrose, high-fructose syrup, sugar alcohol, oligosaccharide, honey, pressed sugarcane juice (brown sugar syrup), starch syrup, Lo Han Kuo (*Siraitia grosvenorii*) powder, Lo Han Kuo extract, licorice powder, licorice extract, *Thaumatococcus daniellii* seed powder and *Thaumatococcus daniellii* seed extract, and artificial sweeteners such as acesulfame potassium, sucralose, neotame, aspartame and saccharin. Among them, natural sweeteners are preferably used from the aspect of imparting clean taste, easy drinkability, natural flavor and moderately rich taste, where fructose, glucose, maltose, sucrose and sugar are particularly preferably used. Either a single type or a plurality of types of these sweetness ingredients may be used.

[0030] 3. Food/beverage containing novel steviol glycoside

In one aspect of the present invention, a food or beverage containing the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof (hereinafter, also referred to as the "food or beverage of the present invention") is provided. The food or beverage of the present invention is not particularly limited as long as it contains the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof, and it may be a food or beverage containing an extract or a sweetener composition containing the compound represented by Formula (1), or a derivative, a salt or a hydrate thereof. As used herein, a food or beverage refers to foods and beverages. Therefore, in some embodiments, the present invention provides a novel food or beverage, and a method for producing said food or beverage.

[0031] While the amount of the glycoside of the present invention contained in the food or beverage of the present invention differs depending on the specific food or beverage, it is preferably around 0.0004%-0.8% and particularly preferably 0.04%-0.4%. As long as the content lies within this range, the lingering aftertaste can advantageously be suppressed.

[0032] The food or beverage of the present invention may further contain other steviol glycosides. For example, the sweetener composition of the present invention may contain, in addition to the glycoside of the present invention, one or more types of steviol glycosides selected from the group consisting of rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside N, rebaudioside M, rebaudioside O, rebaudioside Q, rebaudioside R, dulcoside A, dulcoside C, rubusoside, steviol, steviol monoside, steviol bioside and stevioside.

[0033] In a case where other steviol glycoside is contained, the composition ratio of the glycoside of the present invention and other steviol glycoside is preferably 0.01:9.99-6:4 in a mass ratio.

[0034] The food or beverage of the present invention may further contain a general sweetener. Examples of such a general sweetener include natural sweeteners such as fructose, sugar, fructose-glucose syrup, glucose, maltose, sucrose, high-fructose syrup, sugar alcohol, oligosaccharide, honey, pressed sugarcane juice (brown sugar syrup), starch syrup, Lo Han Kuo (*Siraitia grosvenorii*) powder, Lo Han Kuo extract, licorice powder, licorice extract, *Thaumatococcus daniellii* seed powder and *Thaumatococcus daniellii* seed extract, and artificial sweeteners such as acesulfame potassium, sucralose, neotame, aspartame and saccharin. Among them, natural sweeteners are preferably used from the aspect of imparting clean taste, easy drinkability, natural flavor and moderately rich taste, where fructose, glucose, maltose, sucrose and sugar are particularly preferably used. Either a single type or a plurality of types of these sweetness ingredients may be used.

[0035] Examples of the food of the present invention include, but not particularly limited to, a confection, a bread, cereal flour, noodles, rice, a processed agricultural/forestry food, a processed livestock product, a processed fishery product, a milk/dairy product, an oil-and-fat/processed oil-and-fat product, seasoning or other food material.

[0036] Examples of the beverage of the present invention include, but not particularly limited to, a carbonated beverage, a non-carbonated beverage, an alcoholic beverage, a non-alcoholic beverage, a beer-taste beverage such as beer or non-alcohol beer, a coffee beverage, a tea beverage, a cocoa beverage, a nutritious beverage and a functional beverage.

[0037] The beverage of the present invention may be heat sterilized and packaged to be prepared as a packaged beverage. Examples of such package include, but not particularly limited to, a PET bottle, an aluminum can, a steel can, a paper package, a chilled cup, and a bottle. In a case where heat sterilization is to be performed, the type of heat sterilization is not particularly limited. For example, heat sterilization may be performed by employing a common technique such as UHT sterilization, retort sterilization or the like. While the temperature during the heat sterilization process is not particularly limited, it is, for example, 65-130ºC, and preferably 85-120ºC, for 10-40 minutes. Sterilization, however, can be carried out at an appropriate temperature for a several seconds, for example, 5-30 seconds, without problem as long as the same sterilizing value as that under the above-described conditions can be earned.

[0038] 4. Plant containing novel steviol glycoside and extract thereof

In one aspect of the present invention, a plant containing the novel steviol glycoside and an extract thereof are provided. Furthermore, in another aspect of the present invention, a food or beverage, preferably a beverage, containing the plant of the present invention or an extract of said plant is provided. While the amount of the glycoside of the present invention contained in the plant of the present invention is not particularly limited, it is preferably 0.001%-1.000% and more preferably 0.01%-0.80%.

[0039] Preferably, the plant of the present invention is a plant that contains the glycoside of the present invention in a larger amount than a wild-type stevia species by 0.01% or more. As described above, the steviol glycoside of the present invention is not contained in a wild-type stevia at all or, if any, contained in an amount of the detection limit or less.

[0040] The phrase "contains the glycoside of the present invention in a larger amount than a wild-type stevia species by 0.01% or more" means that, with respect to an amount (concentration) of the glycoside of the present invention contained per unit quantity (e.g., 10 ml) of a liquid extract from fresh leaves (undried leaves) of a wild-type stevia plant, an amount (concentration) of the glycoside of the present invention contained in an equal unit quantity of a liquid extract from fresh leaves (undried leaves) of the plant of the present invention (the same amount as that of the liquid extract from the leaves of the wild-type stevia plant) is higher by 0.01% or more. Here, the plant of the present invention may contain the glycoside of the present invention in a larger amount than a wild-type stevia species by 0.02% or more, 0.03% or more, 0.04% or more, 0.05% or more, 0.07% or more, 0.09% or more, 0.10% or more, 0.15% or more, 0.20% or more, 0.40% or more, 0.60% or more, 0.80% or more, 1.0% or more, 1.50% or more, 2.00% or more, 4.00% or more, 6.00% or more, 8.00% or more, or 10.00% or more.

[0041] Moreover, the phrase "the proportion of the glycoside of the present invention among the total steviol glycosides is 0.01% or more" means that the glycoside of the present invention exists at a percentage of 0.01% or more with respect to the content of the total steviol glycosides existing in the liquid extract from the fresh leaves (undried leaves) of the stevia plant of the present invention. Here, the total steviol glycosides neither contain unknown steviol glycosides nor any steviol glycoside existing in an amount less than the detection limit. Preferably, the total steviol glycosides consist of rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside N, rebaudioside M, rebaudioside O, rebaudioside Q, rebaudioside R, dulcoside A, dulcoside C, rubusoside, steviol, steviol monoside, steviol bioside and stevioside.

[0042] While the content of the glycoside of the present invention in the plant of the present invention is as described above, in a case where dried leaves are obtained from the plant of the present invention, the glycoside of the present invention may exist in an amount of 0.01 wt% or more, 0.02 wt% or more, 0.03 wt% or more, 0.04 wt% or more, 0.05 wt% or more, 0.07 wt% or more, 0.10 wt% or more, 0.15 wt% or more, 0.20 wt% or more, 0.30 wt% or more, 0.50 wt% or more, 0.60 wt% or more, 0.80 wt% or more, 1.00 wt% or more, 2.00 wt% or more, 4.00 wt% or more, 6.00 wt% or more, 8.00 wt% or more, or 10.00 wt% or more with respect to the weight of said dried leaves.

[0043] Here, dried leaves of the plant of the present invention refer to those obtained by drying fresh leaves of the plant of the present invention to reduce their water content to 10 wt% or less, 7 wt% or less, 5 wt% or less, 4 wt% or less, 3 wt% or less, 2 wt% or less, or 1 wt% or less. Preferably, the water content of the dried leaves of the plant of the present invention is 3-4 wt%.

[0044] An example of the plant of the present invention include a plant with abundant Reb.C. As described above, the steviol glycoside of the present invention is not contained in a wild-type stevia at all or, if any, contained in an amount of the detection limit or less. Meanwhile, the present inventors found out that the steviol glycoside of the present invention is contained in a larger amount in a plant having abundant Reb.C. Therefore, the novel steviol glycoside and the extract thereof also comprise such a plant with abundant Reb.C and an extract thereof.

[0045] An example of such a plant with abundant Reb.C includes, but not particularly limited to a high-rebaudioside C-containing non-recombinant stevia plant which contains rebaudioside C in a larger amount than a wild-type stevia species by 20% or more, and whose proportion of rebaudioside C among the total steviol glycosides is 40% or more

(hereinafter, also referred to as a "high-Reb.C plant").

[0046] An example of such a high-Reb.C plant include a high-rebaudioside C-containing non-recombinant stevia plant which contains rebaudioside C in a larger amount than a wild-type stevia species by 20% or more, and whose proportion of rebaudioside C among the total steviol glycosides is 40% or more.

[0047] A high-Reb.C plant is a cultiver derived from a plant of a wild-type stevia, in which genetic mutation has occurred to increase rebaudioside C. Examples of such genetic mutation include, but not particularly limited to, genetic mutation induced under naturally occurring conditions, genetic mutation induced by a non-recombinational technique and genetic mutation induced by genetic recombination.

[0048] A high-Reb.C plant can be screened, for example, by detecting gene polymorphism in the tissue of the plant. Herein, "screening" means to identify and select a high-Reb.C plant among other plant bodies.

[0049] The high-Reb.C plant may also be screened according to a screening method that includes a step of identifying a polymorphism of A in the wild type being altered to T at the 60th nucleotide of the nucleotide sequence represented by SEQ ID NO:11 in the genome of a test plant.

[0050] The plant of the present invention not only comprises the whole plant but may also comprise plant organs (for example, leaf, petal, stem, root, seed, etc.), plant tissues (for example, epidermis, phloem, parenchyma, xylem, vascular bundles, palisade tissue, spongy tissue, etc.), various forms of plant cells (for example, suspension cultured cells), a protoplast, a leaf piece, callus and the like.

[0051] In addition, the plant of the present invention may also comprise a tissue culture or a plant cell culture. This is because such a tissue culture or plant cell culture may be cultured to regenerate a plant. Examples of the tissue culture or the plant cell culture of the plant of the present invention include, but not limited to, an embryo, meristematic cells, pollen, a leaf, a root, a root apex, a petal, a protoplast, a leaf piece and callus.

[0052] An extract of the plant of the present invention can be obtained by reacting a fresh or dried leaf of the plant of the present invention with an appropriate solvent (an aqueous solvent such as water or an organic solvent such as alcohol, ether or acetone). For extraction conditions, see the method described in WO2016/090460 or a method described in the example below.

[0053] Preferably, the extract of the plant of the present invention contains the glycoside of the present invention in a larger amount than a wild-type stevia by 0.01% or more, where the proportion of the glycoside of the present invention among the total steviol

glycosides is 0.01% or more. Here, the phrase "contains the glycoside of the present invention in a larger amount than a wild-type stevia by 0.01% or more" means the same as described above. Similarly, the phrase the "proportion of the glycoside of the present invention among the total steviol glycosides is 0.01% or more" also means the same as described above.

[0054] 5. Flavor controlling agent containing novel steviol glycoside

Although the novel steviol glycoside of the present invention is contained in a stevia extract in a minute quantity, it is considered to have an influence on the flavor the stevia extract. While not wishing to be bound by any theory, addition of a small amount of the steviol glycoside of the present invention is presumably capable of controlling the flavor of a food or beverage. Therefore, in one aspect of the present invention, a flavor controlling agent containing the above-described compound represented by Formula (1) or a derivative, a salt or a hydrate thereof is provided.

[0055] As used herein, a "flavor controlling agent" refers to a substance that can be added to a food or beverage to control the flavor of the food or beverage. Preferably, the flavor controlling agent of the present invention can be added to a food or beverage so as to control the flavor of the food or beverage itself without the consumers recognizing the taste of the flavor controlling agent itself. For example, since the steviol glycoside of the present invention has good sweet lingering as compared to conventional steviol glycosides, it can be used as a flavor controlling agent for controlling the sweet lingering of the food or beverage.

[0056] The flavor controlling agent of the present invention preferably contains, in addition to the above-described compound represented by Formula (1) or a derivative, a salt or a hydrate thereof, one or more types of other sweeteners. Examples of such sweetener include: one or more types of steviol glycosides selected from the group consisting of rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside N, rebaudioside M, rebaudioside O, rebaudioside Q, rebaudioside R, dulcoside A, dulcoside C, rubusoside, steviol, steviol monoside, steviol bioside and stevioside; natural sweeteners such as fructose, sugar, fructose-glucose syrup, glucose, maltose, sucrose, high-fructose syrup, sugar alcohol, oligosaccharide, honey, pressed sugarcane juice (brown sugar syrup), starch syrup, Lo Han Kuo (*Siraitia grosvenorii*) powder, a Lo Han Kuo extract, licorice powder, a licorice extract, *Thaumatococcus daniellii* seed powder and a *Thaumatococcus daniellii* seed extract; and artificial sweeteners such as acesulfame potassium, sucralose, neotame, aspartame and saccharin.

[0057] 6. Method for producing novel steviol glycoside

As described above, the steviol glycoside of the present invention can be produced by (A) isolation/purification from a plant, (B) a chemical synthesis, or (C) a biosynthesis. Hereinafter, each of them will be described.

[0058] (A) Isolation/purification from plant

Since the plant of the present invention contains the novel steviol glycoside of the present invention, the novel steviol glycoside can be isolated/purified from said plant. A fresh or dried leaf of the plant of the present invention is allowed to react with an appropriate solvent (an aqueous solvent such as water or an organic solvent such as alcohol, ether or acetone) to extract the novel steviol glycoside in a liquid extract state. For extraction conditions and else, see the method described in WO2016/090460 or the method described in the example below.

[0059] Furthermore, the resulting liquid extract may be subjected to a known method such as a gradient of ethyl acetate or other organic solvent: water, high performance liquid chromatography (HPLC), or ultra (high) performance liquid chromatography (UPLC) to isolate/purify the novel steviol glycoside.

[0060] The content of the novel steviol glycoside in the plant can be determined by the method described in WO2016/090460 or the method described in the example below. Specifically, the content can be measured by sampling fresh leaves from the plant of the present invention and subjecting the leaves to LC-MS/MS.

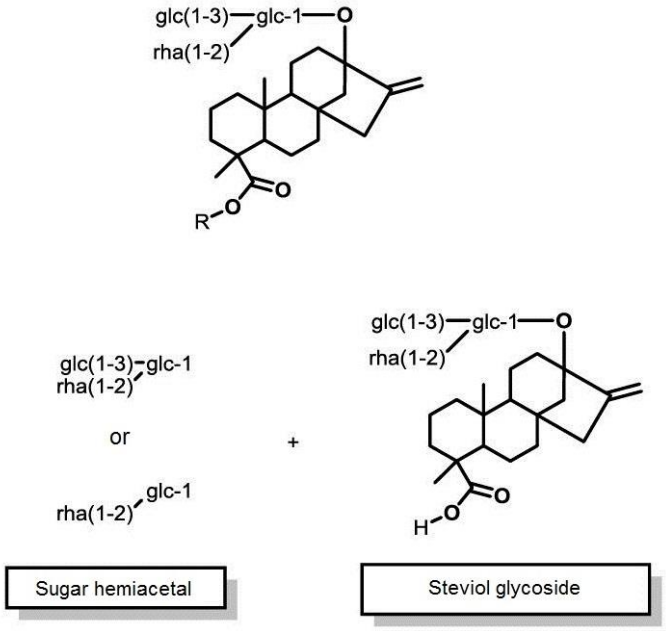
[0061] (B) Chemical synthesis

A method for producing the steviol glycoside of the present invention through chemical synthesis will be described in detail hereinbelow.

[0062] Steviol glycosides have structures in which different sugar moieties (glucose, rhamnose, xylose, etc.) are attached to the aglycone, i.e., steviol, via various linkage forms (linkage positions and conformations). Therefore, a steviol glycoside of interest can be obtained via various synthetic pathways depending on the selected starting material. Those skilled in the art to which the present invention pertains, however, would understand that the time and the yield for obtaining the compound of interest greatly vary depending on the synthetic pathways.

[0063] This time, the present inventors found out a novel method for producing a steviol glycoside of the present invention with higher selectivity and higher yield via a specific synthetic pathway. According to the method for producing the steviol glycoside of the present invention, a chemical synthesis of the steviol glycoside proceeds by separating the steviol glycoside into a "steviol glycoside" and a "sugar hemiacetal" as shown in Scheme 1.

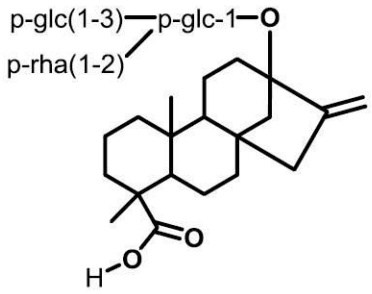
Scheme 1: Novel method for synthesizing steviol glycoside



[0064] The steviol glycoside can be prepared by deriving from an existing natural substance (rebaudioside, dulcoside, stevioside, steviol bioside, rubusoside, etc.). Meanwhile, the sugar hemiacetal can be prepared either from an existing natural substance or by a chemical synthesis. The present inventors found that the steviol glycoside of interest can be obtained with good yield and extremely high β-selectivity by condensing the steviol glycoside and the sugar hemiacetal through the Mitsunobu reaction.

[0065] In one aspect of the present invention, a method for producing the compound represented by Formula (1) is provided where the method comprises the steps of:

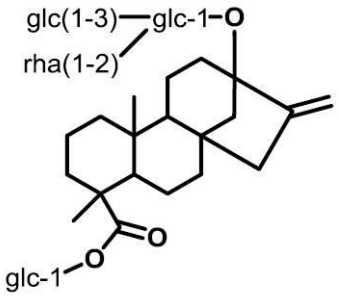
(A) preparing Intermediate 1 represented by Formula (5):



(5)

wherein p-glc represents glucose in which at least one hydroxyl group is protected by a protecting group, and p-rha represents rhamnose in which at least one hydroxyl group is protected by a protecting group,

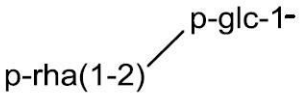
from rebaudioside C represented by Formula (4):



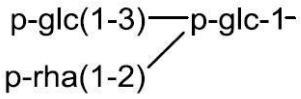
(4)

wherein glc represents glucose and rha represents rhamnose;

(B) preparing Intermediate 2 represented by Formula (6) or Intermediate 3 represented by Formula (7) from a glucopyranoside derivative:



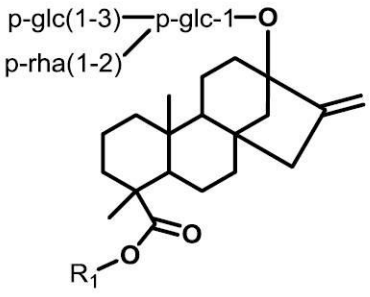
(6)



(7)

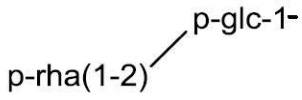
wherein p-glc represents glucose in which at least one hydroxyl group is protected by a protecting group, and p-rha represents rhamnose in which at least one hydroxyl group is protected by a protecting group; and

(C) allowing Intermediate 1 to react with Intermediate 2 or 3 in the presence of a phosphine reagent and an azo compound to obtain Intermediate 4 represented by Formula (8):

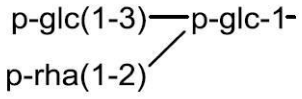


(8)

wherein, R_1 represents a sugar chain of Formula (9) or (10); and



(9)



(10)

p-glc represents glucose in which at least one hydroxyl group is protected by a protecting group, and p-rha represents rhamnose in which at least one hydroxyl group is protected by a protecting group.

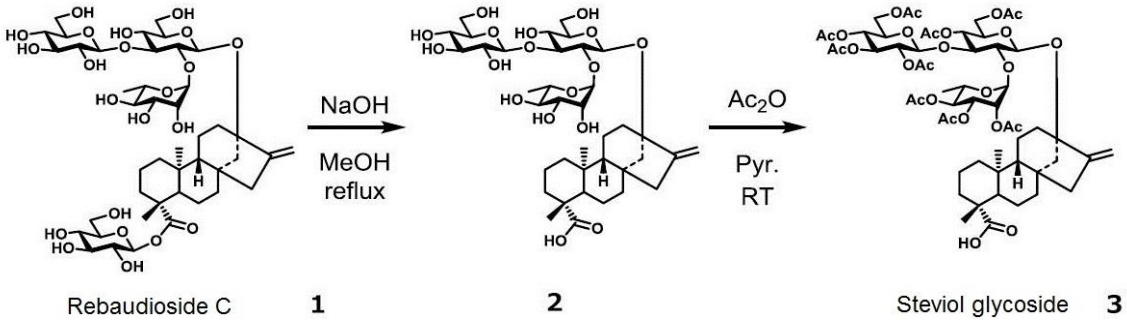
[0066] Herein, examples of the protecting group include an acyl protecting group, a trisubstituted silyl group, an acetal protecting group and an ether protecting group. Preferable examples include a trisubstituted silyl group (a trimethylsilyl group, a triethylsilyl group, a t-butyldimethylsilyl group, etc.) and an acyl protecting group (an acetyl group, a benzoyl group, etc.).

[0067] Hereinafter, one specific aspect of the method for producing the steviol glycoside of the present invention will be described, but the method for producing the glycoside of the present invention should not be limited to this aspect.

[0068] (A) First step (synthesis of steviol glycoside)

A steviol glycoside can be obtained, for example, by following Scheme 2 below using naturally occurring rebaudioside C (dulcoside B) as a starting material.

Scheme 2: Synthesis of steviol glycoside



[0069] First, rebaudioside C is dissolved in a solvent such as methanol and water, added with a strong base such as sodium hydroxide, and refluxed at 60ºC-120ºC for 2 hours or longer so that the glucose molecule is removed from C-19 of rebaudioside C to give Compound 2 above. In doing so, the solvent may be evaporated after neutralizing the reaction solution with a cation exchange resin or the like.

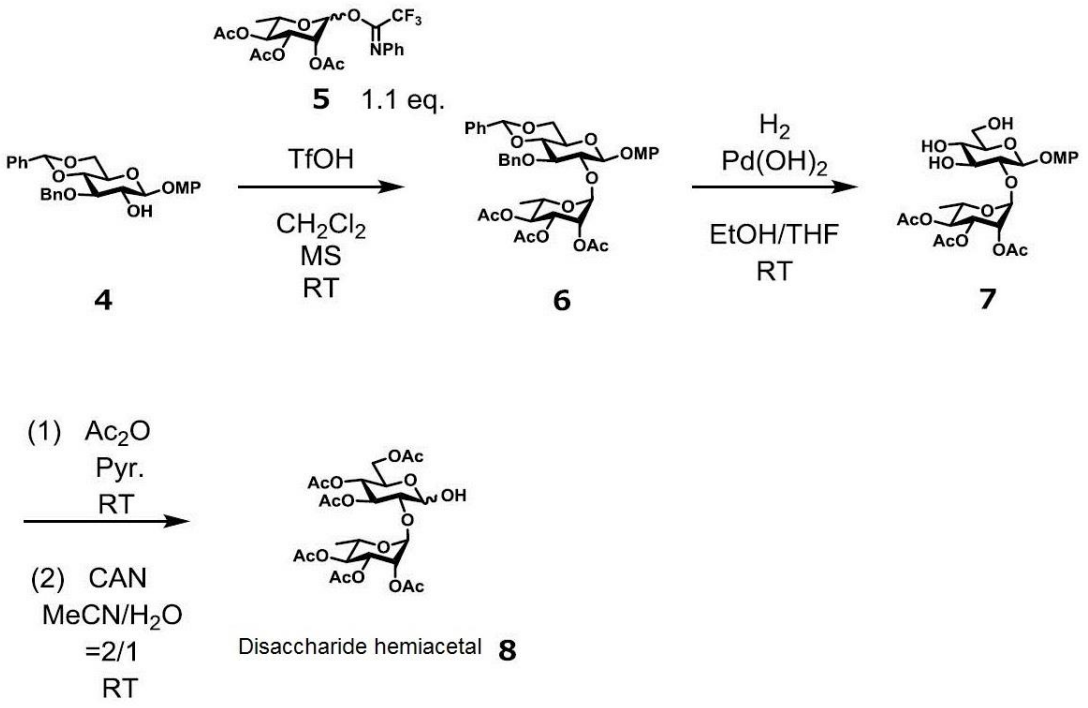
[0070] Compound 2 is further dissolved in a solvent such as pyridine, and added with acetic anhydride or the like to protect the hydroxyl groups contained in Compound 2, thereby obtaining Compound 3 (Intermediate 1).

[0071] (B) Second step (synthesis of disaccharide or trisaccharide hemiacetal)

A disaccharide hemiacetal or a trisaccharide hemiacetal can be obtained, for example, by using a commercially available glucopyranoside derivative as a starting material. Syntheses of a disaccharide hemiacetal (Step 2a) and a trisaccharide hemiacetal (Step 2b) are shown in Schemes 3 and 4, respectively.

[0072] Scheme 3 showing synthesis of a disaccharide hemiacetal (Step 2a) is as follows.

Scheme 3: Synthesis of disaccharide hemiacetal

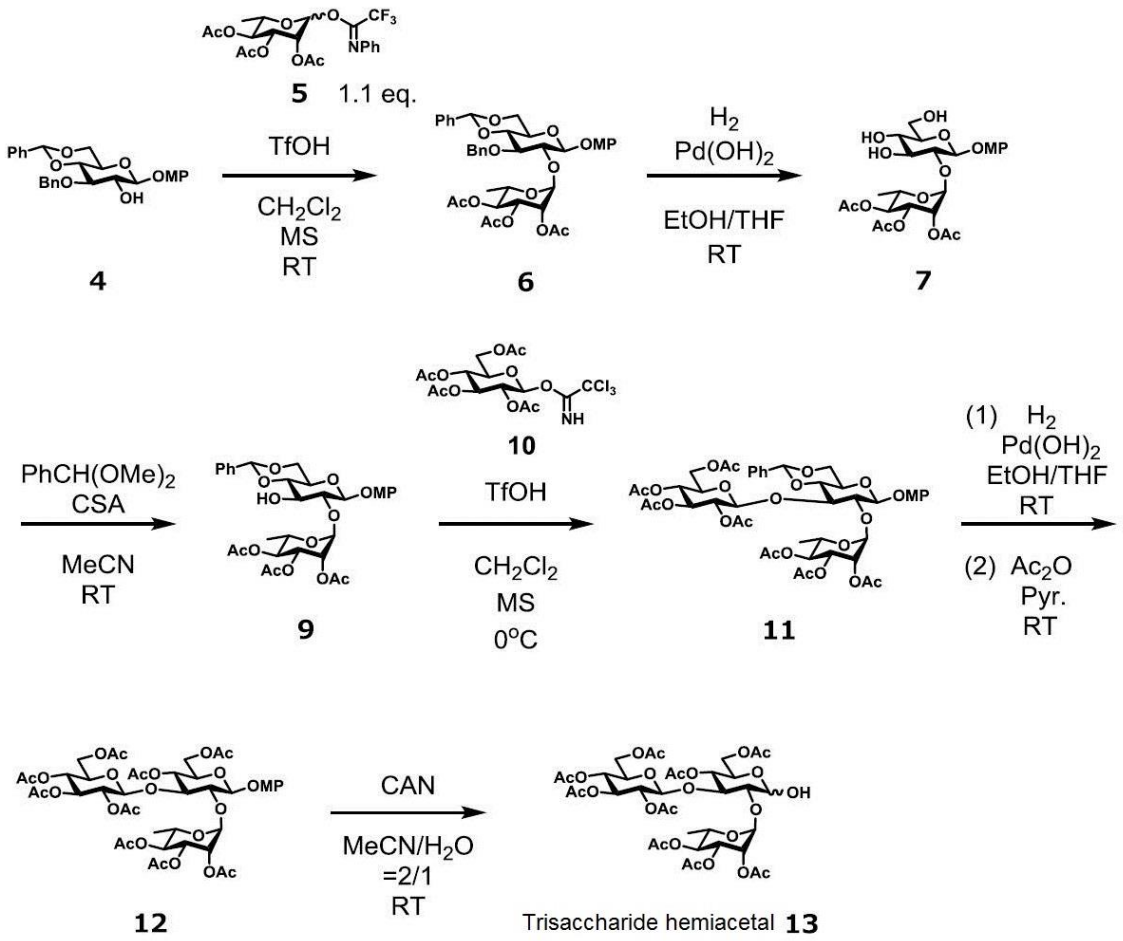


[0073] First, 4-methoxyphenyl 3-O-benzyl-4,6-O-benzylidine-β-D-glucopyranoside (Compound 4), Compound 5 and molecular sieves (MS) are dissolved in a solvent such as dichloromethane, added with trifluoromethanesulfonic acid (acid catalyst), and agitated at 25ºC-80ºC for 2 hours or longer to give Compound 6.

[0074] Subsequently, Compound 6 is dissolved in a solvent such as ethanol or THF, added with a catalyst such as palladium hydroxide at room temperature, and agitated in an hydrogen atmosphere at room temperature for 2 hours or longer to complete the reaction. Then, the catalyst is removed by filtration to give Compound 7. Compound 7 is dissolved in a solvent such as pyridine, added with acetic anhydride at room temperature, and agitated at room temperature for 12-36 hours. Thereafter, the solution is concentrated under a reduced pressure, added with acetonitrile and water, added with an oxidant such as cerium ammonium nitrate, and agitated for 5 minutes to 2 hours to give Compound 8 (Intermediate 2).

[0075] Scheme 4 showing synthesis of a trisaccharide hemiacetal (Step 2b) is as follows.

Scheme 4: Synthesis of trisaccharide hemiacetal



[0076] First, 4-methoxyphenyl 3-O-benzyl-4,6-O-benzylidine-β-D-glucopyranoside (Compound 4), Compound 5 and molecular sieves (MS) are dissolved in a solvent such as dichloromethane, added with trifluoromethanesulfonic acid (acid catalyst), and agitated at 25ºC-80ºC for 2 hours or longer to give Compound 6.

[0077] Subsequently, Compound 6 is dissolved in a solvent such as ethanol or THF, added with a catalyst such as palladium hydroxide at room temperature, and agitated in an hydrogen atmosphere at room temperature for 2 hours or longer to complete the reaction. Then, the catalyst is removed by filtration to give Compound 7. Compound 7 is dissolved in acetonitrile, added with benzaldehyde dimethyl acetal at room temperature, and agitated for 2 hours or longer to give Compound 9.

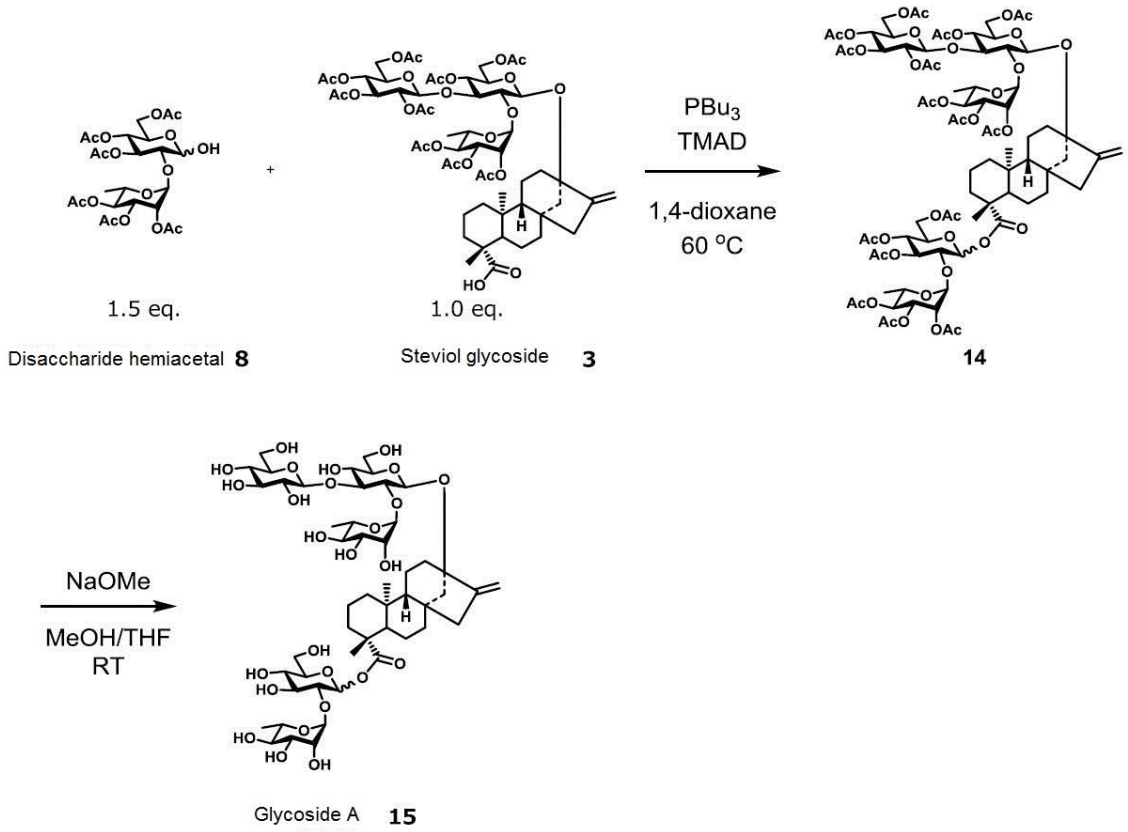
[0078] Compounds 9 and 10 and molecular sieves (MS) are dissolved in a solvent such as dichloromethane, added with trifluoromethanesulfonic acid at room temperature, and agitated at 0ºC for 3-9 hours to give Compound 11. The resulting Compound 11 is dissolved in a solvent such as ethanol or THF, added with a catalyst such as palladium hydroxide at room temperature, and agitated in an hydrogen atmosphere at room

temperature for 2 hours or longer to complete the reaction. Then, the resultant is dissolved in a solvent such as pyridine, added with acetic anhydride at room temperature, and agitated at room temperature for 12-36 hours to give Compound 12. Thereafter, the solution is concentrated under a reduced pressure, added with acetonitrile and water, added with an oxidant such as cerium ammonium nitrate, and agitated for 5 minutes to 2 hours, thereby obtaining Compound 13 (Intermediate 3).

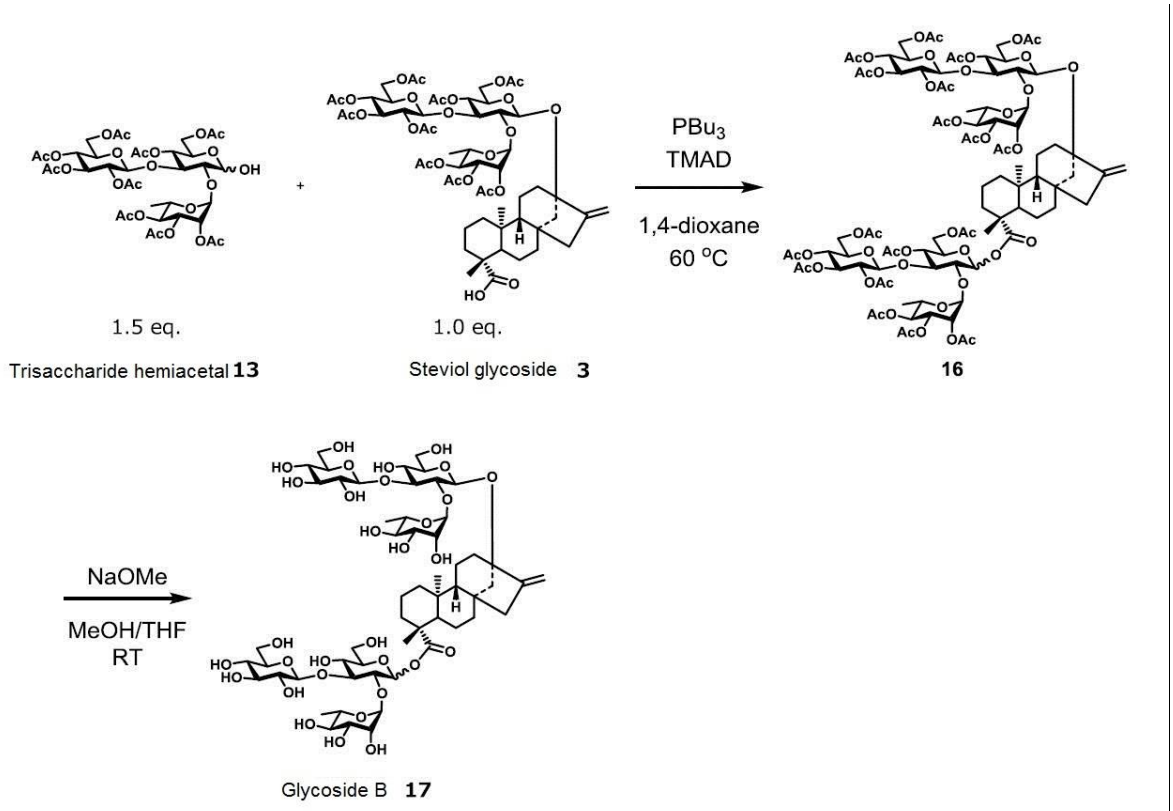
[0079] (C) Third step (Synthesis of compound represented by Formula (1))

The compound represented by Formula (1) can be synthesized, for example, by following Scheme 5 or 6 below using Compound 3 (Intermediate 1) and Compound 8 or 13 (Intermediate 2 or 3) obtained in Steps 1 and 2 (2a or 2b) above.

Scheme 5: Synthesis of compound represented by Formula (1) (Glycoside A)



Scheme 6: Synthesis of compound represented by Formula (1) (Glycoside B)



[0080] First, the disaccharide or trisaccharide hemiacetal and the Steviol glycoside obtained in Steps 1 and 2 (2a or 2b) are allowed to undergo the Mitsunobu reaction so as to obtain a glycoside in which the disaccharide or trisaccharide hemiacetal is selectively attached to the carboxylic group at C-19 of steviol. Specifically, these compounds are dissolved in 1,4-dioxane, added with a phosphine reagent such as tributylphosphine or triphenylphosphine and an azo compound such as 1,1'-azobis (N,N'-dimethylformamide) (TMAD) at room temperature, and agitated at 50ºC-80ºC for 2 hours or longer to give Compound 14 or 16. Finally, the protecting groups of Compound 14 or 16 are deprotected to give the compound represented by Formula (1) (Glycoside A or B).

[0081] (C) Biosynthesis

The steviol glycoside of the present invention can also be generated by transferring a polynucleotide coding for a predetermined protein into a host cell derived from a bacterium, a plant, an insect, a non-human mammal or the like, and using steviol, a steviol glycoside, UDP-glucose and/or UDP-rhamnose as a substrate. Steviol, a steviol glycoside, UDP-glucose or UDP-rhamnose as the substrate may be either provided or biosynthesized in the cell. While examples of the predetermined protein include stevia-derived UGT85C2 (whose amino acid sequence is represented by SEQ ID NO:2),

UGT74G1 (whose amino acid sequence is represented by SEQ ID NO:4), UGT91D2 (whose amino acid sequence is represented by SEQ ID NO:6), UGT76G1 (whose amino acid sequence is represented by SEQ ID NO:8) and *Arabidopsis thaliana*-derived UDP-rhamnose synthase AtRHM2 (whose amino acid sequence is represented by SEQ ID NO:10), it is not limited thereto as long as it has an equivalent activity.

[0082] The above-described protein is an enzyme derived from *Arabidopsis thaliana* or stevia, which is expected to be highly active in an environment outside plant cells such as *Arabidopsis thaliana* and stevia (for example, in an extracellular environment, or inside a host cell other than stevia). In this case, the polynucleotide coding for the above-described protein (for example, UGT85C2 gene is represented by SEQ ID NO:1, UGT74G1 gene is represented by SEQ ID NO:3, UGT91D2 gene is represented by SEQ ID NO:5, UGT76G1 gene is represented by SEQ ID NO:7 and AtRHM2 gene is represented by SEQ ID NO:9) is transferred into a host cell derived from a bacterium, a fungus, a plant, an insect or a non-human mammal so as to allow expression of the protein of the present invention, to which steviol, a steviol glycoside, UDP-glucose or UDP-rhamnose as the substrate is provided to generate the compound of the present invention. Alternatively, depending in the host, the above-described protein is expressed in the host cell, to which an appropriate substrate is provided to generate the compound of the present invention.

[0083] In one aspect of the present invention, a method for producing the novel steviol glycoside of the present invention is provided, where the method is characterized by use of a non-human transformant that has been introduced with at least one of polynucleotides (a) to (g).

(a) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:2 and that has an activity of adding glucose to the hydroxyl group at C-13 of the steviol glycoside.

(b) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:4 and that has an activity of adding glucose to the carboxylic acid at C-19 of the steviol glycoside.

(c) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:6 and that has an activity of adding rhamnose to glucose attached to C-13 of the steviol glycoside via a 1→2 linkage.

(d) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:8 and that has an activity of adding glucose to C-3 of glucose at C-13 of the steviol glycoside via a 1→3 linkage.

(e) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:6 and that has an activity of adding glucose to glucose at C-19 of the steviol glycoside via a 1→2 linkage.

(f) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:8 and that has an activity of adding glucose to glucose at C-19 of the steviol glycoside via a 1→3 linkage.

(g) A polynucleotide coding for a protein that has 90% or higher identity with the amino acid sequence of SEQ ID NO:10 and that has an activity of generating UDP-rhamnose from UDP-glucose.

[0084] In a preferable aspect of the present invention, polynucleotides independently having 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher, or 99.9% or higher sequence identity with the nucleotide sequences of the sequence numbers mentioned in (a) to (g) above can be used.

[0085] In another preferable aspect of the present invention, proteins that independently have an amino acid sequence having 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher, or 99.9% or higher sequence identity with the amino acid sequences of the sequence number mentioned in (a) to (g) above and that has the predetermined activity described in (a) to (g) above can be used.

[0086] Preferably, a polynucleotide coding for the above-described protein is introduced into a host while being inserted into an appropriate expression vector. The polynucleotides may individually be inserted into separate vectors.

[0087] An appropriate expression vector is generally made to contain:

(i) a promoter that allows transcription in the host cell;

(ii) a polynucleotide of the present invention linked to said promoter; and

(iii) an expression cassette that is involved in transcription termination and polyadenylation of RNA molecules and that contains, as a component thereof, a signal that functions in the host cell.

[0088] Examples of a method for preparing an expression vector include, but not particularly limited to, a method that uses a plasmid, a phage, a cosmid or the like, and DNA molecules having necessary components.

[0089] The specific type of the vector is not particularly limited, and any vector that

allows expression in the host cell can suitably be selected. Specifically, a promoter sequence is suitably selected according to the type of the host cell to ensure expression of the polynucleotide of the present invention, and a vector obtained by integrating this promoter sequence and the polynucleotide of the present invention into a plasmid or the like is used as an expression vector.

[0090] The expression vector of the present invention includes expression controlling regions (for example, a promoter, a terminator and/or an origin of replication and the like) depending on the type of the host into which it is introduced. A promoter used in a bacterial expression vector may be a common promoter (for example, a trc promoter, a tac promoter, a lac promoter, etc.), a promoter used for a yeast may be, for example, a glyceraldehyde-3-phosphate dehydrogenase promoter, a PH05 promoter, a GAL1/10 promoter or the like, and a promoter for filamentous fungi may be, for example, amylase, trpC or the like. Moreover, examples of a promoter for expressing the gene of interest in a plant cell include a cauliflower mosaic virus 35S RNA promoter, a rd29A gene promoter, a rbcS promoter, and a mac-1 promoter in which the enhancer sequence of the cauliflower mosaic virus 35S RNA promoter is provided at the 5' end of a promoter sequence of *Agrobacterium*-derived mannopine synthase. A promoter for an animal cell host may be a viral promoter (for example, a SV40 early promoter, a SV40 late promoter, etc.). Examples of a promoter that is inducibly activated in response to external stimuli include a mouse mammary tumor virus (MMTV) promoter, a tetracycline responsive promoter, a metallothionein promoter and a heat shock protein promoter.

Preferably, the expression vector contains at least one selectable marker. As such a marker, an auxotrophic marker (LEU2, URA3, HIS3, TRP1, ura5, niaD), a drug resistance marker (hygromycin, zeocin), a geneticin resistance gene (G418r), a copper resistance gene (CUP1) (Marin et al., Proc. Natl. Acad. Sci. USA, vol. 81, p. 337, 1984), a cerulenin resistance gene (fas2m, PDR4) (Junji Inokoshi et al., Journal of Japanese Biochemical Society, vol. 64, p. 660, 1992; Hussain et al., Gene, vol. 101, p. 149, 1991, respectively) or the like can be used.

[0091] As a method for transforming a host cell, a generally employed known method can be employed. For example, an electroporation method (Mackenxie, D. A. et al., Appl. Environ. Microbiol., vol. 66, p. 4655-4661, 2000), a particle delivery method (Japanese Unexamined Patent Application Publication No. 2005-287403), a spheroplast method (Proc. Natl. Acad. Sci. USA, vol. 75, p. 1929, 1978), a lithium acetate method (J. Bacteriology, vol. 153, p. 163, 1983), a method described in Methods in yeast genetics, 2000 Edition: A Cold Spring Harbor Laboratory Course Manual, or the like can be

performed although the present invention is not limited thereto.

[0092] In addition, as to general molecular biological processes, see "Sambrook and Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor Laboratory Press 2001", "Methods in Yeast Genetics, A laboratory manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)" and the like.

[0093] A non-human transformant obtained as described above can be cultured so as to allow the non-human transformant to produce a steviol glycoside. Such a non-human transformant is preferably a yeast. Moreover, the non-human transformant is preferably cultured in a medium containing steviol. The accumulated steviol glycoside can be extracted/purified to obtain the steviol glycoside of the present invention.

EXAMPLES

[0094] [Isolation of novel steviol glycoside]

Extracts obtained from the leaves of four lines of novel stevia plant bodies (Sample 1 (EM3-4), Sample 2 (EM2-27-8), Sample 3 (EM2-27-15) and Sample 4 (EM2-11)) developed at Suntory Global Innovation Center (SIC) were subjected to high performance liquid chromatography (HPLC) separation-mass spectrometry (MS) for the screening analysis of the steviol glycosides contained in the stevia plant bodies based on the molecular weights of steviol glycosides that had a sugar chain formed of D-glucopyranosyl (Glc), L-rhamnopyranosyl (Rha) and xylopyranosyl (Xyl). Here, Sample 1 is a high-Reb.C plant having a genome polymorphism of A in the wild type being altered to T at the 60th nucleotide of the nucleotide sequence represented by SEQ ID NO:11 in the genome. A statistical analysis of the correlation between the phenotype having a high-Reb.C concentration and the polymorphism of SEQ ID NO:11 revealed that said polymorphism had a statistic correlation with the phenotype having a high-Reb.C concentration.

[0095] A process for preparing a test liquid was as follows: 10.0 mg each of lyophilized dried stevia leaves was weighed into a glass vial, to which 1.0 mL of water/methanol (1/1 vol/vol) was added as an extracting solvent, and then the resultant was subjected to ultrasonic irradiation in an ultrasonic cleaner (AS ONE, AS52GTU) at a set temperature of 25°C for 20 minutes, thereby obtaining a liquid extract of a steviol glycoside from the stevia leaves. The resultant was further 10-fold diluted with water/methanol and filtrated through a filter with a pore size of 0.45 μm (Nacalai tesque, Cosmonice filter S (solvent)) before being subjected to HPLC-MS.

[0096] For the HPLC part of HPLC-MS, Nexera LC-30AD (Shimadzu Corporation) was

used as a liquid delivery unit LC pump, and SM-C18 (4.6 x 250 mm) (from Imtakt) as a separation column. Liquid delivery of the LC mobile phase was carried out by using 0.2% acetic acid-containing Milli-Q water as mobile phase A and methanol as mobile phase B, where the binary gradient was such that the concentration of the mobile phase B was constantly maintained at 10% during 0-5 minutes, shifted from 10% to 70% in the next 15 minutes, and then shifted from 70% to 100% in the following 5 minutes, and the gradient was finally ended by maintaining the concentration of the mobile phase B at 100% for 5 minutes. The flow rate of the mobile phase was 0.4 mL/min, and the stevia leaf liquid extract that had been diluted and filtrated with a filter was injected for 5 μL.

[0097] For the MS part, triple quadrupole mass spectrometer LCMS-8030 (Shimadzu Corporation) equipped with an electrospray ionization (ESI) ion source was used. The mass spectrometry measurement was carried out in a selected ion monitoring (SIM) mode by selecting the negative ion measurement mode and the m/z values. The m/z values were selected by calculation based on the molecular weights of the steviol glycosides that had sugar chains formed of D-glucopyranosyl (Glc), L-rhamnopyranosyl (Rha) and xylopyranosyl (Xyl). Accordingly, m/z = 641.2 (Glc (2)), 773.2 (Glc (2), Xyl (1)), 787.2 (Glc (2), Rha (1)), 803.2 (Glc (3)), 935.3 (Glc (3), Xyl (1)), 949.3 (Glc (3), Rha (1)), 965.3 (Glc (4)), 1095.4 (Glc (3), Rha (2)), 1097.4 (Glc (4), Xyl (1)), 1111.4 (Glc (4), Rha (1)), 1127.4 (Glc (5)), 1257.5 (Glc (4), Rha (2)), 1259.5 (Glc (5), Xyl (1)), 1273.5 (Glc (5), Rha (1)), 1289.5 (Glc (6)), 1435.6 (Glc (6), Rha (1)) were selected. Furthermore, a high purity reagent, rebaudiosides A, B, D, F, M, N and O, stevioside, and dulcosides A and B available were also measured under the same conditions so as to confirm the negative ion m/z values and the retention time in HPLC. The peak areas (arbitrary unit) of the mainly detected steviol glycosides are shown in Table 1.

[0098]

Table 1: Peak areas (arbitrary unit) observed by STM measurement in HPLC-MS

Compound name	Rebaudioside A	Rebaudioside C	Rebaudioside D	Rebaudioside M	Dulcoside C	Novel steviol glycoside 1 *m/z* 1095.4	Novel steviol glycoside 2 *m/z* 1257.5	Rebaudioside N
Retention time (min)	29.60	29.96	28.00	28.66	27.70	28.73	28.50	28.18
Peak area (Sample 1)	29,669,582	30,122,062	1,428,384	1,030,603	140,947	76,369	242,070	772,570
	46.92%	47.63%	2.26%	1.63%	0.22%	0.12%	0.38%	1.22%
Peak area (Sample 2)	23,762,676	24,201,473	2,253,735	1,029,837	97,388	94,330	292,157	1,211,504
	45.13%	45.97%	4.28%	1.96%	0.18%	0.18%	0.55%	2.30%
Peak area (Sample 3)	15,386,726	5,872,656	3,585,775	3,296,579	89,988	0	86.845	896.549
	52.82%	20.16%	12.31%	11.32%	0.31%	0.00%	0.30%	3.08%
Peak area (Sample 4)	16,070,017	10,339,094	1,404,429	74,413	0	17,634	0	308,709
	56.96%	36.64%	4.98%	0.26%	0.00%	0.06%	0.00%	1.09%

[0099] Figure 2 shows a selected ion chromatogram of Sample 1 (EM3-4) at m/z of 1095.4. A peak of a molecular weight that had never been reported was observed in the selected ion chromatogram of the steviol glycoside (m/z 1095.4) in which the modified sugar chain contained three glucose moieties (Glc) and two rhamnose moieties (Rha). Specifically, the peak at Rt 28.73 minutes shown in Figure 2 was a unknown substance. For Sample 3 whose rebaudioside C content was lower than the content of rebaudioside A and whose sugar chain elongation was shorter than other samples, the peak value at Rt 28.73 minutes was lower than the detection limit.

[0100] Figure 3 shows a selected ion chromatogram of Sample 1 (EM3-4) at m/z of 1257.5. A peak of a molecular weight that had never been reported was observed in the selected ion chromatogram of the steviol glycoside (m/z 1257.5) in which the modified sugar chain contained four glucose moieties (Glc) and two rhamnose moieties (Rha). Specifically, the peak at Rt 28.50 minutes shown in Figure 3 was a unknown substance. For Sample 4 whose rebaudioside C content was lower than the content of rebaudioside A and whose sugar chain elongation was shorter than other samples, the peak value at Rt 28.50 minutes was lower than the detection limit.

[0101] [Structural analysis of novel steviol glycoside]

According to the present invention, structural analyses of Novel steviol glycosides 1 and 2 detected in a cultiver with high rebaudioside C content were performed according to the following procedure.

(i) Structural deduction by a fragmentation analysis through high performance liquid chromatography (HPLC)-high resolution mass spectrometry (MS), MS/MS, and three-stage ion fragmentation (MS^3 fragmentation).

(ii) Chemical synthesis of the deduced steviol glycoside standard products via chemical reaction.

(iii) Structural confirmation by matching with the chemically synthesized standard products with respect to the retention time and the fragmented pattern from HPLC-high resolution MS and MS^3 fragmentation

[0102] Hereinafter, each of Steps (i)-(iii) above will be described in detail.

[0103] (i) Structural deduction by a fragmentation analysis through high performance liquid chromatography (HPLC)-high resolution mass spectrometry (MS), MS/MS, and three-stage ion fragmentation (MS^3 fragmentation)

A process for preparing test liquids were as follows: 10.0 mg each of lyophilized dried stevia leaves was weighed into a glass vial, to which 1.0 mL of water/methanol (1/1 vol/vol) was added as an extracting solvent, and then the resultant was subjected to

ultrasonic irradiation in an ultrasonic cleaner (AS ONE, AS52GTU) at a set temperature of 25°C for 20 minutes, thereby obtaining a liquid extract of a steviol glycoside from the stevia leaves. The resultant was further 10-fold diluted with water/methanol and filtrated through a filter with a pore size of 0.45 μm (Nacalai tesque, Cosmonice filter S (solvent)) before being subjected to HPLC-MS.

[0104] In an equipment configuration for high performance liquid chromatography-electrospray ionization-high resolution mass spectrometry (HPLC-ESI-HRMS), equipment for HPLC was configured by using Prominence LC-20AD (Shimadzu Corporation) as a liquid delivery unit LC pump and SM-C18 (4.6 x 250 mm) (from Imtakt) as a separation column. The LC mobile phase was delivered using 0.2% acetic acid-containing Milli-Q water as mobile phase A and methanol as mobile phase B, where the binary gradient was such that the concentration of the mobile phase B was constantly maintained at 10% during 0-5 minutes, shifted from 10% to 70% in the next 15 minutes, and further shifted from 70% to 100% in the following 5 minutes. Finally, the concentration of the mobile phase B was maintained at 100% for 5 minutes to end. The flow rate of the mobile phase was 0.4 mL/min, and the stevia leaf liquid extract that had been diluted and subsequently filtrated with a filter was injected for 20 μL. For the mass spectrometry part, Orbitrap Elite MS (from Thermo Fisher Scientific) equipped with an ESI ion source was used. The mass spectrometry measurement was carried out in a negative ion measurement mode at m/z in a range of 150-2000 with resolution set to 60,000. The MS/MS measurement was carried out by selecting the targeted m/z of 1095.4 or 1257.5 and in a CID mode where fragmentation was induced by collision with an inert gas. The ion with the highest intensity in the MS/MS spectrum was targeted for MS^3. Irradiation of energy required for fragmentation was performed at the standard collision energy unique to the apparatus, i.e., 35.

[0105] In order to study the fragmented pattern of Novel steviol glycosides 1 and 2, standard samples rebaudiosides A, D and M with known structures were subjected to MS/MS and MS^3 fragmentation pattern analyses. As a result, MS/MS of the novel steviol glycoside gave data showing that the highest ion intensity appeared at the peak where all sugar chains attached to C-19 via an ester bond were released. This result represents the total molecular weight of the sugar chains attached to the carbon of C-19 via an ester bond.

[0106] The MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 1 (corresponding to m/z 1095.4, Rt:28.73) are shown in Figure 4. In the MS/MS spectrum of the novel steviol glycoside, the main peak was detected at m/z of 787.38 corresponding to release of one Glc moiety and one Rha moiety. From these results, the number of

sugar chains attached to the carbon of C-19 via an ester bond was found to be one Glc moiety and one Rha moiety. In order to acquire further structural information, a MS^3 spectrum was acquired by fragmenting the main peak at m/z of 787.4 obtained by MS/MS. As a result, a spectrum having the same peak pattern as the MS^3 spectrum of rebaudioside C (949.4→787.4→) was acquired. Accordingly, the sugar chains attached to C-13 were presumed to be the same as rebaudioside C. The deduced structure is shown in Figure 4.

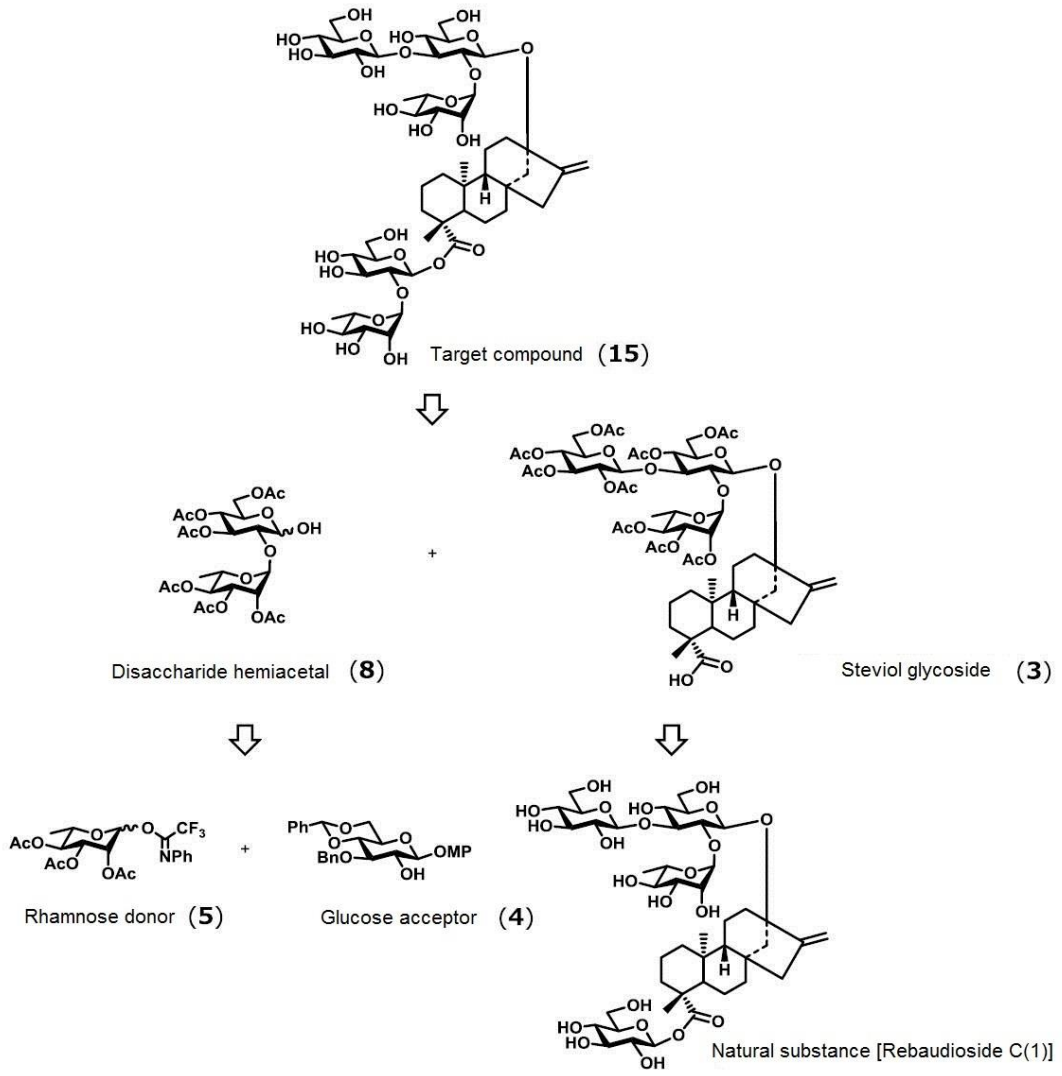
[0107] The MS/MS and MS^3 fragmented mass spectra of Novel steviol glycoside 2 (corresponding to m/z 1257.5, Rt:28.50) are shown in Figure 5. In the MS/MS spectrum of the novel steviol glycoside, the main peak was detected at m/z of 787.38 corresponding to release of two Glc moieties and one Rha moiety. From these results, the number of sugar chains attached to the carbon of C-19 via an ester bond was found to be two Glc moieties and one Rha moiety. In order to acquire further structural information, a MS^3 spectrum was acquired by fragmenting the main peak at m/z of 787.4 obtained by MS/MS. As a result, a spectrum having the same peak pattern as the MS^3 spectrum of rebaudioside C (949.4→787.4→) was acquired. Accordingly, the sugar chains attached to C-13 were presumed to be the same as rebaudioside C. The deduced structure is shown in Figure 5.

[0108] (ii) Chemical synthesis of deduced steviol glycoside standard product by chemical reaction

[Synthesis of Novel steviol glycoside 1]

(1) Outline of synthetic pathways

Scheme 7: Strategy for synthesizing Novel steviol glycoside 1



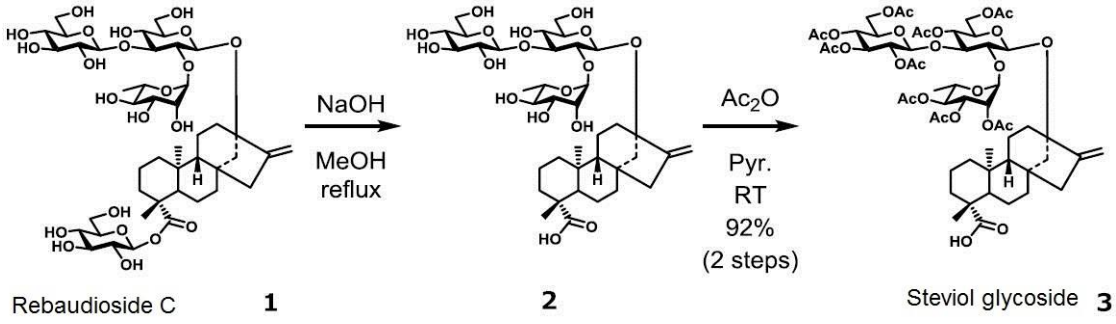
As can be appreciated from Scheme 7, for the synthesis of Novel steviol glycoside 1 (Compound 15), the steviol glycoside (Compound 3) and the disaccharide hemiacetal (Compound 8) were condensed via the Mitsunobu reaction to obtain the backbone of Novel steviol glycoside 1 (Compound 15). For synthesis of the steviol glycoside, a known natural substance, rebaudioside C (Compound 1), was purchased from Ark Pharm, the ester bond at C-19 of steviol was subjected to alkaline hydrolysis and then the hydroxyl groups of the sugar chain were protected with acetyl (Ac) groups to obtain the steviol glycoside. For synthesis of the disaccharide hemiacetal, a disaccharide backbone was produced by condensation reaction between an appropriately protected glucose acceptor (Compound 4) and a rhamnose donor (Compound 5), and the protecting group at the anomeric carbon of the reducing end was deprotected to give the disaccharide hemiacetal.

The resulting steviol glycoside and disaccharide hemiacetal were subjected to condensation via the Mitsunobu reaction, where the reaction proceeded with good yield and high β-selectivity of 75% (α/β= 1/20). The protecting groups of the resulting compound were deprotected, thereby obtaining Novel steviol glycoside 1 (15).

Next, each of the synthesis steps will be described.

[0109] (2) Synthesis of steviol glycoside

Scheme 8: Synthesis of steviol glycoside



As can be appreciated from Scheme 8, for synthesis of the steviol glycoside (Compound 3), rebaudioside C (Compound 1) (1.0 g, 1.05 mmol) purchased from Ark Pharm was dissolved in methanol (10 mL) and water (10 mL), added with 4 mol/L of sodium hydroxide (2.6 mL, 10.4 mmol) at room temperature, and refluxed at 100ºC for 20 hours. After confirming the completion of the reaction by TLC (chloroform/methanol/water = 5/4/0.1, Rf value = 0.9), the reaction solution was neutralized with cation exchange resin Dowex MAC-3 hydrogen form (SIGMA-ALDRICH) (pH 7). After the resin was removed by filtration, the resultant was concentrated under a reduced pressure. The resulting syrup was dried for 18 hours by using a vacuum pump to give Compound 2 (828 mg, quant.).

[0110] Compound 2 (828 mg, 1.05 mmol) was dissolved in pyridine (20 mL), added with acetic anhydride (5 mL) at room temperature and agitated at room temperature for 48 hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 2/1, Rf value = 0.5), a saturated sodium hydrogen carbonate solution (5 mL) was added, and the reaction solution was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate (ethyl acetate/hexane = 2/1) was used to give Compound 3 (1.1 g, 92 %).

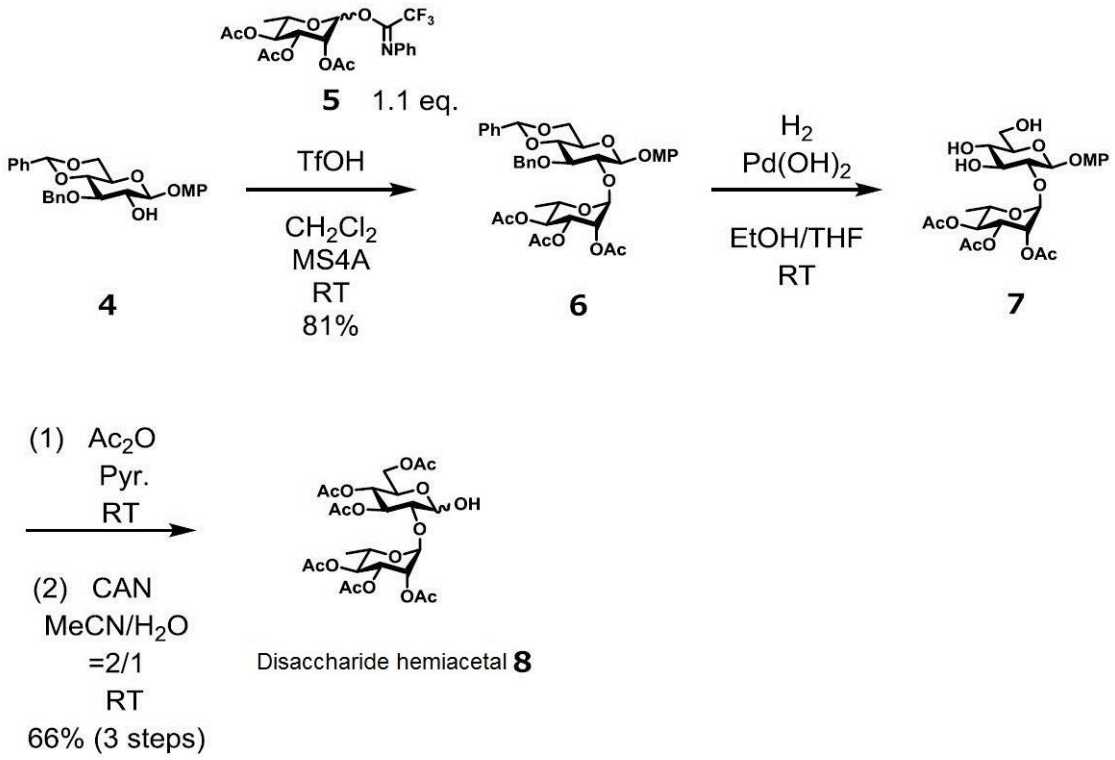
[Compound 3]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.81 (m, 2H), 0.83-1.45 (complex, 19H), 1.39-1.91

(complex, 24H), 1.91-2.35 (s, 30H), 3.58 (m, 1H), 3.71-3.81 (complex, 4H), 3.95-4.12 (complex, 7H), 4.34-4.46 (complex, 3H), 4.56-4.66 (complex, 4H), 4.69-4.92 (complex, 7H), 5.05-5.14 (complex, 5H), 5.23-5.38 (complex, 6H), 5.45 (s, 1H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 15.9, 17.3, 19.1, 20.5, 20.7, 20.8, 20.9, 21.1, 21.5, 21.7, 29.1, 37.8, 38.0, 39.5, 40.7, 41.4, 42.2, 43.8, 48.4, 53.8, 56.8, 61.6, 63.0, 65.5, 66.8, 68.0, 68.6, 69.3, 69.6, 69.8, 70.5, 70.9, 71.6, 71.9, 72.4, 72.8, 73.9, 74.9, 81.3, 87.3, 96.6, 96.8, 99.2, 99.4, 125.4, 128.3, 129.1, 137.9, 151.9, 168.9, 169.2, 169.5, 169.6, 169.8, 170.1, 170.2, 170.3, 170.6, 170.9, 176.8, 183.4.

[0111] (3) Synthesis of disaccharide hemiacetal

Scheme 9: Synthesis of disaccharide hemiacetal



As can be appreciated from Scheme 9, for synthesis of the disaccharide hemiacetal (Compound 8), 4-methoxyphenyl 3-O-benzyl-4,6-O-benzylidine-β-D-glucopyranoside (Compound 4) (3.0 g, 6.46 mmol) purchased from Tokyo Chemical Industry, Compound 5 (3.3 g, 7.10 mmol) and 4Å molecular sieves (6.0 g) were dissolved in dichloromethane (136 mL), added with trifluoromethanesulfonic acid (114 μL, 1.29 mmol) at room temperature, and agitated at room temperature for 18 hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 1/2, Rf value = 0.5), the resultant was neutralized with triethylamine (100 μL) (pH 8), 4Å molecular sieves was removed by filtration, and the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate

(ethyl acetate/hexane = 1/1.5) was used to give Compound 6 (3.9 g, 81%).

[Compound 6]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.18 (d, J = 6.4 Hz, 3H, H-6 of Rha), 1.96 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.07 (s, 3H, OAc), 3.51 (m, 1H, H-5), 3.73-3.81 (complex, 5H, H-4, H-6, OMe), 3.83-3.96 (complex, 2H, H-2, H-3), 4.28 (m, 1H, H-5 of Rha), 4.36 (m, 1H, H-6'), 4.70 (d, 1H, CH_2Ph), 4.95 (d, 1H, CH_2Ph), 4.99 (d, J = 7.2 Hz, 1H, H-1), 5.03 (t, 1H, H-4 of Rha), 5.20 (dd, 1H, H-3 of Rha), 5.32 (s, 1H, H-1 of Rha), 5.34 (m, 1H, H-2 of Rha), 5.57 (s, 1H, CHPh), 6.90 (dd, 4H, OMePh), 7.21-7.49 (complex, 10H, Ph); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 14.2, 17.4, 20.8, 20.9, 21.0, 22.8, 31.7, 55.8, 66.2, 66.7, 68.8, 69.4, 69.5, 71.0, 75.2, 76.7, 77.5, 81.7, 81.8, 98.4, 100.8, 101.4, 114.8, 118.3, 126.1, 127.9, 128.4, 128.5×2, 129.2, 137.2, 137.9, 150.8, 155.7, 169.9, 170.1, 170.2.

[0112] Compound 6 (3.9 g, 5.29 mmol) was dissolved in ethanol (27 mL) and THF (27 mL), added with palladium hydroxide (2.0 g) at room temperature, and agitated in an hydrogen atmosphere at room temperature for 18 hours. After confirming the completion of the reaction by TLC (chloroform/methanol = 10/1, Rf value = 0.2), palladium hydroxide was removed by filtration and the filtrate was concentrated under a reduced pressure to give Compound 7 (2.9 g, quant.).

[0113] Compound 7 (1.1 g, 1.97 mmol) was dissolved in pyridine (20 mL), added with acetic anhydride (740 μL, 7.88 mmol) at room temperature, and agitated at room temperature for 24 hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 1/1, Rf value = 0.6), azeotropic distillation with toluene (30 mL) was repeated for three times. Subsequently, the resultant was concentrated under a reduced pressure and the resulting syrup was dissolved in acetonitrile (14 mL) and water (7.0 mL), added with cerium ammonium nitrate (3.2 g, 5.91 mmol) at room temperature, and agitated at room temperature for 30 minutes. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 1.5/1, Rf value = 0.5), the resultant was diluted with ethyl acetate, the organic layer was washed with water and a saturated aqueous sodium hydrogen carbonate solution, and dried with magnesium sulfate. Magnesium sulfate was removed by filtration and the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate (ethyl acetate/hexane = 1/1) and (toluene/ethyl acetate = 3/1) were used to give Compound 8 (721 mg, 66%, 3 steps).

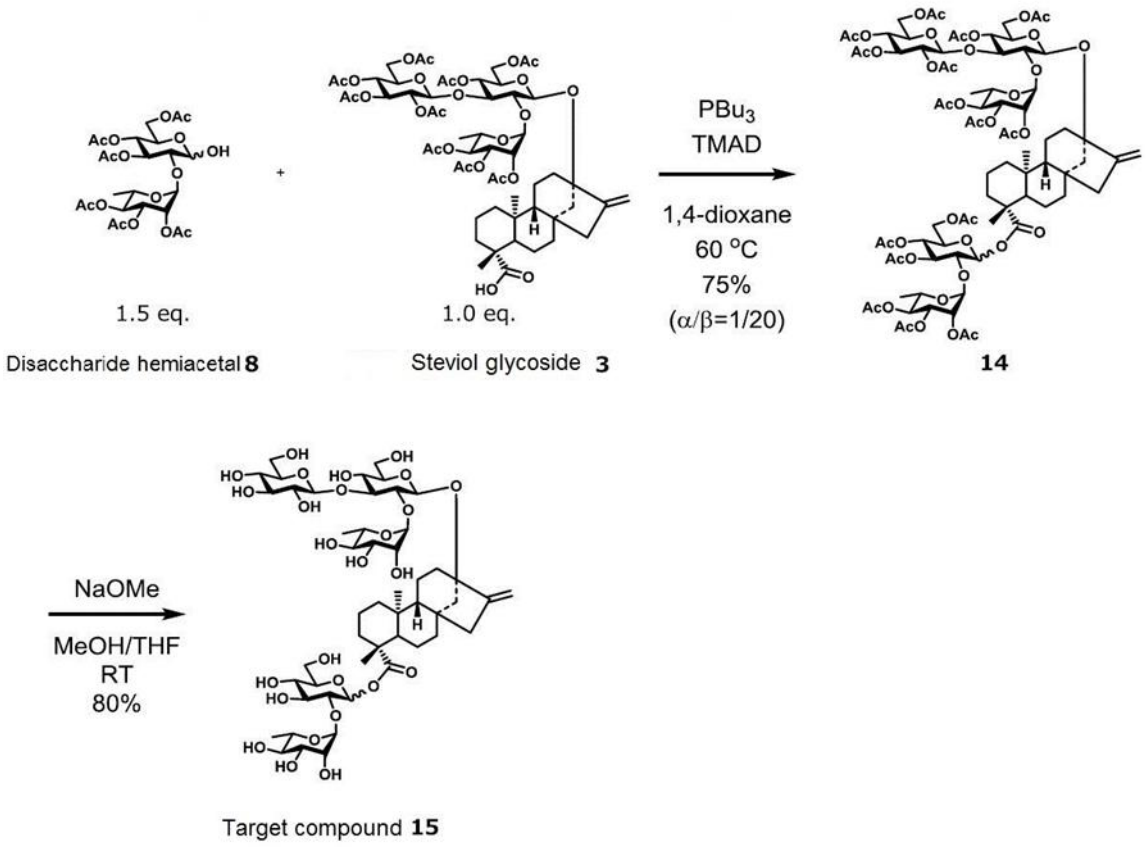
[Compound 8]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.16-1.19 (complex, 4.5H, H-6α of Rha, H-6β of Rha), 1.97-2.34 (complex, 27 H, OAc), 3.58 (t, 0.5H, H-2β), 3.72-3.75 (complex, 1.5H, H-

2α, H-5β), 4.00 (m, 1H, H-4α of Rha), 4.05-4.16 (complex, 1.5H), 4.21-4.27 (complex, 3H), 4.76 (d, J = 7.6 Hz, 0.5H, H-1β), 4.86 (s, 1H, H-1α of Rha), 4.91 (s, 0.5H, H-1β of Rha), 4.98-5.08 (complex, 4.6H), 5.23-5.26 (complex, 2H), 5.34 (d, J = 3.2 Hz, 1H, H-1α), 5.48 (t, 1H, H-3α); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 17.2, 17.5, 20.7×2, 20.8×3, 20.9×2, 21.0, 21.6, 62.1, 62.2, 67.2, 67.3, 67.5, 68.5, 68.6, 68.7, 70.0, 70.4, 71.0, 74.2, 77.4, 77.9, 79.3, 92.0, 75.7, 98.4, 99.2, 125.4, 128.3, 129.1, 137.9, 169.8, 169.9×2, 170.0, 170.1×2, 170.2, 170.4, 170.9×2.

[0114] (4) Synthesis of Compound 15

Scheme 10: Synthesis of Novel steviol glycoside 1 (Target compound 15)



As can be appreciated from Scheme 10, for synthesis of Compound 15, Compound 8 (291 mg, 0.503 mmol) and Compound 3 (391 mg, 0.335 mmol) were dissolved in 1,4-dioxane (17 mL), added with tributylphosphine (252 μL, 1.01 mmol) and 1,1'-azobis (N,N'-dimethylformamide) (TMAD) (173 mg, 1.01 mmol) at room temperature, and agitated at 60ºC for 6 hours. After confirming the completion of the reaction by TLC (toluene/ethyl acetate = 1/1, Rf value = 0.4), the resultant was diluted with ethyl acetate, the organic layer was washed with water, a saturated aqueous sodium hydrogen carbonate solution and saturated saline, and dried with magnesium sulfate. Magnesium sulfate was removed by filtration, and the resultant was concentrated under a

reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate (toluene/ethyl acetate = 1.5/1) was used to give Compound 14 (435 mg, 75%, α/β= 1/20).

[Compound 14]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.50-1.18 (complex, 7H), 1.15 (d, 3H, H-6 of Rham), 1.24 (s, 3H), 1.40-2.32 (complex, 70H), 3.60 (m, 1H), 3.73 (m, 2H), 3.82-4.28 (complex, 10H), 4.40-4.48 (complex, 2H), 4.63 (d, J = 7.6 Hz, 1H), 4.72 (d, J = 8.0 Hz, 1H), 4.75-4.88 (complex, 3H), 4.98 (s, 1H), 5.01-5.18 (complex, 8H), 5.24-5.31 (complex, 4H), 5.32 (s, 1H), 5.71 (d, J = 7.6 Hz, 1H, H-1β), 6.31 (d, J = 3.0 Hz, 0.05H, H-1α); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 16.6, 17.4, 17.6, 20.5, 20.7×2, 20.8×3, 20.9×3, 21.6, 29.0, 39.5, 42.5, 44.1, 53.8, 57.9, 61.7, 66.6, 67.4, 68.0, 68.3, 68.5, 68.7, 69.7, 69.8, 70.8, 71.1, 71.4, 71.9×2, 72.4, 72.9, 74.2, 75.1, 86.6, 92.2, 96.4, 96.9, 97.7, 99.3, 125.4, 128.3, 129.1, 152.9, 169.0, 169.5, 169.8×2, 169.9, 170.0, 170.1, 170.2×2, 170.3, 170.5, 170.6, 170.9×2, 174.6.

[0115] Compound 14 (435 mg, 0.252 mmol) was dissolved in methanol (2.0 mL) and THF (2.0 mL), added with sodium methoxide (0.5M in MeOH) (0.5 mL, 0.252 mmol) at room temperature, and agitated at room temperature for 18 hours. After confirming the completion of the reaction by TLC (chloroform/methanol/water = 5/4/1, Rf value = 0.4), the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to gel filtration column (GE Healthcare, Sephadex LH-20, ethanol) to give Compound 15 (220 mg, 80%, α/β= 1/20). Subsequently, the β-form of Compound 15 was isolated by preparative HPLC (C18 YMC HPLC column, 20 mM aqueous ammonium acetate solution/90% acetonitrile/10% water = 70/30-10/90, 45 minutes), and subjected to lyophilization. The structural analysis data of Compound 15 by nuclear magnetic resonance (NMR) method is shown in Figures 6-9.

[Compound 15 (the β-form)]

^{1}H-NMR (pyridine-d5, 800 MHz) δ 0.68 (m, 1H), 0.86 (m, 1H), 0.98 (m, 1H), 1.11-1.15 (complex, 4H), 1.24 (m, 2H), 1.39 (m, 2H), 1.49 (s, 3H), 1.62 (m, 3H), 1.71 (d, 3H), 1.75 (d, 3H), 1.88 (m, 1H), 1.93 (m, 1H), 2.00 (m, 2H), 2.11 (m, 2H), 2.19 (m, 2H), 2.46 (m, 1H), 2.66 (m, 1H), 3.62 (m, 1H), 3.91 (m, 1H), 3.97-4.10 (complex, 5H), 4.17-4.42 (complex, 11H), 4.49 (m, 1H), 4.51-4.59 (complex, 3H), 4.73 (m, 1H), 4.82 (m, 1H), 4.87 (m, 1H), 4.98 (d, J = 8.0 Hz, 1H), 5.08 (s, 1H), 5.10 (d, J = 7.2 Hz, 1H), 5.66 (s, 1H), 6.26 (d, J = 7.2 Hz, 1H), 6.41 (s, 1H), 6.47 (s, 1H); ^{13}C-NMR (pyridine-d5, 200 MHz) δ 17.1, 19.2×2, 20.1, 20.9, 22.3, 29.6, 37.9, 38.2, 39.9, 40.9, 41.9, 42.8, 43.7, 44.5, 48.4, 54.1, 58.3, 62.3, 62.4, 62.5, 69.8×2, 70.2, 71.2, 71.6, 72.4, 72.5, 72.6, 74.0, 74.1, 75.2, 76.4, 76.8, 77.4, 78.5, 78.8, 79.0, 79.5, 86.9, 89.8, 94.0, 98.4, 101.8, 101.9, 104.4, 105.3, 154.5, 176.2.

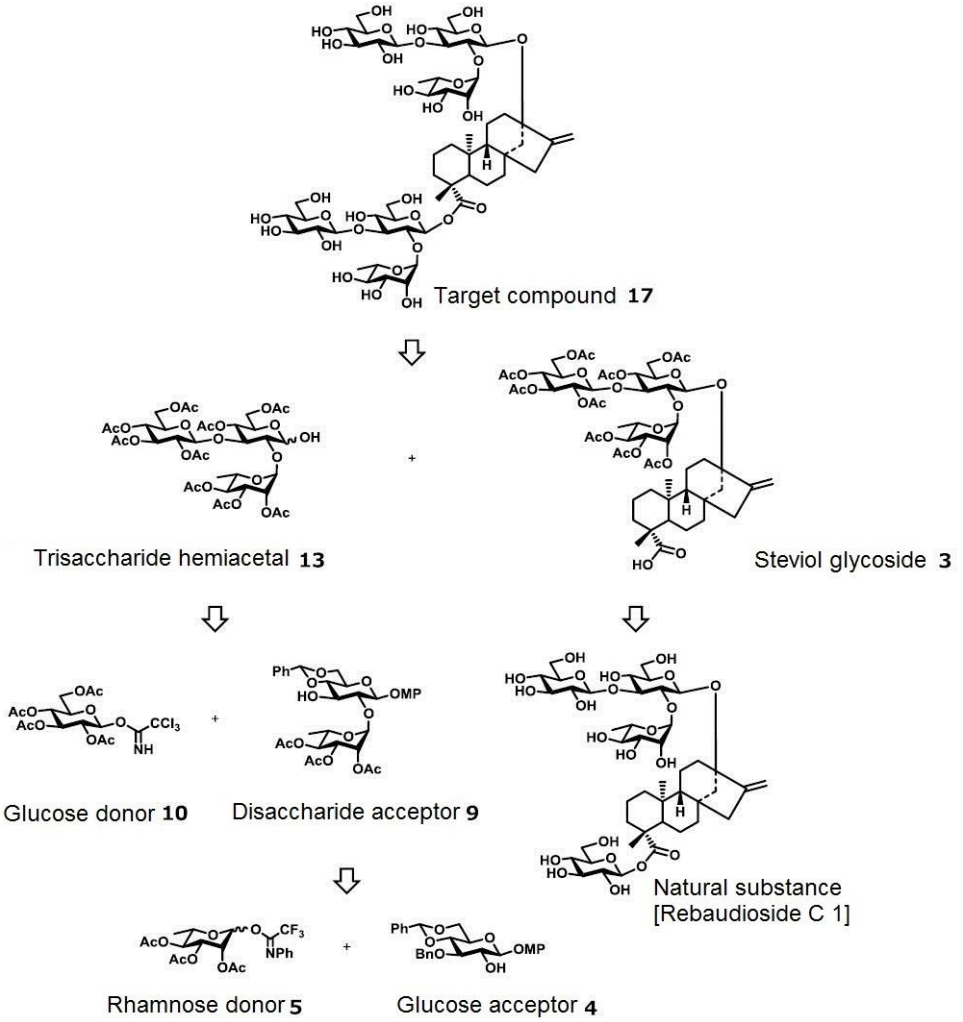
$[\alpha]_D$ = -46.3º (c 0.05, MeOH)

MALDI-TOF-MS m/z found $[M + Na]^+$ 1119.5, $C_{50}H_{80}O_{26}$ calcd for $[M + Na]^+$ 1119.5.

[0116] [Synthesis of Novel steviol glycoside 2]

(1) Outline of synthetic pathways

Scheme 11: Strategy for synthesizing Novel steviol glycoside 2



As can be appreciated from Scheme 11, for the synthesis of Novel steviol glycoside 2 (Compound 17), the steviol glycoside (Compound 3) and the trisaccharide hemiacetal (Compound 13) were condensed via the Mitsunobu reaction to obtain the backbone of Novel steviol glycoside 2. For synthesis of the steviol glycoside, a known natural substance, rebaudioside C (Compound 1), was purchased from Ark Pharm, the ester bond at C-19 of steviol was subjected to alkaline hydrolysis and then the hydroxyl groups of the sugar chain were protected with acetyl (Ac) groups to obtain the steviol glycoside. For synthesis of the trisaccharide hemiacetal, a disaccharide acceptor (Compound 9) was

synthesized by condensation reaction between an appropriately protected glucose acceptor (Compound 4) and a rhamnose donor (Compound 5), which was subjected to condensation reaction with a glucose donor (Compound 10) to give the trisaccharide backbone. The protecting group at the anomeric carbon of the reducing end of the resulting trisaccharide was deprotected to give the trisaccharide hemiacetal. The steviol glycoside and the trisaccharide hemiacetal were subjected to condensation via the Mitsunobu reaction, where the reaction proceeded with good yield and high β-selectivity of 44% (α/β= 1/10). The protecting groups of the resulting compound were deprotected, thereby obtaining Novel steviol glycoside 2.

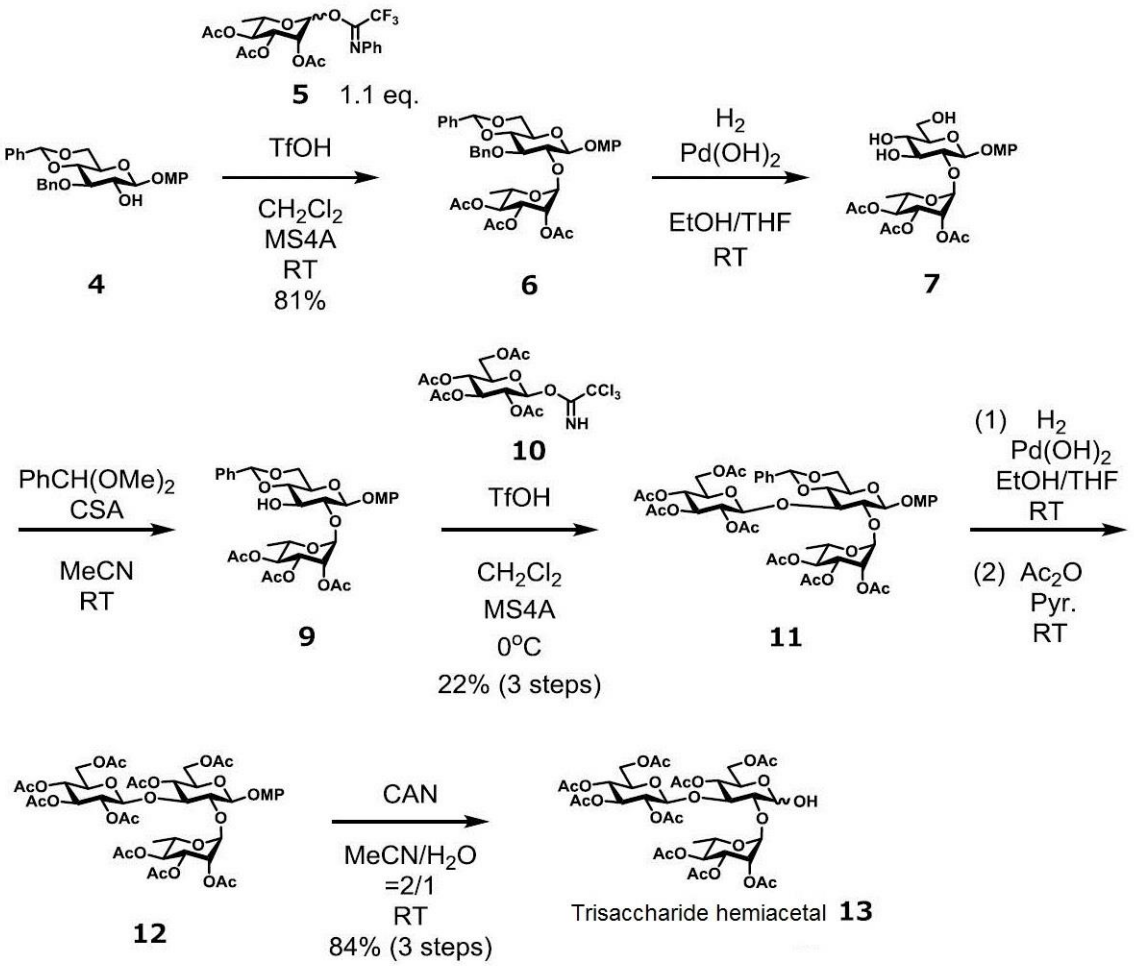
Next, each of the synthesis steps will be described.

[0117] (2) Synthesis of steviol glycoside

Steviol glycoside was synthesized in the same manner as "Synthesis of Novel steviol glycoside 1".

[0118] (3) Synthesis of trisaccharide hemiacetal

Scheme 12: Synthesis of trisaccharide hemiacetal



As can be appreciated from Scheme 12, Compounds 6 and 7 were obtained in the

same manner as "Synthesis of Novel steviol glycoside 1".

[0119] Compound 7 (1.7 g, 3.04 mmol) was dissolved in acetonitrile (30 mL), added with benzaldehyde dimethyl acetal (681 μL, 4.57 mmol) at room temperature, and agitated at room temperature for 2 hours. The completion of the reaction was confirmed by TLC (ethyl acetate/hexane = 1/1, Rf value = 0.7). The resultant was neutralized with triethylamine (2 mL) (pH 8), and then concentrated under a reduced pressure to give Compound 9 (2.0 g).

[0120] Compound 9 (2.0 g, 3.04 mmol), Compound 10 (1.6 g, 3.34 mmol) and 4Å molecular sieves (4.0 g) were dissolved in dichloromethane (64 mL), added with trifluoromethanesulfonic acid (114 μL, 1.29 mmol) at room temperature, and agitated at 0ºC for 6 hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 1/1, Rf value = 0.4), the resultant was neutralized with triethylamine (100 μL) (pH 8), 4Å molecular sieves was removed by filtration, and the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate (ethyl acetate/hexane = 1/2) was used to give Compound 11 (650 mg, 22%, 3 steps).

[Compound 11]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.16 (d, J = 6.0 Hz, 1H, H-6 of Rha), 1.94-2.19 (complex, 21H, OAc), 3.43-3.52 (complex, 2H), 3.62-3.81 (complex, 5H, OMe), 3.96-4.04 (complex, 2H), 4.07-4.18 (complex, 3H), 4.28-4.35 (complex, 2H), 4.86 (d, J = 8.0 Hz, 1H, H-1), 4.94-4.98 (complex, 2H, H-1), 5.01-5.11 (complex, 2H), 5.16-5.21 (complex, 2H), 5.28 (s, 2H, H-1 of Rha), 5.52 (s, 1H, PhCH), 6.90 (dd, 4H, PhOMe), 7.31-7.49 (complex, 5H, Ph); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 14.3, 17.3, 20.4, 20.7, 20.9×3, 21.0, 21.1, 55.8, 60.5, 62.0, 66.3, 66.9, 68.3, 68.7, 69.3, 69.4, 70.7, 71.6, 71.9, 72.9, 78.9, 81.0, 97.7, 99.3, 100.7, 101.6, 114.8, 118.4, 126.2, 128.4, 129.4, 137.1, 150.7, 155.8, 169.4, 169.5, 170.0, 170.2, 170.4, 170.5, 170.8, 171.2.

[0121] Compound 11 (627 mg, 0.642 mmol) was dissolved in ethanol (3 mL) and THF (3 mL), added with palladium hydroxide (1.0 g) at room temperature, and agitated in an hydrogen atmosphere at room temperature for two hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 2/1, Rf value = 0.2), palladium hydroxide was removed by filtration, and the filtrate was concentrated under a reduced pressure. Subsequently, the resultant was dissolved in pyridine (6.4 mL), added with acetic anhydride (182 μL, 1.93 mmol) at room temperature, and agitated at room temperature for 18 hours. After confirming the completion of the reaction by TLC (ethyl acetate/hexane = 2/1, Rf value = 0.7), azeotropic distillation with toluene (20 mL) was

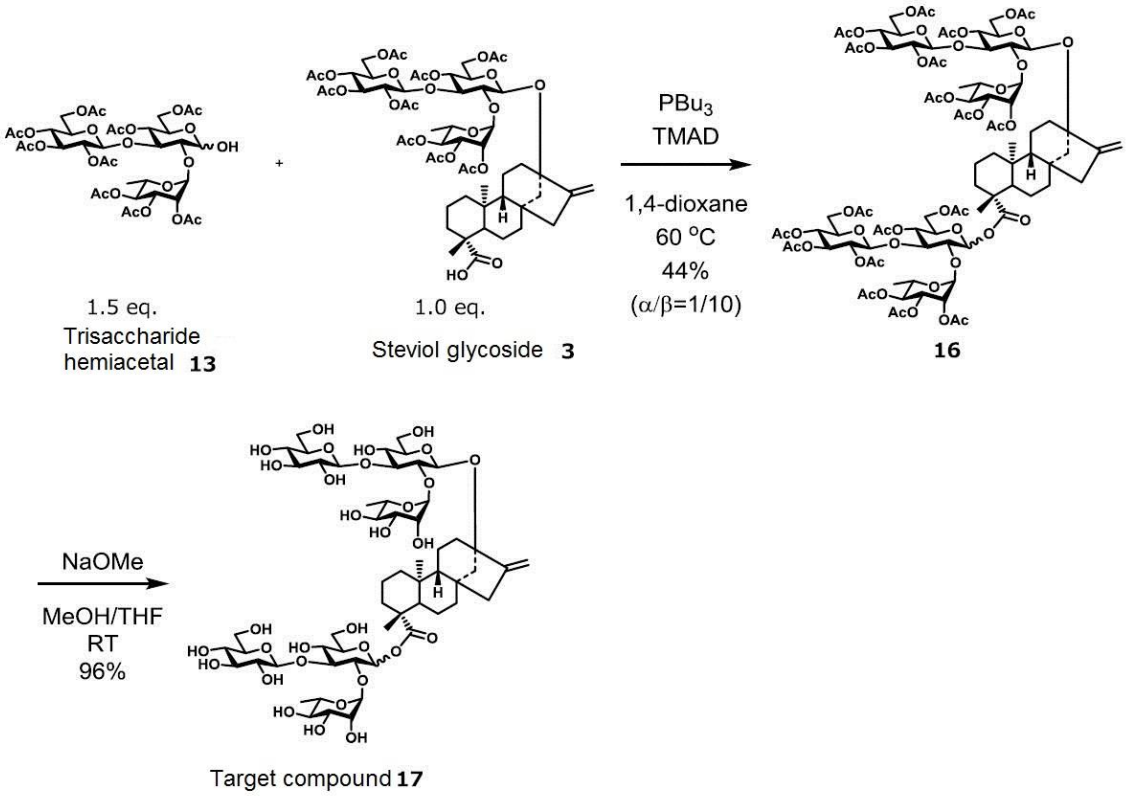
repeated for three times. Subsequently, the resultant was concentrated under a reduced pressure. The resulting syrup was dissolved in acetonitrile (4 mL) and water (2 mL), added with cerium ammonium nitrate (1.0 g, 1.87 mmol) at room temperature, and agitated at room temperature for 30 minutes. After confirming the completion of the reaction by TLC (toluene/ethyl acetate = 1/1, Rf value = 0.3), the resultant was diluted with ethyl acetate, the organic layer was washed with water and a saturated aqueous sodium hydrogen carbonate solution, and dried with magnesium sulfate. Magnesium sulfate was removed by filtration, and the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography and an eluate (toluene/ethyl acetate = 1/1) was used to give Compound 13 (468 mg, 84%, 3 steps).

[Compound 13]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.18 (d, J = 6.4 Hz, 1H, H-6 of Rha), 1.93-2.19 (complex, 27H, OAc), 3.60 (t, 1.6H, H-2β), 3.68 (m, 1.2H, H-5β), 3.78 (m, 1.4H, H-5'β), 3.97 (t, 1H, H-3β), 4.03 (dd, 1H, H-6'β), 4.15 (m, 2.3H, H-6β, H-6β), 4.22 (m, 1.2H, H-5β of Rha), 4.43 (dd, 1.2H, H-6'β), 4.67 (d, J = 7.6 Hz, 1H, H-1β), 4.77 (d, J = 8.0 Hz, 1H, H-1'β), 4.82-4.91 (complex, 2.4H, H-4β, H-2'β), 5.06-5.14 (complex, 2.3H, H-4'β, H-4β of Rha), 5.16 (s, 1H, H-1 of Rha), 5.26-5.33 (complex, 2.4H, H-3'β, H-3β of Rha), 5.39 (m, 1.1H, H-2β of Rha); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 17.3, 17.5, 20.6, 20.7×2, 20.8×3, 20.9×2, 21.6, 29.8, 31.1, 61.7, 61.8, 62.4, 67.4, 67.6, 67.9, 68.0, 68.1×2, 68.3, 68.8×2, 69.5, 69.9, 70.6×2, 71.5, 71.8, 71.9, 72.1, 72.3, 72.8, 73.0, 75.5, 79.9, 80.1, 80.5, 91.9, 95.8, 98.1, 98.9, 99.6, 99.8, 125.4, 128.4, 129.2, 169.1, 169.4, 169.5, 169.6, 169.7, 170.0×3, 170.1, 170.4, 170.5×2, 170.7.

[0122] (4) Synthesis of Compound 17

Scheme 13: Synthesis of Novel steviol glycoside 2 (Compound 17)



As can be appreciated from Scheme 13, for synthesis of Compound 17, Compound 13 (468 mg, 0.540 mmol) and Compound 3 (420 mg, 0.360 mmol) were dissolved in 1,4-dioxane (18 mL), added with tributylphosphine (270 μL, 1.08 mmol) and 1,1'-azobis (N,N'-dimethylformamide) (TMAD) (186 mg, 1.08 mmol) at room temperature, and agitated at 60ºC for 6 hours. After confirming the completion of the reaction by TLC (toluene/ethyl acetate = 1/1, Rf value = 0.4), the resultant was diluted with ethyl acetate, the organic layer was washed with water, a saturated aqueous sodium hydrogen carbonate solution and saturated saline, and dried with magnesium sulfate. Magnesium sulfate was removed by filtration, and the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to silica gel column chromatography, and an eluate (toluene/ethyl acetate = 1.5/1) and (toluene/acetone = 3/1) were used to give Compound 16 (320 mg, 44%, α/β= 1/10).

[Compound 16]

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.45-1.18 (complex, 8H), 1.28 (d, 3H, H-6 of Rham), 1.40-1.81 (complex, 20H), 1.81-2.35 (complex, 54H, OAc), 3.60 (m, 1.2H), 3.71-3.78 (complex, 3H), 3.81-3.91 (complex, 2.4H), 3.98-4.20 (complex, 10H), 4.40-4.50 (complex, 3.4H), 4.62 (d, J = 8.0 Hz, 1.1H, H-1), 4.73 (d, J = 8.0 Hz, 1H, H-1), 4.75-4.91

(complex, 7H), 4.96-5.12 (complex, 7H), 5.17 (s, 1H, H-1 of Rha), 5.21-5.31 (complex, 6H), 5.32 (s, 1H, H-1 of Rha), 5.38 (t, 1.1H), 5.60 (d, J = 8.0 Hz, 1H, H-1β), 6.22 (d, J = 3.0 Hz, 0.1H, H-1α); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 16.7, 17.5, 20.5, 20.7×2, 20.8×2, 20.9×2, 21.0, 21.6, 29.0, 39.6, 42.5, 44.0, 53.9, 58.1, 61.7, 66.7, 67.6, 68.0, 68.1, 68.3, 68.5, 69.8, 70.2, 70.7, 71.2, 71.4, 71.8, 71.9, 72.2, 72.4, 72.8, 72.9, 75.2, 80.3, 81.4, 86.6, 92.2, 96.4, 96.9, 99.3, 99.8, 125.4, 128.4, 129.2, 138.0, 152.9, 169.0, 169.3, 169.5×2, 169.6×2, 169.8, 170.1×3, 170.2, 170.5, 170.6, 170.9×2, 174.7.

[0123] Compound 16 (300 mg, 0.149 mmol) was dissolved in methanol (2.0 mL) and THF (2.0 mL), added with sodium methoxide (0.5 M in MeOH) (0.3 mL, 0.149 mmol) at room temperature, and agitated at room temperature for 3 hours. After confirming the completion of the reaction by TLC (chloroform/methanol/water = 5/4/1, Rf value = 0.5), the resultant was concentrated under a reduced pressure. The resulting syrup was subjected to gel filtration column (GE Healthcare, Sephadex LH-20, ethanol) to give Compound 17 (188 mg, 96%, α/β= 1/10). Subsequently, the β-form of Compound 17 was isolated by preparative HPLC (C18 YMC HPLC column, 20 mM aqueous ammonium acetate solution/90% acetonitrile/10% water = 70/30-10/90, 45 minutes), and subjected to lyophilization. The structural analysis data of Compound 17 by nuclear magnetic resonance (NMR) method is shown in Figures 10-13.

[Compound 17 (the β-form)]

^{1}H-NMR (pyridine-d5, 800 MHz) δ 0.67 (m, 1H), 0.86 (m, 1H), 0.96 (m, 1H), 1.07 (m, 1H), 1.14 (s, 3H), 1.26 (m, 1H), 1.36 (m, 1H), 1.41 (m, 1H), 1.48 (s, 3H), 1.61 (m, 3H), 1.70 (m, 6H), 1.87-2.21 (complex, 11H), 2.46 (m, 1H), 2.57 (m, 1H), 3.63 (m, 1H), 3.85 (m, 1H), 3.95-4.09 (complex, 8H), 4.14-4.31 (complex, 14H), 4.33 (m, 1H), 4.45 (m, 2H), 4.55 (m, 3H), 4.78 (m, 1H), 4.82 (m, 1H), 4.86 (m, 1H), 4.98 (d, J = 8.0 Hz, 1H), 5.05-5.11 (complex, 3H), 5.66 (s, 1H), 6.17 (d, J = 8.0 Hz, 1H), 6.24 (s, 1H), 6.46 (s, 1H); ^{13}C-NMR (pyridine-d5, 200 MHz) δ 17.0, 19.2, 20.1, 20.9, 22.3, 29.6, 37.8, 38.3, 39.9, 40.8, 41.9, 42.7, 43.7, 44.5, 48.4, 54.2, 58.4, 61.9, 62.4, 62.5, 69.1, 69.8×2, 70.5, 71.6, 71.7, 72.3, 72.4, 72.5×2, 73.9, 74.1, 75.2, 76.4, 76.5, 77.5, 78.3, 78.4, 78.5, 78.8×2, 86.9, 88.8, 89.8, 93.8, 98.4, 101.8, 101.9, 104.4, 104.5, 105.3, 154.4, 176.0.

$[\alpha]_D$ = -44.5° (c 0.1, MeOH)

MALDI-TOF-MS m/z found $[M + Na]^+$ 1281.4, $C_{56}H_{90}O_{31}$ calcd for $[M + Na]^+$ 1281.5.

[0124] (iii) Structural determination by matching with chemically synthesized standard product with respect to retention time and fragmented pattern from HPLC-high resolution MS/MS and MS^3 fragmentation

The chemically synthesized product of Novel steviol glycoside 1 (the β-form of Compound 15) and stevia leaf liquid extracts were compared by HPLC-high resolution MS/MS and MS^3-fragmentation under the same conditions as (i). As a result, the peaks of the chemically synthesized product and the stevia leaf liquid extract were detected at the peak at the retention time of 29.1 minutes (Figure 14). Moreover, they also matched in the respective MS/MS and MS^3 fragmented mass spectra (Figure 15). From this result, Novel steviol glycoside 1 obtained from the liquid extract of the plant was confirmed to have the same structure as the β-form of Compound 15.

[0125] In addition, the chemically synthesized product of Novel steviol glycoside 2 (the β-form of Compound 17) and the stevia leaf liquid extract were compared by HPLC-high resolution MS/MS and MS^3-fragmentation under the same conditions as (i). As a result, the peaks of the chemically synthesized product and the stevia leaf liquid extract were detected at the peak at the retention time of 28.9 minutes (Figure 16). Moreover, they also matched in the respective MS/MS and MS^3 fragmented mass spectra (Figure 17). From this result, Novel steviol glycoside 2 obtained from the liquid extract of the plant was confirmed to have the same structure as the β-form of Compound 17.

[0126] [Biosynthesis of novel steviol glycoside]

A novel steviol glycoside was generated from steviol in yeast. First, a yeast that could coexpress four types of stevia-derived glycosylated enzyme genes UGT85C2, UGT91D2, UGT74G1 and UGT76G1 and *Arabidopsis thaliana*-derived UDP-rhamnose synthase gene AtRHM2 was bred.

[0127] Unless otherwise specified, the molecular biological processes employed in this example followed the methods described in Molecular Cloning (Sambrook et al., Cold Spring Harbour Laboratory Press, 2001).

[0128] In order to clone the four stevia-derived glycosylated enzyme genes, the following primer sets were synthesized to perform PCR using cDNA prepared from stevia leaves as a template.

Primer set for UGT85C2 gene amplification

CACC-NdeI-SrUGT85C2-Fw (NdeI-recognizing site underlined):

5’-CACC<u>CATATG</u>GATGCAATGGCTACAACTGAGAA-3’ (SEQ ID NO:12)

BglII-SrUGT85C2-Rv (BglII-recognizing site underlined):

5’-<u>AGATCT</u>CTAGTTTCTTGCTAGCACGGTGATTT-3’ (SEQ ID NO:13)

Primer set for UGT91D2 gene amplification

SrUGT91D2-pET15b-FW
5’-TGCCGCGCGGCAGCCATATGTACAACGTTACTTATCATC-3’ (SEQ ID NO:35)
SrUGT91D2-pET15b-RV
5’-GTTAGCAGCCGGATCCTTAACTCTCATGATCGATGGCAA-3’ (SEQ ID NO:36)

Primer set for UGT74G1 gene amplification
CACC-NdeI-SrUGT74G1-Fw (NdeI-recognizing site underlined):
5’-CACCCATATGGCGGAACAACAAAAGATCAAGAAAT-3’ (SEQ ID NO:14)
BamHI-SrUGT74G1-Rv (BamHI-recognizing site underlined):
5’-GGATCCTTAAGCCTTAATTAGCTCACTTACAAATT-3’ (SEQ ID NO:15)

Primer set for UGT76G1 gene amplification
CACC-NdeI-SrUGT76G1-Fw (NdeI-recognizing site underlined):
5’-CACCCATATGGAAAATAAAACGGAGACCA-3’ (SEQ ID NO:16)
BamHI-SrUGT76G1-Rv (BamHI-recognizing site underlined):
5’-GGATCCTTACAACGATGAAATGTAAGAAACTA-3’ (SEQ ID NO:17)

[0129] Stevia leaf cDNA was obtained by extracting total RNA from stevia leaves using RNeasy Plant Mini kit (QIAGEN), and subjecting 0.5 μg of them to reverse transcription (RT) reaction using Random Oligo-dT primer.

[0130] The PCR reaction solution (50 μl) had the following composition: 1 μl of stevia leaf-derived cDNA, 1 x KOD plus buffer (TOYOBO), 0.2 mM dNTPs, 0.4 pmol/μl of each primer, 1 mM $MgSO_4$ and 1U heat resistant KOD plus polymerase. PCR reaction consisted of reaction at 95ºC for 5 minutes, followed by amplification by a total of 30 cycles of reaction at 94ºC for 0.5 minutes, 50ºC for 0.5 minutes and 68ºC for 2 minutes. Each PCR product was subjected to electrophoresis with 0.8% agarose gel and ethidium bromide staining, by which an amplification band of nearly 1.4 kb in size was obtained as presumed from each template DNA.

This PCR product was subcloned into pENTR-TOPO Directional vector (Invitrogen) according to a method recommended by the manufacturer. DNA Sequencer model 3100 (Applied Biosystems) was used for sequencing by a primer walking process with a synthesized oligonucleotide primer, thereby confirming that all of the UGT genes of

interest, namely, UGT85C2, UGT91D2, UGT74G1 and UGT76G1 were cloned.

[0131] Construction of yeast expression vector

The following primer sets were designed to integrate these UGT genes and *Arabidopsis thaliana*-derived UDP-rhamnose synthase gene AtRHM2 (J Biol Chem 2007, Oka et. al) into a yeast expression vector.

SrUGT85C2 set

Bgl2-UGT85C2-F (BglII-recognizing site underlined):

5’-ACAGATCTATGGATGCAATGGCTACAACTGAGA-3’ (SEQ ID NO:18)

Sal-UGT85C2-R (SalI-recognizing site underlined):

5’-TAGTCGACTAGTTTCTTGCTAGCACGGTGATTTC-3’ (SEQ ID NO:19)

SrUGT91D2 set

NotI-UGT91DIL3-F (NotI-recognizing site underlined):

5’-AAGCGGCCGCATGTACAACGTTACTTATCATCAAAATTCAAA-3’ (SEQ ID NO:20)

Pac-UGT91D1L3-R (PacI-recognizing site underlined):

5’-CGTTAATTAACTCTCATGATCGATGGCAACC-3’ (SEQ ID NO:21)

SrUGT74G1 set

Not-UGT74G1-F (NotI-recognizing site underlined):

5’-AAGCGGCCGCATGGCGGAACAACAAAAGATCAAG-3’ (SEQ ID NO:22)

Pac-UGT74G1-R (PacI-recognizing site underlined):

5’-CGTTAATTAAGCCTTAATTAGCTCACTTACAAATTCG-3’ (SEQ ID NO:23)

SrUGT76G1 set

Bam-UGT76G1-F (BamHI-recognizing site underlined):

5’-AAGGATCCATGGAAAATAAAACGGAGACCACCG-3’ (SEQ ID NO:24)

Sal-UGT76G1-R (SalI-recognizing site underlined):

5’-GCGTCGACTTACAACGATGAAATGTAAGAAACTAGAGACTCTAA-3’ (SEQ ID NO:25)

AtRHM2 set

Bam-AtRHM2-F (BamHI-recognizing site underlined):

5’-GGATCCATGGATGATACTACGTATAAGCCAAAG-3’ (SEQ ID NO:26)

Xho-AtRHM2-R (XhoI-recognizing site underlined):

5’-CTCGAGTTAGGTTCTCTTGTTTGGTTCAAAGA-3’ (SEQ ID NO:27)

[0132] The combinations of templates and primers, namely, template UGT85C2 and SrUGT85C2 set, template UGT91D2 and SrUGT91D2 set, template UGT74G1 and SrUGT74G1 set, template UGT76G1 and SrUGT76G1 set, and template AtAHM2 and AtAHM2 set, were used for PCR amplification using heat resistant KOD DNA polymerase (TOYOBO) and introduction of the restriction enzyme sites at both ends of each ORF. The resulting DNA fragment was subcloned using Zero Blunt-TOPO PCR cloning kit (Invitrogen), and sequenced using DNA Sequencer model 3100 (Applied Biosystems) by a primer walking process with a synthesized oligonucleotide primer to confirm that each of the UGT genes of interest was cloned.

[0133] In order to allow expressions of the above-described genes in yeasts by using pESC yeast expression system (Stratagene), the following expression vectors were constructed.

(1) Construction of plasmid pESC-URA-UGT56

UGT85C2 was cleaved with restriction enzymes BglII and SalI, and linked to vector pESC-URA (Stratagene) that had been cleaved with restriction enzymes BamHI and SalI to give plasmid pESC-URA-UGT-5. This plasmid pESC-URA-UGT-5 was cleaved with restriction enzymes NotI and PacI while UGT91D2 was also cleaved with restriction enzymes NotI and PacI. The resultants were linked to give pESC-URA-UGT56.

(2) Construction of plasmid pESC-HIS-UGT78

UGT76G1 was cleaved with restriction enzymes BamHI and SalI, and linked to vector pESC-HIS (Stratagene) that had been cleaved with the same restriction enzymes to give plasmid pESC-HIS-UGT-8. This plasmid pESC-HIS-UGT-8 was cleaved with restriction enzymes NotI and PacI while UGT74G1 was also cleaved with NotI and PacI. The resultants were linked to give pESC-HIS-UGT78.

(3) Construction of plasmid pESC-TRP-AtRHM2

AtAHM2 was cleaved with restriction enzymes BamHI and XhoI while vector pESC-TRP (Stratagene) was cleaved with the same restriction enzymes. The resultants were linked to give plasmid pESC-TRP-AtAHM2.

[0134] Transformation of yeast

Plasmids shown in Table 2 were introduced into *Saccharomyces cerevisiae* YPH499 strain (ura3-52 lys2-801^{amber}ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 a) as a host by lithium acetate technique. As transformed strains, those that survived in a SC-

Trp-Ura-His agar medium (6.7 g of yeast nitrogen base without amino acids, 20 g of glucose, 1.3 g of amino acid powder mix-Trp-Ura-His and 20 g of Bacto agar, per 1L) were selected.

Table 2

Transformed strain	Plasmids introduced	Genes introduced
A-5678	pESC-URA-UGT-56 pESC-HIS-UGT-78 pESC-TRP-AtAHM2	SrUGT85C2, SrUGT91D2 SrUGT74G1, SrUGT76G1 AtAHM2

[0135] Here, the amino acid powder mix-Trp-Ura-His was prepared by mixing adenine sulfate (2.5 g), L-arginine hydrochloride (1.2 g), L-aspartic acid (6.0 g), L-glutamic acid (6.0 g), L-leucine (3.6 g), L-lysine (1.8 g), L-methionine (1.2 g), L-phenylalanine (3.0 g), L-serine (22.5 g), L-threonine (12 g), L-tyrosine (1.8 g) and L-valine (9.0 g).

[0136] Induction and analysis of expression of transgene

The resulting transformed strain was cultured as follows.

First, for preliminary culture, each transformed strain was seeded in 10 ml of a SC-Trp-Ura-His liquid medium (SC-Trp-Ura-His agar medium without Bacto agar) and shake cultured at 30ºC for a day. Subsequently, for main culture, 1 ml of the preliminary culture solution was seeded into 10 ml of SG-Trp-Ura-His liquid medium (6.7 g of yeast nitrogen base without amino acids, 20 g of galactose and 1.3 g of amino acid powder mix-Trp-Ura-His, per 1L) and shake cultured at 30ºC for two days.

[0137] In order to confirm whether or not the introduced gene was expressed in the transformed strain, cells were harvested from the culture solution to purify total RNA with RNeasy Mini Kit.

[0138] With 1 μg of total RNA, cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fischer Scientific) and random hexamer as a primer.

[0139] In order to confirm expression of the transgene, the following primers were prepared.

For confirming expression of UGT85C2

UGT85C2-r1:

5’-CAAGTCCCCAACCAAATTCCGT-3’ (SEQ ID NO:28)

For confirming expression of UGT91D2

UGT91D1L3-r1:

5’-CACGAACCCGTCTGGCAACTC-3’ (SEQ ID NO:29)

For confirming expression of UGT74G1
UGT74G1-r1:
5’-CCCGTGTGATTTCTTCCACTTGTTC-3’ (SEQ ID NO:30)

For confirming expression of UGT76G1
UGT76G1-r1:
5’-CAAGAACCCATCTGGCAACGG-3’ (SEQ ID NO:31)

For confirming expression of AtAHM2
AtAHM2-r1
5’-GCTTTGTCACCAGAATCACCATT-3’ (SEQ ID NO:32)

GAL10p region (promoter region)
PGAL10-f3:
5’-GATTATTAAACTTCTTTGCGTCCATCCA-3’ (SEQ ID NO:33)

GAL1p region (promoter region)
PGAL1-f3:
5’-CCTCTATACTTTAACGTCAAGGAGAAAAAACC-3’ (SEQ ID NO:34)

[0140] Expression of each transgene was confirmed by performing PCR by using ExTaq (Taraka Bio) with the following combination of primers and the previously synthesized cDNA as a template and subjecting the resulting product to agarose gel electrophoresis.

UGT85C2: UGT85C2-r1 (SEQ ID NO:28) and PGAL1-f3 (SEQ ID NO:34)
UGT91D2 or UGT91D2L3: UGT91D1L3-r1 (SEQ ID NO:29) and PGAL10-f3 (SEQ ID NO:33)
UGT74G1: UGT74G1-r1 (SEQ ID NO:30) and PGAL1-f3 (SEQ ID NO:34)
UGT76G1: UGT76G1-r1 (SEQ ID NO:31) and PGAL10-f3 (SEQ ID NO:33)
AtAHM2: AtAHM2-r1 (SEQ ID NO:32) and PGAL10-f3 (SEQ ID NO:33)

Accordingly, expression of the transgene in the transformed strain was confirmed.

[0141] Production of novel steviol glycoside

Culturing was performed under the same conditions as described above except that 0.5 μg or 2 μg of steviol (ChromaDex Inc.) was added to the liquid medium for the main culture per 1 ml of the medium. After culturing, the culture solution was separated

into supernatant and cells by centrifugation. The culture supernatant was washed with acetonitrile, then subjected to a water-equilibrated Sep-Pak C18 column, washed with 20% acetonitrile, eluted with 80% acetonitrile, dried to solidify, and then dissolved in a small amount of 80% acetonitrile to prepare a glycoside sample. This glycoside sample was subjected to the following analysis.

[0142] Analysis by LC-MS

An analysis by LC-MS was carried out as described in the example under "Isolation of novel steviol glycoside".

[0143] The results are shown in Figures 18 and 19. Generation of Novel steviol glycosides 1 and 2 was confirmed in A-5678 strain. These results corresponded with the steviol glycosides resulting from the above-described chemical synthesis.

[0144] Evaluation of sweetness level of novel steviol glycoside

In order to evaluate the sweetness level of the novel steviol glycoside, samples were prepared by adding sucrose to pure water to give Brix of 0.5 to 3 in 0.5 increments. A sample was prepared by adding Compound 17 having the same structure as Novel steviol glycoside 2 to pure water to 1,700 ppm. Here, the ratio of the α-form and the β-form contained in Compound 17 was 1: 10 (α: β, molar ratio).

[0145] Evaluation was conducted by selecting the sucrose-added sample having a sweetness intensity equivalent to that of the sample added with the novel steviol glycoside, where sensory evaluation was conducted by panelists trained about sensory attributes of sweeteners (5 members). As a result, the sample prepared by adding Novel glycoside 2 was found to have sweetness equivalent to that of the sucrose-added sample with Brix of 1. Therefore, the novel steviol glycoside of the invention was found to have a sweetness level of 14.7 with respect to sucrose. Although an accurate sweetness level was not acquired for Novel steviol glycoside 1 since it did not dissolve in water sufficiently, Novel steviol glycoside 1 was also confirmed to have sweetness as will be described below.

[0146] Sensory evaluation of novel steviol glycoside (Compound 17)

In order to evaluate the taste quality of various steviol glycosides, Reb.A, Reb.D and Compound 17 having the same structure as Novel steviol glycoside 2 were added to pure water at amounts indicated in Figure 20 to prepare beverage samples. All of the beverage samples were adjusted to have final Brix of 2 in terms of sucrose, provided that the sweetness levels were Reb.A: 300, Reb.D: 250 and Novel glycoside 2 (Compound 17): 14.7.

[0147] The resulting beverage samples were subjected to sensory evaluation for rating attributes, namely, sweetness onset, sweet lingering, total sweetness, unrefined taste, and

unrefined taste lingering. Unrefined taste as used herein refers to undesirable flavor other than sweetness such as bitterness and astringency. Panelists trained about sensory attributes of sweeteners (7 members: Suntory Beverage and Food Ltd.) evaluated based on the following evaluation criteria. Very weak (-3), weak (-2), slightly weak (-1), normal (0), slightly strong (+1), strong (+2) and very strong (+3). The results are shown in Figure 20. The evaluation scores shown in the diagram are the average scores of the scores from the 7 panelists.

[0148] As a result of the sensory evaluation, Compound 17 having the same structure as Novel steviol glycoside 2 was found to have faster sweetness onset than the conventional sweeteners Reb.A and Reb.D and sweet lingering as good as sugar. It was also found to have total sweetness comparative to Reb.D.

[0149] Evaluation of flavors of novel steviol glycosides (Compounds 15 and 17) in powder forms

Flavors of Novel steviol glycosides 1 and 2 in powder forms were evaluated. Specifically, Compounds 15 (corresponding to Novel steviol glycoside 1) and 17 (corresponding to Novel steviol glycoside 2) obtained by chemical syntheses were subjected to high performance liquid chromatography (HPLC) to isolate only the β-form, which was made into a powder form and subjected to flavor evaluation. Panelists trained about sensory attributes of sweeteners (3 members: Suntory Beverage and Food Ltd.) evaluated. The results are shown in Table 3.

Table 3: Results from evaluating flavors of novel steviol glycosides (only β-form) in powder forms

	Presence of sweetness	Flavor characteristics
Novel steviol glycoside 1 (Compound 15)	Present	·Sweetness level was weaker than Novel steviol glycoside 2 ·Lingering aftertaste was stronger than Steviol glycoside 2, but with good finish
Novel steviol glycoside 2 (Compound 17)	Present	·Flavor resembled the mixture ·No bitterness

[0150] From the above results, both of Novel steviol glycosides 1 and 2 had sweetness and were found to be useful as sweeteners. Moreover, both glycosides had less undesirable flavor such as bitterness. Compound 17 had a flavor that resembled a mixture of α- and β-forms used for evaluating the sweetness level.