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DESCRIPTION

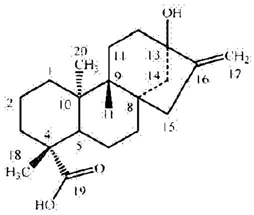
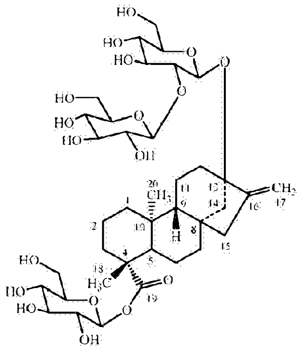
BACKGROUND OF THE DISCLOSURE

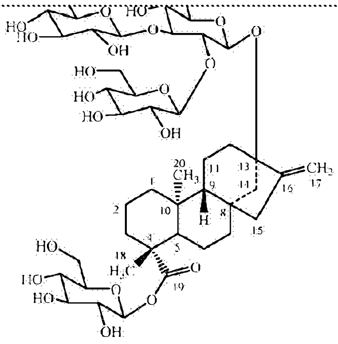
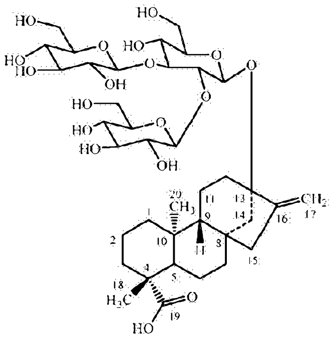
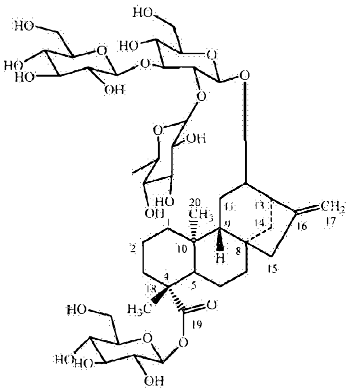
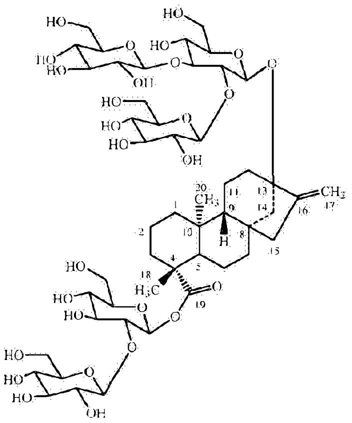
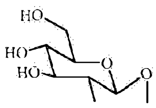
[0001] The present disclosure relates generally to natural sweeteners. More particularly, the present invention relates to methods for synthesizing the non-caloric sweeteners rebaudioside KA and rebaudioside E.

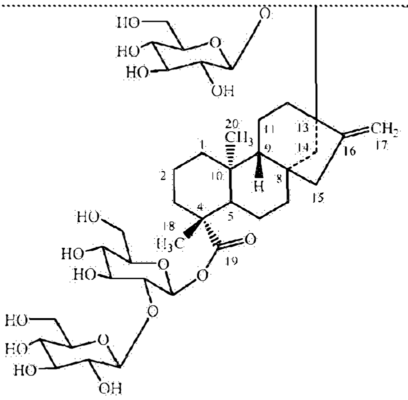
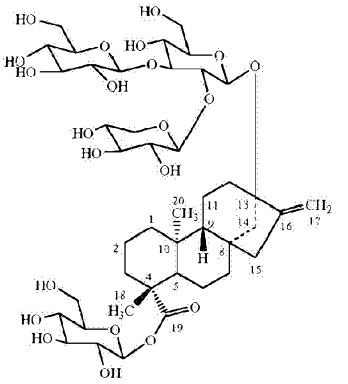
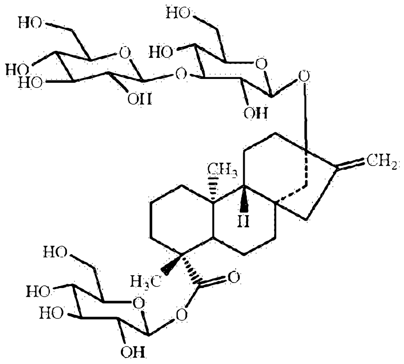
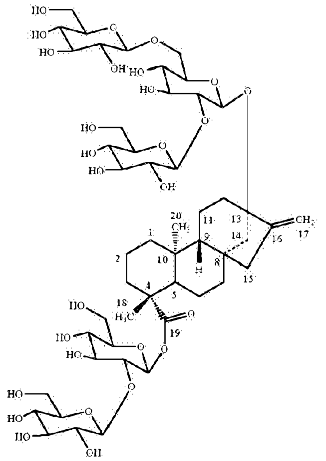
[0002] Steviol glycosides are natural products isolated from *Stevia rebaudiana* leaves. Steviol glycosides are widely used as high intensity, low-calorie sweeteners and are significantly sweeter than sucrose. As natural sweeteners, different steviol glucosides have different degrees of sweetness and after-taste. The sweetness of steviol glycosides is significantly higher than that of sucrose. For example, stevioside is 100-150 times sweeter than sucrose with bitter after-taste. Rebaudioside C is between 40-60 times sweeter than sucrose. Dulcoside A is about 30 times sweeter than sucrose.

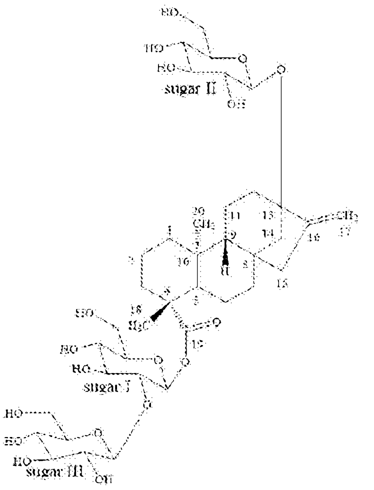
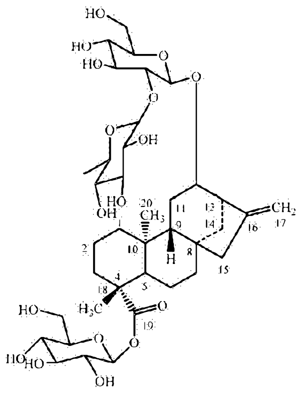
[0003] Naturally occurring steviol glycosides share the same basic steviol structure, but differ in the content of carbohydrate residues (e.g., glucose, rhamnose and xylose residues) at the C13 and C19 positions. Steviol glycosides with known structures include, steviol, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F and dulcoside A (see e.g., Table 1). Other steviol glycosides are rebaudioside M, rebaudioside N and rebaudioside O.

Table 1. Steviol glycosides.

Name	Structure	Molecular Formula	Molecular Weight
Steviol		$C_{20}H_{30}O_3$	318
Stevioside		$C_{38}H_{60}O_{18}$	804
Rebaudioside A		$C_{44}H_{70}O_{23}$	966

Name	Structure	Molecular Formula	Molecular Weight
			
Rebaudioside-B		$C_{38}H_{60}O_{18}$	804
Rebaudioside C		$C_{44}H_{70}O_{22}$	950
Rebaudioside D		$C_{50}H_{80}O_{28}$	1128
Rebaudioside E		$C_{44}H_{70}O_{23}$	966

Name	Structure	Molecular Formula	Molecular Weight
			
Rebaudioside F		$C_{43}H_{68}O_{22}$	936
Rebaudioside G		$C_{38}H_{60}O_{18}$	804
Rebaudioside D2 (Note: the designation Rebaudioside D2 has been used in some prior art documents, e.g. WO2014/193888 for a different rebaudioside structure)		$C_{50}H_{80}O_{28}$	1128

Name	Structure	Molecular Formula	Molecular Weight
Rebaudioside KA		$C_{38}H_{60}O_{18}$	804
Dulcoside A		$C_{38}H_{60}O_{17}$	788

[0004] On a dry weight basis, stevioside, rebaudioside A, rebaudioside C, and dulcoside A, account for 9.1, 3.8, 0.6, and 0.3% of the total weight of the steviol glycosides in the leaves, respectively, while the other steviol glycosides are present in much lower amounts. Extracts from the *Stevia rebaudiana* plant are commercially available, which typically contain stevioside and rebaudioside A as primary compounds. The other steviol glycosides typically are present in the stevia extract as minor components. For example, the amount of rebaudioside A in commercial preparations can vary from about 20% to more than 90% of the total steviol glycoside content, while the amount of rebaudioside B can be about 1-2%, the amount of rebaudioside C can be about 7-15%, and the amount of rebaudioside D can be about 2% of the total steviol glycosides.

[0005] The majority of steviol glycosides are formed by several glycosylation reactions of steviol, which are typically catalyzed by the UDP-glycosyltransferases (UGTs) using uridine 5'-diphosphoglucose (UDP-glucose) as a donor of the sugar moiety. UGTs in plants make up a very diverse group of enzymes that transfer a glucose residue from UDP-glucose to steviol. For example, glycosylation of the C-3' of the C-13-O-glucose of stevioside yields rebaudioside A; and glycosylation of the C-2' of the 19-O-glucose of the stevioside yields rebaudioside E.

Further glycosylation of rebaudioside A (at C-2' -19-O-glucose) or rebaudioside E (at C-3' -13-O-glucose) produces rebaudioside D. (FIG. 1).

[0006] Alternative sweeteners are receiving increasing attention due to awareness of many diseases in conjunction with the consumption of high-sugar foods and beverages. Although artificial sweeteners are available, many artificial sweeteners such as dulcin, sodium cyclamate and saccharin have been banned or restricted by some countries due to concerns over their safety. Therefore, non-caloric sweeteners of natural origin are becoming increasingly popular. One of the main obstacles for the widespread use of stevia sweeteners are their undesirable taste attributes. Accordingly, there exists a need to develop alternative sweeteners and methods for their production to provide the best combination of sweetness potency and flavor profile.

SUMMARY OF THE INVENTION

[0007] The present invention now provides a method of producing rebaudioside KA and /or rebaudioside E from rubusoside, the method comprising:

providing a reaction mixture comprising:

1. (A)
    1. (i) rubusoside,
    2. (ii) uridine diphosphate-glucose (UDP-glucose) as a substrate; and
    3. (iii) the HVI glycosyltransferase having the amino acid sequence of SEQ. ID. NO:5; or
2. (B)

    (i) rubusoside,

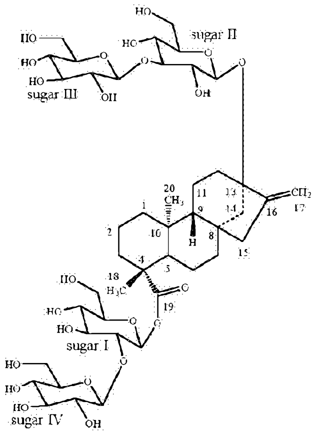
    (ii) sucrose, UDP and UDP-glucose as substrates and

    (ii) the HVI glycosyltransferase having the amino acid sequence of SEQ. ID. NO:5 with a sucrose synthase;

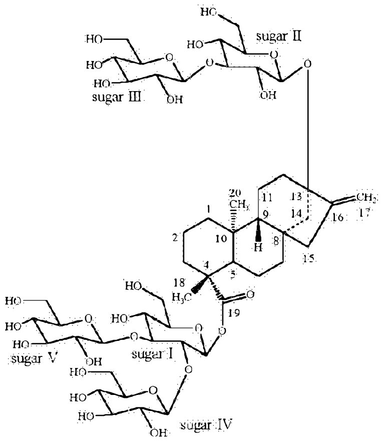
incubating the reaction mixture so as to produce rebaudioside KA, wherein a glucose is covalently coupled to the C2'of the 19-0 glucose of rubusoside to produce rebaudioside KA and /or rebaudioside E, wherein glucose is further covalently coupled by said glycosyltransferase to the C2' of the 13-O-glucose of rebaudioside KA; and obtaining rebaudioside KA from the reaction mixture for use as a sweetener.

[0008] A method of the invention may further comprise incorporating rebaudioside KA as a sweetener into an orally-consumable product, which is a beverage or other orally-consumable product.

[0009] Also disclosed herein and referred to below, but not claimed in the present claims, are sweeteners designated rebaudioside V and rebaudioside W consisting of the chemical structures shown below:



Reb V



Reb W

[0010] In certain embodiments rebaudioside KA can be obtained for use as a sole sweetener, and the product has a sweetness intensity equivalent to about 1% to about 4% (w/v-%) sucrose solution. In certain embodiments that can be combined with any of the preceding embodiments, the orally consumable product further can include an additional sweetener, where the product has a sweetness intensity equivalent to about 1% to about 10% (w/v-%) sucrose solution. In certain embodiments that can be combined with any of the preceding embodiments, every sweetening ingredient in the product can be a high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, every sweetening ingredient in the product can be a natural high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, the additional sweetener can be one or more sweeteners selected from a stevia extract, a steviol glycoside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside D2, rebaudioside E, rebaudioside F, rebaudioside G, rebaudioside M, dulcoside A, rubusoside, steviolbioside, sucrose, high fructose corn syrup, fructose, glucose, xylose, arabinose, rhamnose, erythritol, xylitol, mannitol, sorbitol, inositol, AceK, aspartame, neotame, sucralose, saccharine, naringin dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside, mogroside IV, siamenoside I, mogroside V, monatin, thaumatin, monellin, brazzein, L-alanine, glycine, Lo Han Guo, hernandulcin, phyllodulcin, trilobtain, and combinations thereof. In certain embodiments that can be combined with any of the preceding embodiments, the beverage product and consumable product can further include one or more additives selected from a carbohydrate, a polyol, an amino acid or salt thereof, a poly-amino acid or salt thereof, a sugar acid or salt thereof, a nucleotide, an organic acid, an inorganic acid, an organic salt, an organic acid salt, an organic base salt, an inorganic salt, a bitter compound, a flavorant, a flavoring ingredient, an astringent compound, a protein, a protein hydrolysate, a surfactant, an emulsifier, a flavonoid, an alcohol, a polymer, and combinations thereof. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside V has a purity of about 50% to about 100% by weight before it is added into the product. In certain embodiments that can be combined with any of the preceding embodiments, the W has a purity of about 50% to about 100% by weight before it is added into the product. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside V in the product is a rebaudioside V polymorph or amorphous rebaudioside V. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside V in the product is a rebaudioside V stereoisomer. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside W in the product is a rebaudioside W polymorph or amorphous rebaudioside W. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside W in the product is a rebaudioside W stereoisomer.

[0011] Other aspects of the present disclosure relate to a method of preparing a beverage product and a consumable product by including synthesized rebaudioside KA into the product or into the ingredients for making the beverage product and the consumable product, where rebaudioside KA is present in the product at a concentration of from about 5 ppm to about 100 ppm. Other aspects of the present disclosure relate to a method for enhancing the sweetness of a beverage product and a consumable product by adding from about 5 ppm to about 100 ppm of synthesized rebaudioside KA, where the added synthesized rebaudioside KA enhances the sweetness of the beverage product and the consumable product as compared to a corresponding beverage product or consumable product lacking the synthesized rebaudioside KA.

[0012] Other aspects of the present disclosure relate to a method for preparing a sweetened beverage product or a sweetened consumable product by: a) providing a beverage product or a consumable product containing one or more sweetener; and b) adding from about 5 ppm to about 100 ppm of one or more synthesized rebaudiosides including rebaudioside KA and selected from rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, and rebaudioside G, and combinations thereof into the beverage product or the consumable product.

[0013] In certain embodiments that can be combined with any of the preceding embodiments, the method further includes adding one or more additives to the beverage product or the consumable product. In certain embodiments that can be combined with any of the preceding embodiments, the orally consumable product further contains one or more additives. In certain embodiments that can be combined with any of the preceding embodiments, the one or more additives are selected from a carbohydrate, a polyol, an amino acid or salt thereof, a poly-amino acid or salt thereof, a sugar acid or salt thereof, a nucleotide, an organic acid, an inorganic acid, an organic salt, an organic acid salt, an organic base salt, an inorganic salt, a bitter compound, a flavorant, a flavoring ingredient, an astringent compound, a protein, a protein hydrolysate, a surfactant, an emulsifier, a flavonoid, an alcohol, a polymer, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments, every sweetening ingredient in the product is a high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, every sweetening ingredient in the product is a natural high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, the sweetener is selected from a stevia extract, a steviol glycoside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside D2, rebaudioside E, rebaudioside F, rebaudioside G, rebaudioside M, dulcoside A, rubusoside, steviolbioside, sucrose, high fructose corn syrup, fructose, glucose, xylose, arabinose, rhamnose, erythritol, xylitol, mannitol, sorbitol, inositol, AceK, aspartame, neotame, sucralose, saccharine, naringin dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside, mogroside IV, siamenoside I, mogroside V, monatin, thaumatin, monellin, brazzein, L-alanine, glycine, Lo Han Guo, hernandulcin, phyllodulcin, trilobtain, and combinations thereof. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside V has a purity of about 50% to about 100% by weight before it is added into the product. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside V in the product is a rebaudioside V polymorph or amorphous rebaudioside V. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside W has a purity of about 50% to about 100% by weight before it is added into the product. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside W in the product is a rebaudioside W polymorph or amorphous rebaudioside W.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The disclosure will be better understood, and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIG. 1 depicts a steviol glycosides biosynthesis pathway from steviol.

FIG. 2 depicts SDS-PAGE analysis purified recombinant proteins indicated by arrows: A: HV1, B: UGT76G1, C: EUGT11, D: AtSUS1, E: UGT76G1-SUS1 (GS), F: EUGT11-SUS1 (EUS).

FIG. 3 depicts the HV1 catalysis reaction to produce rebaudioside KA ("Reb KA") and rebaudioside E ("Reb E") from rubusoside. A-C: showing the HPLC retention times of rubusoside ("Rub"), stevioside ("Ste") and rebaudioside E ("Reb E") standards. Reb KA enzymatically produced by HV1 alone at 6hr (D), 12hr (F) and 24 hours (H); Reb KA and Reb E enzymatically produced by the UGT-SUS (HV1-AtSUS1) coupling system at 6hr (E), 12hr (G) and 24 hr (I).

FIG. 4 depicts the conversion of Reb E to rebaudioside Z by HV1. (A): shows the HPLC retention time of rebaudioside E ("Reb E"). Rebaudioside Z ("Reb Z") enzymatically produced by HV1 in the HV1-AtSUS1 coupling system at 3hr (B), 7hr (C), 24hr (D) and 44hr (E).

FIG. 5 depicts the conversion of Reb KA to Reb E by HV1. (A-B): show the HPLC retention times of rebaudioside KA ("Reb KA") and rebaudioside E ("Reb E") standards. Reb E enzymatically produced by HV1 alone at 12hr (C); Reb E enzymatically produced by the UGT-SUS (HV1-AtSUS1) coupling system at 12hr (D).

FIG. 6 depicts the EUGT11 catalysis reaction to produce Reb KA and stevioside from rubusoside. (A-F): show the HPLC retention times of rubusoside ("Rub"), stevioside ("Ste"), rebaudioside G ("Reb G"), rebaudioside E ("Reb E"), rebaudioside D ("Reb D") and rebaudioside D2 ("Reb D2") standards. Enzymatic reaction by EUGT11 alone at 12hr (G) and 48hr (J); enzymatic reaction by the UGT-SUS (EUGT11-AtSUS1) coupling system at 12hr (H) and 48hr (K); enzymatic reaction by EUS fusion protein at 12hr (I) and 48hr (L).

FIG. 7 depicts the conversion of Reb KA to Reb E and Reb D2 by EUGT11 and EUS fusion proteins. (A-C): showing the HPLC retention times of rebaudioside KA ("Reb KA"), rebaudioside E ("Reb E"), and rebaudioside D2 ("Reb D2") standards. Enzymatic reaction by EUGT11 alone at 12hr (D) and 48hr (G); enzymatic reaction by the UGT-SUS (EUGT11-AtSUS1) coupling system at 12hr (E) and 48hr (H); enzymatic reaction by EUS fusion protein at 12hr (F) and 48hr (I).

FIG. 8 depicts the UGT76G1 production of rebaudioside G *in vitro*. (A-B): show the HPLC retention times of rubusoside ("Rub") and rebaudioside G ("Reb G") standards. Enzymatic reaction by UGT76G1 alone at 12hr (C) and 24hr (F); enzymatic reaction by the UGT-SUS (EUGT11-AtSUS1) coupling system at 12hr (D) and 24hr (G); enzymatic reaction by GS fusion protein at 12hr (E) and 48hr (H).

FIG. 9 depicts the UGT76G1 catalysis reaction to produce the steviol glycosides Reb V and Reb W from rebaudioside KA. (A-D): show the HPLC retention times of rubusoside ("Rub"), rebaudioside D ("Reb D"), rebaudioside E ("Reb E") and rebaudioside KA ("Reb KA") standards. Enzymatic reaction by UGT76G1 alone at 6hr (E) and 12hr (H); enzymatic reaction by the UGT-SUS (UGT76G1-AtSUS1) coupling system at 6hr (F) and 12hr (I); enzymatic reaction by GS fusion protein at 6hr (G) and 12hr (J).

FIG. 10 depicts the UGT76G1 conversion of Reb V to Reb W *in vitro*. (A-B): showing the HPLC retention times of Reb V and Reb W. (C): Enzymatic reaction by the UGT76G1-AtSUS1 coupling system at 6hr.

FIG. 11 depicts the HV1 conversion of Reb G to Reb V. (A-C): showing the HPLC retention times of rebaudioside G ("Reb G"), rebaudioside A ("Reb A") and rebaudioside E ("Reb E") standards. Enzymatic reaction by HV1 alone at 12hr (D) and 24hr (F); enzymatic reaction by the UGT-SUS (HV1-AtSUS1) coupling system at 12hr (E) and 24hr (G).

FIG. 12 depicts the EUGT11 conversion of Reb G to Reb V. (A-D): showing the HPLC retention times of rebaudioside G ("Reb G"), rebaudioside A ("Reb A"), rebaudioside E ("Reb E") and rebaudioside D ("Reb D") standards. Enzymatic reaction by EUGT11 alone at 12hr (E) and 24hr (H); enzymatic reaction by the UGT-SUS (EUGT11-AtSUSl) coupling system at 12hr (F) and 24hr (I); enzymatic reaction by EUS fusion enzyme at 12hr (G) and 24hr (J).

FIG. 13 depicts the *in vitro* production of Reb W from rubusoside catalyzed by a combination of a recombinant HV1 polypeptide, a recombinant UGT76G1, a GS fusion enzyme, and a recombinant AtSUS1. (A-F): show the standards of rubusoside ("Rub"), stevioside ("Ste"), Rebaudioside G ("Reb G"), rebaudioside A ("Reb A"), Rebaudioside D ("Reb D") and rebaudioside E ("Reb E"). Reb W enzymatically produced by HV1, UGT76G1 and AtSUS1 at 6 hours (G), 12 hr (I) and 24hr (K); Reb W enzymatically produced by HV1 and GS fusion protein at 6 hours (H), 12 hr (J) and 24hr (L).

FIG. 14 depicts the *in vitro* production of Reb W from rubusoside catalyzed by a combination of a recombinant EUGT11 polypeptide, a recombinant UGT76G1, a GS fusion enzyme, and a recombinant AtSUS1. (A-E): show the standards of rubusoside ("Rub"), stevioside ("Ste"), rebaudioside G ("Reb G"), rebaudioside E ("Reb E") and rebaudioside D ("Reb D"). Reb W enzymatically produced by EUGT11, UGT76G1 and AtSUS1 at 12 hours (F) and 48hr (H); Reb W enzymatically produced by EUGT11 and GS fusion protein at 12 hours (G) and 48hr (I).

FIG. 15 depicts the *in vitro* production of Reb W from Reb G catalyzed by a combination of a recombinant HV1 polypeptide, a recombinant UGT76G1, a GS fusion enzyme and a recombinant AtSUS1. A-D shows the standards of rebaudioside G ("Reb G"), rebaudioside A ("Reb A"), Rebaudioside D ("Reb D"), rebaudioside and rebaudioside E ("Reb E"). Reb V and Reb W enzymatically produced by HV1, UGT76G1 and AtSUS1 at 6 hours (E), 12 hr (G)and 36hr (I); Reb V and Reb W enzymatically produced by HV1 and GS fusion protein at 6 hours (F), 12 hr (H) and 36hr (J).

FIG. 16 depicts the *in vitro* production of Reb W from Reb G catalyzed by a combination of a recombinant EUGT11 polypeptide, a recombinant UGT76G1, a GS fusion enzyme, and a recombinant AtSUS1. (A-D): show the standards of rebaudioside G ("Reb G"), rebaudioside A ("Reb A"), rebaudioside E ("Reb E") and rebaudioside D ("Reb D"). Reb W enzymatically produced by EUGT11, UGT76G1 and AtSUS1 at 12 hours (E) and 48hr (G); Reb W enzymatically produced by EUGT11 and GS fusion protein at 12 hours (F) and 48hr (H).

FIG. 17 depicts the structures of Reb V and Reb G.

FIG. 18 depicts the key TOCSY and HMBC correlations of Reb V.

FIG. 19 depicts the structures of Reb W and Reb V.

FIG. 20 depicts the key TOCSY and HMBC correlations of Reb W.

FIG. 21 depicts the biosynthesis pathway of steviol glycosides.

FIG. 22 depicts the *in vitro* production of Reb M from Reb D catalyzed by UGT76G1 and GS fusion enzyme. (A-B): showing the HPLC retention times of rebaudioside D ("Reb D") and rebaudioside M ("Reb M") standards. Enzymatic reaction by UGT76G1 alone at 3hr (C) and 6hr (F); enzymatic reaction by the UGT-SUS (UGT76G1-AtSUS1) coupling system at 3hr (D) and 6hr (G); enzymatic reaction by the GS fusion enzyme at 3hr (E) and 6hr (H).

FIG. 23 depicts the *in vitro* production of Reb D and Reb M from Reb E catalyzed by UGT76G1 and GS fusion enzyme. (A-C): showing the HPLC retention times of rebaudioside E ("Reb E"), rebaudioside D ("Reb D") and rebaudioside M ("Reb M") standards. Enzymatic reaction by UGT76G1 alone at 3hr (D), 12hr (G) and 24hr (J); enzymatic reaction by the UGT-SUS (UGT76G1-AtSUS1) coupling system at 3hr (E), 12hr (H) and 24hr (K); enzymatic reaction by the GS fusion enzyme at 3hr (F), 12hr (I) and 24hr (L).

FIG. 24 depicts the *in vitro* production of Reb D and Reb M from stevioside catalyzed by a combination of a recombinant HV1, a recombinant UGT76G1, a GS fusion enzyme, and/or a recombinant AtSUS1. (A-D): showing the HPLC retention times of stevioside ("Ste"), rebaudioside A ("Reb A"), rebaudioside D ("Reb D") and rebaudioside M ("Reb M) standards. Enzymatic reaction by HV1 and UGT76G1 in the UGT-SUS coupling system at 6hr (E), 12hr (H) and 24hr (K); enzymatic reaction by HV1 and GS fusion enzyme at 6hr (F), 12hr (I) and 24hr (L). Enzymatic reaction by UGT76G1 and HV1 at 6hr (G), 12hr (J) and 24hr (M).

FIG. 25 depicts the *in vitro* production of Reb D and Reb M from rebaudioside A catalyzed by a combination of recombinant HV1, a recombinant UGT76G1, a GS fusion enzyme, and/or a recombinant AtSUS1. (A-C): showing the HPLC retention times of rebaudioside A ("Reb A"), rebaudioside D ("Reb D") and rebaudioside M ("Reb M) standards. Enzymatic reaction by HV1 and UGT76G1 in the UGT-SUS coupling system at 6hr (D), 12hr (G) and 24hr (J); enzymatic reaction by HV1 and GS fusion enzyme at 6hr (E), 12hr (H) and 24hr (K). Enzymatic reaction by UGT76G1 and HV1 at 6hr (F), 12hr (I) and 24hr (J).

FIG. 26 depicts the structure of Reb M.

FIG. 27 depicts the key TOCSY and HMBC correlations of Reb M.

DETAILED DESCRIPTION

[0015] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to those described herein may be used in the practice or testing of the present disclosure, the preferred materials and methods are described below.

[0016] The term "complementary" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to describe the relationship between nucleotide bases that are capable to hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the subject technology also includes isolated nucleic acid fragments that are complementary to the complete sequences as reported in the accompanying Sequence Listing as well as those substantially similar nucleic acid sequences.

[0017] The terms "nucleic acid" and "nucleotide" are used according to their respective ordinary and customary meanings as understood by a person of ordinary skill in the art, and are used without limitation to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified or degenerate variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated.

[0018] The term "isolated" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and when used in the context of an isolated nucleic acid or an isolated polypeptide, is used without limitation to refer to a nucleic acid or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as, for example, in a transgenic host cell.

[0019] The terms "incubating" and "incubation" as used herein refers to a process of mixing two or more chemical or biological entities (such as a chemical compound and an enzyme) and allowing them to interact under conditions favorable for producing a steviol glycoside composition.

[0020] The term "degenerate variant" refers to a nucleic acid sequence having a residue sequence that differs from a reference nucleic acid sequence by one or more degenerate codon substitutions. Degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed base and/or deoxyinosine residues. A nucleic acid sequence and all of its degenerate variants will express the same amino acid or polypeptide.

[0021] The terms "polypeptide," "protein," and "peptide" are used according to their respective ordinary and customary meanings as understood by a person of ordinary skill in the art; the three terms are sometimes used interchangeably, and are used without limitation to refer to a polymer of amino acids, or amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "polypeptide" as used herein refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms "protein," "polypeptide," and "peptide" are used interchangeably herein when referring to a polynucleotide product. Thus, exemplary polypeptides include polynucleotide products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing.

[0022] The terms "polypeptide fragment" and "fragment," when used in reference to a reference polypeptide, are used according to their ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both.

[0023] The term "functional fragment" of a polypeptide or protein refers to a peptide fragment that is a portion of the full length polypeptide or protein, and has substantially the same biological activity, or carries out substantially the same function as the full length polypeptide or protein (e.g., carrying out the same enzymatic reaction).

[0024] The terms "variant polypeptide," "modified amino acid sequence" or "modified polypeptide," which are used interchangeably, refer to an amino acid sequence that is different from the reference polypeptide by one or more amino acids, e.g., by one or more amino acid substitutions, deletions, and/or additions. In an aspect, a variant is a "functional variant" which retains some or all of the ability of the reference polypeptide.

[0025] The term "functional variant" further includes conservatively substituted variants. The term "conservatively substituted variant" refers to a peptide having an amino acid sequence that differs from a reference peptide by one or more conservative amino acid substitutions, and maintains some or all of the activity of the reference peptide. A "conservative amino acid substitution" is a substitution of an amino acid residue with a functionally similar residue. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one charged or polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between threonine and serine; the substitution of one basic residue such as lysine or arginine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another; or the substitution of one aromatic residue, such as phenylalanine, tyrosine, or tryptophan for another. Such substitutions are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. The phrase "conservatively substituted variant" also includes peptides wherein a residue is replaced with a chemically-derivatized residue, provided that the resulting peptide maintains some or all of the activity of the reference peptide as described herein.

[0026] The term "variant," in connection with the polypeptides of the subject technology, further includes a functionally active polypeptide having an amino acid sequence at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical to the amino acid sequence of a reference polypeptide.

[0027] The term "homologous" in all its grammatical forms and spelling variations refers to the relationship between polynucleotides or polypeptides that possess a "common evolutionary origin," including polynucleotides or polypeptides from superfamilies and homologous polynucleotides or proteins from different species (Reeck et al., Cell 50:667, 1987). Such polynucleotides or polypeptides have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or the presence of specific amino acids or motifs at conserved positions. For example, two homologous polypeptides can have amino acid sequences that are at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical.

[0028] "Percent (%) amino acid sequence identity" with respect to the variant polypeptide sequences of the subject technology refers to the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues of a reference polypeptide after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

[0029] Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For example, the % amino acid sequence identity may be determined using the sequence comparison program NCBI-BLAST2. The NCBI-BLAST2 sequence comparison program may be downloaded from ncbi.nlm.nih.gov. NCBI BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask yes, strand=all, expected occurrences 10, minimum low complexity length=15/5, multi-pass e-value=0.01, constant for multi-pass=25, dropoff for final gapped alignment=25 and scoring matrix=BLOSUM62. In situations where NCBI-BLAST2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y where X is the number of amino acid residues scored as identical matches by the sequence alignment program NCBI-BLAST2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

[0030] In this sense, techniques for determining amino acid sequence "similarity" are well known in the art. In general, "similarity" refers to the exact amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" may then be determined between the compared polypeptide sequences. Techniques for determining nucleic acid and amino acid sequence identity also are well known in the art and include determining the nucleotide sequence of the mRNA for that gene (usually via a cDNA intermediate) and determining the amino acid sequence encoded therein, and comparing this to a second amino acid sequence. In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more polynucleotide sequences can be compared by determining their "percent identity", as can two or more amino acid sequences. The programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, Wis.), for example, the GAP program, are capable of calculating both the identity between two polynucleotides and the identity and similarity between two polypeptide sequences, respectively. Other programs for calculating identity or similarity between sequences are known by those skilled in the art.

[0031] An amino acid position "corresponding to" a reference position refers to a position that aligns with a reference sequence, as identified by aligning the amino acid sequences. Such alignments can be done by hand or by using well-known sequence alignment programs such as ClustalW2, Blast 2, etc.

[0032] Unless specified otherwise, the percent identity of two polypeptide or polynucleotide sequences refers to the percentage of identical amino acid residues or nucleotides across the entire length of the shorter of the two sequences.

[0033] "Coding sequence" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence that encodes for a specific amino acid sequence.

[0034] "Suitable regulatory sequences" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

[0035] "Promoter" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different cell types, or at different stages of development, or in response to different environmental conditions. Promoters, which cause a gene to be expressed in most cell types at most times, are commonly referred to as "constitutive promoters." It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0036] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0037] The term "expression" as used herein, is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to refer to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the subject technology. "Over-expression" refers to the production of a gene product in transgenic or recombinant organisms that exceeds levels of production in normal or non-transformed organisms.

[0038] "Transformation" is used according to its ordinary and customary meaning as understood by a person of ordinary skill in the art, and is used without limitation to refer to the transfer of a polynucleotide into a target cell. The transferred polynucleotide can be incorporated into the genome or chromosomal DNA of a target cell, resulting in genetically stable inheritance, or it can replicate independent of the host chromosomal. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[0039] The terms "transformed," "transgenic," and "recombinant," when used herein in connection with host cells, are used according to their ordinary and customary meanings as understood by a person of ordinary skill in the art, and are used without limitation to refer to a cell of a host organism, such as a plant or microbial cell, into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host cell, or the nucleic acid molecule can be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or subjects are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

[0040] The terms "recombinant," "heterologous," and "exogenous," when used herein in connection with polynucleotides, are used according to their ordinary and customary meanings as understood by a person of ordinary skill in the art, and are used without limitation to refer to a polynucleotide (e.g., a DNA sequence or a gene) that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of site-directed mutagenesis or other recombinant techniques. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position or form within the host cell in which the element is not ordinarily found.

[0041] Similarly, the terms "recombinant," "heterologous," and "exogenous," when used herein in connection with a polypeptide or amino acid sequence, means a polypeptide or amino acid sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, recombinant DNA segments can be expressed in a host cell to produce a recombinant polypeptide.

[0042] The terms "plasmid," "vector," and "cassette" are used according to their ordinary and customary meanings as understood by a person of ordinary skill in the art, and are used without limitation to refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[0043] Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described, for example, by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1989 (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1984; and by Ausubel, F. M. et al., in 'Current Protocols in Molecular Biology', published by Greene Publishing and Wiley-Interscience, 1987.

[0044] As used herein, "synthetic" or "organically synthesized" or "chemically synthesized" or "organically synthesizing" or "chemically synthesizing" or "organic synthesis" or "chemical synthesis" are used to refer to preparing the compounds through a series of chemical reactions; this does not include extracting the compound, for example, from a natural source.

[0045] The term "orally consumable product" as used herein refers to any beverage, food product, dietary supplement, nutraceutical, pharmaceutical composition, dental hygienic composition and cosmetic product which are contacted with the mouth of man or animal, including substances that are taken into and subsequently ejected from the mouth and substances which are drunk, eaten, swallowed, or otherwise ingested; and that are safe for human or animal consumption when used in a generally acceptable range of concentrations.

[0046] The term "food product" as used herein refers to fruits, vegetables, juices, meat products such as ham, bacon and sausage; egg products, fruit concentrates, gelatins and gelatin-like products such as jams, jellies, preserves, and the like; milk products such as ice cream, sour cream, yogurt, and sherbet; icings, syrups including molasses; corn, wheat, rye, soybean, oat, rice and barley products, cereal products, nut meats and nut products, cakes, cookies, confectionaries such as candies, gums, fruit flavored drops, and chocolates, chewing gum, mints, creams, icing, ice cream, pies and breads. "Food product" also refers to condiments such as herbs, spices and seasonings, flavor enhancers, such as monosodium glutamate. "Food product" further refers to also includes prepared packaged products, such as dietetic sweeteners, liquid sweeteners, tabletop flavorings, granulated flavor mixes which upon reconstitution with water provide non-carbonated drinks, instant pudding mixes, instant coffee and tea, coffee whiteners, malted milk mixes, pet foods, livestock feed, tobacco, and materials for baking applications, such as powdered baking mixes for the preparation of breads, cookies, cakes, pancakes, donuts and the like. "Food product" also refers to diet or low-calorie food and beverages containing little or no sucrose.

[0047] As used herein, the term "stereoisomer" is a general term for all isomers of individual molecules that differ only in the orientation of their atoms in space. "Stereoisomer" includes enantiomers and isomers of compounds with more than one chiral center that are not mirror images of one another (diastereomers).

[0048] As used herein, the term "amorphous rebaudioside V" refers to a non-crystalline solid form of rebaudioside V. As used herein, the term "amorphous rebaudioside W" refers to a non-crystalline solid form of rebaudioside W.

[0049] As used herein, the term "sweetness intensity" refers to the relative strength of sweet sensation as observed or experienced by an individual, e.g., a human, or a degree or amount of sweetness detected by a taster, for example on a Brix scale.

[0050] As used herein, the term "enhancing the sweetness" refers to the effect of rebaudioside V and/or rebaudioside W in increasing, augmenting, intensifying, accentuating, magnifying, and/or potentiating the sensory perception of one or more sweetness characteristics of a beverage product or a consumable product of the present disclosure without changing the nature or quality thereof, as compared to a corresponding orally consumable product that does not contain rebaudioside V and/or rebaudioside W.

[0051] As used herein, the term "off-taste(s)" refers to an amount or degree of taste that is not characteristically or usually found in a beverage product or a consumable product of the present disclosure. For example, an off-taste is an undesirable taste of a sweetened consumable to consumers, such as, a bitter taste, a licorice-like taste, a metallic taste, an aversive taste, an astringent taste, a delayed sweetness onset, a lingering sweet aftertaste, and the like, etc.

[0052] As used herein, the term "w/v-%" refers to the weight of a compound, such as a sugar, (in grams) for every 100 ml of a liquid orally consumable product of the present disclosure containing such compound. As used herein, the term "w/w-%" refers to the weight of a compound, such as a sugar, (in grams) for every gram of an orally consumable product of the present disclosure containing such compound.

[0053] As used herein, the term "ppm" refers to part(s) per million by weight, for example, the weight of a compound, such as rebaudioside V and/or rebaudioside W (in milligrams) per kilogram of an orally consumable product of the present disclosure containing such compound (i.e., mg/kg) or the weight of a compound, such as rebaudioside V and/or rebaudioside W (in milligrams) per liter of an orally consumable product of the present disclosure containing such compound (i.e., mg/L); or by volume, for example the volume of a compound, such as rebaudioside V and/or rebaudioside W (in milliliters) per liter of an orally consumable product of the present disclosure containing such compound (i.e., ml/L).

[0054] The following disclosure relates to various non-caloric sweeteners and methods for synthesizing non- caoric sweeteners. However, the claimed invention should be understood by reference to the present claims.

Synthetic Non-Caloric Sweeteners: Synthetic Rebaudioside V

[0055] In one aspect, the present disclosure is directed to a synthetic non-caloric sweetener. The synthetic non-caloric sweetener is a synthetic rebaudioside-type steviol glycoside and has been given the name, "Rebaudioside V". Rebaudioside V ("Reb V") is a steviol glycoside having four β-D-glucosyl units in its structure connected to the aglycone steviol, a steviol aglycone moiety with a Glc β1-3-Glc β1 unit at C-13 in the form of ether linkage and another Glc β1-2-Glc β1 unit at C-19 position in the form of an ester linkage.

[0056] Rebaudioside V has the molecular formula $C_{44}H_{70}O_{23}$ and the IUPAC name, 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

Synthetic Non-Caloric Sweeteners: Synthetic Rebaudioside W

[0057] In one aspect, the present disclosure is directed to a synthetic non-caloric sweetener. The synthetic non-caloric sweetener is a synthetic rebaudioside-type steviol glycoside and has been given the name, "Rebaudioside W". Rebaudioside W ("Reb W") is a steviol glycoside having five β-D-glucosyl units in its structure connected to the aglycone steviol, a steviol aglycone moiety with a Glc β1-3-Glc β1 unit at C-13 in the form of ether linkage and a Glc β1-2(Glc β1-3)-Glc β1 unit at C-19 position in the form of an ester linkage.

[0058] Rebaudioside W has the molecular formula $C_{50}H_{80}O_{28}$ and the IUPAC name, 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester.

Synthetic Non-Caloric Sweeteners: Synthetic Rebaudioside KA

[0059] In one aspect, the present disclosure is directed to a synthetic non-caloric sweetener. The synthetic non-caloric sweetener is a synthetic rebaudioside-type steviol glycoside and has been given the name, "Rebaudioside KA". Rebaudioside KA ("Reb KA") is a steviol glycoside having three β-D-glucosyl units in its structure connected to the aglycone steviol, a steviol aglycone moiety with a Glc β1 unit at C-13 in the form of ether linkage and a Glc β1-2-Glc β1 unit at C-19 in the form of ether linkage. Rebaudioside KA has the molecular formula $C_{38}H_{60}O_{18}$ and the IUPAC name, 13-β-D-glucopyranosyloxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

Synthetic Non-Caloric Sweeteners: Synthetic Rebaudioside G

[0060] In one aspect, the present disclosure is directed to a synthetic non-caloric sweetener. The synthetic non-caloric sweetener is a synthetic rebaudioside-type steviol glycoside and has been given the name, "Rebaudioside G". Rebaudioside G ("Reb G") is a steviol glycoside having three β-D-glucosyl units in its structure connected to the aglycone steviol, a steviol aglycone moiety with a Glc β1-3-Glc β1 unit at C-13 in the form of ether linkage and a Glc β1unit at C-19 in the form of ether linkage.

[0061] Rebaudioside G has the molecular formula $C_{38}H_{60}O_{18}$ and the IUPAC name, 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-β-D-glucopyranosyl) ester on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

Synthetic Non-Caloric Sweeteners: Synthetic Rebaudioside M

[0062] In one aspect, the present disclosure is directed to a synthetic non-caloric sweetener. The synthetic non-caloric sweetener is a synthetic rebaudioside-type steviol glycoside and has been given the name, "Rebaudioside M". Rebaudioside M ("Reb M") is a steviol glycoside having six β-D-glucosyl units in its structure connected to the aglycone steviol, a steviol aglycone moiety with a Glc β1-2(Glc β1-3)-Glc β1unit at the C-13 position in the form of an ether linkage and a Glc β1-2(Glc β1-3)-Glc β1unit at the C-19 position in the form of an ester linkage.

[0063] Rebaudioside M has the molecular formula $C_{56}H_{90}O_{33}$ and the IUPAC name, 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)ester on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

Methods of Synthesizing Steviol Glycosides

[0064] Method of Producing Rebaudioside V from Rebaudioside G. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside V from rebaudioside G. The method comprises preparing a reaction mixture comprising rebaudioside G; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and a HV1 UDP-glycosyltransferase; with or without sucrose synthase (SUS) and incubating the reaction mixture for a sufficient time to produce rebaudioside V, wherein a glucose is covalently coupled to the rebaudioside G to produce rebaudioside V.

[0065] Method of Producing Rebaudioside V from Rebaudioside G. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside V from rebaudioside G. The method comprises preparing a reaction mixture comprising rebaudioside G; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a uridine dipospho glycosyltransferase (UDP-glycosyltransferase) selected from the group consisting of an EUGT11, a UDP-glycosyltransferase-Sucrose synthase (SUS) fusion enzyme; with or without sucrose synthase (SUS) and incubating the reaction mixture for a sufficient time to produce rebaudioside V, wherein a glucose is covalently coupled to the rebaudioside G to produce rebaudioside V.

[0066] Method of producing Rebaudioside V from Rebaudioside KA. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside V from rebaudioside KA. The method comprises preparing a reaction mixture comprising rebaudioside KA; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a uridine dipospho glycosyltransferases (UDP-glycosyltransferase) selected from the group consisting of a UDP-glycosyltransferase (UGT76G1) and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase (SUS) and incubating the reaction mixture for a sufficient time to produce rebaudioside V, wherein a glucose is covalently coupled to the rebaudioside KA to produce rebaudioside V.

[0067] Method of Producing Rebaudioside V from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside V from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); uridine dipospho glycosyltransferase(s) (UDP-glycosyltransferase) selected from the group consisting of a UDP-glycosyltransferase (UGT76G1), HV1 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase (SUS) and incubating the reaction mixture for a sufficient time to produce rebaudioside V, wherein a glucose is covalently coupled to the rubusoside to produce rebaudioside KA. Continually, a glucose is covalently coupled to the rebaudioside KA to produce rebaudioside V.

[0068] Method of Producing of Rebaudioside V from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing a mixture of rebaudioside A and rebaudioside V from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); uridine dipospho glycosyltransferase(s) (UDP-glycosyltransferase) selected from the group consisting of a UDP-glycosyltransferase (UGT76G1), EUGT11 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside V, wherein a glucose is covalently coupled to the rubusoside to produce rebaudioside KA and a glucose is covalently coupled to the rebaudioside KA to produce rebaudioside V. A glucose is covalently coupled to the rubusoside to produce rebaudioside G. Continually, a glucose is covalently coupled to the rebaudioside G to produce rebaudioside V.

[0069] Method of producing Rebaudioside W from Rebaudioside V. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rebaudioside V. The method comprises preparing a reaction mixture comprising rebaudioside V; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); an uridine dipospho glycosyltransferase (UDP-glycosyltransferase) selected from the group consisting of a UDP-glycosyltransferase (UGT76G1) and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase and incubating the reaction mixture for a sufficient time to produce rebaudioside W, wherein a glucose is covalently coupled to the rebaudioside V to produce rebaudioside W.

[0070] Method of Producing Rebaudioside W from Rebaudioside G. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rebaudioside G. The method comprises preparing a reaction mixture comprising rebaudioside G; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); an uridine dipospho glycosyltransferase (UDP-glycosyltransferase) selected from the group consisting of a uridine diphospho glycosyltransferase (UGT76G1), a UDP-glycosyltransferase-Sucrose synthase fusion enzyme and a HV1; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside W, wherein a glucose is covalently coupled to the rebaudioside G to produce rebaudioside V by HV1. Continually, a glucose is covalently coupled to the rebaudioside V to produce rebaudioside W by UGT76G1.

[0071] Method of Producing Rebaudioside W from Rebaudioside G. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rebaudioside G. The method comprises preparing a reaction mixture comprising rebaudioside G; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); an uridine diphospho glycosyltransferase (UDP-glycosyltransferase) selected from the group consisting of a UGT76G1, an EUGT11, and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; and incubating the reaction mixture for a sufficient time to produce rebaudioside W, wherein a glucose is covalently coupled to the rebaudioside G to produce rebaudioside V by EUGT11. Continually, a glucose is covalently coupled to the rebaudioside V to produce rebaudioside W by UGT76G1.

[0072] Method of Producing Rebaudioside W from Rebaudioside KA. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rebaudioside KA. The method comprises preparing a reaction mixture comprising rebaudioside KA; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); an uridine dipospho glycosyltransferase (UDP-glycosyltransferase) selected from the group consisting of a uridine diphospho glycosyltransferase (UGT76G1), and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside W, wherein a glucose is covalently coupled to the rebaudioside KA to produce rebaudioside V. Continually, a glucose is covalently coupled to the rebaudioside V to produce rebaudioside W.

[0073] Method of Producing of Rebaudioside W from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); uridine diphospho glycosyltransferases selected from the group consisting of a UGT76G1, an HV1, and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase and incubating the reaction mixture for a sufficient time to produce a mixture of rebaudioside W.

[0074] Method of Producing of Rebaudioside W from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside W from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); uridine diphospho glycosyltransferases selected from the group consisting of a UGT76G1, an EUGT11, and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase and incubating the reaction mixture for a sufficient time to produce rebaudioside W.

[0075] Method of Producing a Mixture of Stevioside and Rebaudioside KA from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing a mixture of stevioside and rebaudioside KA from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a UDP-glycosyltransferase selected from the group consisting of EUGT11 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce a mixture of stevioside and rebaudioside KA, wherein a glucose is covalently coupled to C2'-19-O-glucose of rubusoside to produce rebaudioside KA; a glucose is convalently coupled to C2'-13-O-glucose of rubusoside to produce stevioside.

[0076] Method of Producing Rebaudioside KA from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing a rebaudioside KA from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and HV1 UDP-glycosyltransferase; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside KA, wherein a glucose is covalently coupled to the C2'-19-O-glucose of rubusoside to produce a rebaudioside KA.

[0077] Method of Producing Rebaudioside G from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing a rebaudioside G from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a UDP-glycosyltransferase selected from the group consisting of UGT76G1 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside G, wherein a glucose is covalently coupled to the C3'-13-O-glucose of rubusoside to produce a rebaudioside G.

[0078] Method of Producing Rebaudioside E from Rebaudioside KA. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside E from rebaudioside KA. The method comprises preparing a reaction mixture comprising rebaudioside KA; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and HV1 UDP-glycosyltransferase; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside E, wherein a glucose is covalently coupled to the C2' 13-O-glucose of rebaudioside KA to produce rebaudioside E.

[0079] Method of Producing Rebaudioside E from Rebaudioside KA. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside E from rebaudioside KA. The method comprises preparing a reaction mixture comprising rebaudioside KA; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a UDP-glycosyltransferase from a group of EUGT11 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme; with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside E, wherein a glucose is covalently coupled to the C2' 13-O-glucose of rebaudioside KA to produce rebaudioside E.

[0080] Method of Producing Rebaudioside E from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside E from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; a substrate selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and a UDP-glycosyltransferase from the group of EUGT11 and a UDP-glycosyltransferase-Sucrose synthesis fusion enzyme; with or without sucrose synthase; incubating the reaction mixture for a sufficient time to produce rebaudioside E, wherein a glucose is covalently coupled to rubusoside to produce a mixture of rebaudioside KA and stevioside. Continually, a glucose is covalently coupled to rebaudioside KA and stevioside to produce rebaudioside E.

[0081] Method of Producing Rebaudioside E from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside E from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and HV1 UDP-glycosyltransferase; with or without sucrose synthase; incubating the reaction mixture for a sufficient time to produce rebaudioside E, wherein a glucose is covalently coupled to the rubusoside to produce rebaudioside KA; and further incubating the rebaudioside KA with HV1 to produce rebaudioside E.

[0082] Method of Producing Rebaudioside D2 from Rubusoside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside D2 from rubusoside. The method comprises preparing a reaction mixture comprising rubusoside; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); a UDP-glycosyltransferase from the group of EUGT11 and a UDP-glycosyltransferase-Sucrose synthesis fusion enzyme; with or without sucrose synthase; incubating the reaction mixture for a sufficient time to produce rebaudioside D2, wherein a glucose is covalently coupled to the rubusoside to produce a mixture of stevioside and rebaudioside KA; further incubating the mixture of stevioside and rebaudioside KA with

EUGT11 to produce rebaudioside E, wherein a glucose is covalently coupled to the stevioside and the rebaudioside KA to produce rebaudioside E; and further incubating the rebaudioside E with EUGT11 to produce rebaudioside D2, wherein a glucose is covalently coupled to the rebaudioside E to produce rebaudioside D2.

[0083] Method of Producing Rebaudioside D2 from Rebaudioside KA. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside D2 from rebaudioside KA. The method includes preparing a reaction mixture comprising rebaudioside KA, substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose), a UDP-glycosyltransferase selected from the group consisting of an EUGT11 and a UDP-glycosyltransferase-Sucrose synthase fusion enzyme, with or without sucrose synthase; incubating the reaction mixture for a sufficient time to produce rebaudioside D2, wherein a glucose is covalently coupled to the rebaudioside KA to produce rebaudioside E; further incubating the mixture of rebaudioside E with EUGT11 to produce rebaudioside D2, wherein a glucose is covalently coupled to the rebaudioside E to produce rebaudioside D2.

[0084] Method of Producing Rebaudioside Z from Rebaudioside E. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside Z from rebaudioside E. The method comprises preparing a reaction mixture comprising rebaudioside E; substrates selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose); and HV1 UDP-glycosyltransferase; and sucrose synthase, incubating the reaction mixture for a sufficient time to produce rebaudioside Z, wherein a glucose is covalently coupled to the rebaudioside E to produce rebaudioside Z, wherein a glucose is covalently coupled to the C2'-13-O-glucose of rebaudioside E to produce rebaudioside Z1. A glucose is convalently coupled to C2'-19-O-glucose of rebaudioside E to produce rebaudioside Z2.

[0085] Method of Producing Rebaudioside M from Rebaudioside D. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside M from rebaudioside D. The method includes preparing a reaction mixture comprising rebaudioside D, substrates selected from the group consisting of sucrose, uridine diphosphate (UDP), uridine diphosphate-glucose (UDP-glucose), and combinations thereof, and a UDP-glycosyltransferase selected from the group consisting of UGT76G1, a UDP-glycosyltransferase-Sucrose synthase fusion enzyme, and combinations thereof, with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside M, wherein a glucose is covalently coupled to the rebaudioside D to produce rebaudioside M.

[0086] Method of Producing Rebaudioside D and Rebaudioside M from Stevioside. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside D and rebaudioside M from stevioside. The method includes preparing a reaction mixture comprising stevioside, substrates selected from the group consisting of sucrose, uridine diphosphate (UDP), uridine diphosphate-glucose (UDP-glucose), and combinations thereof, and a UDP-glycosyltransferase selected from the group consisting of HV1, UGT76G1, a UDP-glycosyltransferase-Sucrose synthase fusion enzyme, and combinations thereof, with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside D and/or rebaudioside M. For instance, in embodiments, the reaction mixture may be incubated for a sufficient time to produce rebaudioside D, and the reaction mixture comprising rebaudioside D further incubated (e.g., with UGT76G1 and/or the fusion enzyme) to produce rebaudioside M. In certain embodiments, the reaction mixture will comprise HV1 and UGT76G1. In other embodiments, the reaction mixture will comprise HV1 and the fusion enzyme.

[0087] In certain embodiments, a glucose is covalently coupled to the stevioside to produce rebaudioside A and/or rebaudioside E. For example, a glucose may be covalently coupled to the stevioside by UGT76G1 or the fusion enzyme to produce rebaudioside A and/or a glucose may be covalently coupled to the stevioside by HV1 to produce rebaudioside E. Continually, a glucose may be covalently coupled to the rebaudioside A by HV1 to produce rebaudioside D and/or a glucose may be covalently coupled to the rebaudioside E by UGT76G1 or the fusion enzyme to produce rebaudioside D. A glucose may further be covalently coupled to the rebaudioside D by UGT76G1 or the fusion enzyme to produce rebaudioside M.

[0088] Method of Producing Rebaudioside D and Rebaudioside M from Rebaudioside A. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside D and rebaudioside M from rebaudioside A. The method includes preparing a reaction mixture comprising rebaudioside A, substrates selected from the group consisting of sucrose, uridine diphosphate (UDP), uridine diphosphate-glucose (UDP-glucose), and combinations thereof, and a UDP-glycosyltransferase selected from the group consisting of HV1, UGT76G1, a UDP-glycosyltransferase-Sucrose synthase fusion enzyme, and combinations thereof, with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside D and/or rebaudioside M. For instance, in embodiments, the reaction mixture (e.g., comprising HV1) may be incubated for a sufficient time to produce rebaudioside D, and the reaction mixture comprising rebaudioside D further incubated (e.g., with UGT76G1 and/or the fusion enzyme) to produce rebaudioside M. In certain embodiments, the reaction mixture will comprise HV1 and UGT76G1. In other embodiments, the reaction mixture will comprise HV1 and the fusion enzyme.

[0089] A glucose is covalently coupled to the rebaudioside A to produce rebaudioside D. For example, a glucose may be covalently coupled to the rebaudioside A by HV1 to produce rebaudioside D. Continually, a glucose may be covalently coupled to the rebaudioside D by UGT76G1 or the fusion enzyme to produce rebaudioside M.

[0090] Method of Producing Rebaudioside D and Rebaudioside M from Rebaudioside E. In another aspect, the present disclosure is directed to a method for synthesizing rebaudioside D and rebaudioside M from rebaudioside E. The method includes preparing a reaction mixture comprising rebaudioside E, substrates selected from the group consisting of sucrose, uridine diphosphate (UDP), uridine diphosphate-glucose (UDP-glucose), and combinations thereof, and a UDP-glycosyltransferase selected from the group consisting of an UGT76G1, a UDP-glycosyltransferase-Sucrose synthase fusion enzyme, and combinations thereof, with or without sucrose synthase; and incubating the reaction mixture for a sufficient time to produce rebaudioside D and/or rebaudioside M. For instance, in embodiments, the reaction mixture (e.g., comprising UGT76G1 and/or the fusion enzyme) may be incubated for a sufficient time to produce rebaudioside D, and the reaction mixture comprising rebaudioside D further incubated to produce rebaudioside M.

[0091] A glucose is covalently coupled to the rebaudioside E to produce rebaudioside D. For example, a glucose may be covalently coupled to the rebaudioside E by UGT76G1 or the fusion enzyme to produce rebaudioside D. Continually, a glucose may be covalently coupled to the rebaudioside D by UGT76G1 or the fusion enzyme to produce rebaudioside M.

[0092] The majority of the steviol glycosides are formed by several glycosylation reactions of steviol, which typically are catalyzed by the UDP-glycosyltransferases (UGTs) using uridine 5'-diphosphoglucose (UDP-glucose) as a donor of the sugar moiety. In plants, UGTs are a very divergent group of enzymes that transfer a glucose residue from UDP-glucose to steviol.

[0093] Uridine diphospho glycosyltransferase (UGT76G1) is a UGT with a 1,3-13-O-glucose glycosylation activity to produce related glycoside (rebaudioside A and D). Surprisingly and unexpectedly, it was discovered that UGT76G1 also has 1,3-19-O-glucose glycosylation activity to produce rebaudioside G from rubusoside, and to produce rebaudioside M from rebaudioside D. UGT76G1 can convert rebaudioside KA to Reb V and continue to form Reb W. A particularly suitable UGT76G1 has an amino acid sequence of SEQ ID NO:1.

[0094] EUGT11 (described in WO 2013022989) is a UGT having 1,2-19-0-glucose and 1,2-13-O-glucose glycosylation activity. EUGT11 is known to catalyze the production of stevioside to rebaudioside E and rebaudioside A to rebaudioside D. Surprisingly and unexpectedly, it was discovered that EUGT11 can be used *in vitro* to synthesize rebaudioside D2 from rebaudioside E by a new enzyme activity (β1,6-13-O-glucose glycosylation activity) (U.S. Patent Application Ser. No. 14/269,435, assigned to Conagen, Inc.). EUGT11 has 1,2-19-O-glucose glycosylation activity to produce rebaudioside KA from rubusoside. A particularly suitable EUGT11 has the amino acid sequence of SEQ ID NO:3.

[0095] HV1 is a UGT with a 1,2-19-O-glucose glycosylation activity to produce related steviol glycosides (rebaudioside E, D and Z). Surprisingly and unexpectedly, it was discovered that HV1 also has 1,2-19-O-glucose glycosylation activity to produce rebaudioside KA from rubusoside. HV1 also can convert Reb G to Reb V and Reb KA to Reb E. A particularly suitable HV1 has the amino acid sequence of SEQ ID NO:5.

[0096] The method can further include adding a sucrose synthase to the reaction mixture that contains the uridine diphospho (UDP) glycosyltransferase. Sucrose synthase catalyzes the chemical reaction between NDP-glucose and D-fructose to produce NDP and sucrose. Sucrose synthase is a glycosyltransferase. The systematic name of this enzyme class is NDP-glucose:D-fructose 2-alpha-D-glucosyltransferase. Other names in common use include UDP glucose-fructose glucosyltransferase, sucrose synthetase, sucrose-UDP glucosyltransferase, sucrose-uridine diphosphate glucosyltransferase, and uridine diphosphoglucose-fructose glucosyltransferase. Addition of the sucrose synthase to the reaction mixture that includes a uridine diphospho glycosyltransferase creates a "UGT-SUS coupling system". In the UGT-SUS coupling system, UDP-glucose can be regenerated from UDP and sucrose, which allows for omitting the addition of extra UDP-glucose to the reaction mixture or using UDP in the reaction mixture. Suitable sucrose synthases can be for example, an *Arabidopsis* sucrose synthase 1; an *Arabidopsis* sucrose synthase 3; and a *Vigna radiate* sucrose synthase. A particularly suitable sucrose synthase can be, for example, *Arabidopsis* sucrose synthase 1. A particularly suitable *Arabidopsis* sucrose synthase 1 is *Arabidopsis thaliana* sucrose synthase 1 (AtSUS1), having the amino acid sequence of SEQ ID NO:7.

[0097] In another aspect, uridine dipospho glycosyltransferase fusion enzyme can be used in the methods. A particularly suitable uridine dipospho glycosyltransferase fusion enzyme can be a UGT-SUS 1 fusion enzyme. The UDP-glycosyltransferase can be a UDP-glycosyltransferase fusion enzyme that includes a uridine diphospho glycosyltransferase domain coupled to a sucrose synthase domain. In particular, the UDP-glycosyltransferase fusion enzyme includes a uridine diphospho glycosyltransferase domain coupled to a sucrose synthase domain. Additionally, the UGT-SUS1 fusion enzyme has sucrose synthase activity, and thus, can regenerate UDP-glucose from UDP and sucrose. A particularly suitable UGT-SUS1 fusion enzyme can be, for example, a UGT76G1-AtSUS1 fusion enzyme (named as: "GS") having the amino acid sequence of SEQ ID NO:9. Another particularly suitable UGT-SUS1 fusion enzyme can be, for example, a EUGT11-SUS1 (named as: "EUS") having the amino acid sequence of SEQ ID NO:11.

[0098] Suitable sucrose synthase domains can be for example, an *Arabidopsis* sucrose synthase 1; an *Arabidopsis* sucrose synthase 3; and a *Vigna radiate* sucrose synthase. A particularly suitable sucrose synthase domain can be, for example, *Arabidopsis* sucrose synthase 1. A particularly suitable *Arabidopsis* sucrose synthase 1 is *Arabidopsis thaliana* sucrose synthase 1 (AtSUS1), having the amino acid sequence of SEQ ID NO:7.

[0099] The UGT76G1-AtSUS1 ("GS") fusion enzyme can have a polypeptide sequence with at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% identical to the amino acid sequence set forth in SEQ ID NO:9. Suitably, the amino acid sequence of the UGT-AtSUS1 fusion enzyme has at least 80% identity to SEQ ID NO:9. More suitably, the amino acid sequence of the UGT-AtSUS1 fusion enzyme has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:9.

[0100] The isolated nucleic acid can include a nucleotide sequence encoding a polypeptide of the UGT-AtSUS1 fusion enzyme having a nucleic acid sequence that has at least 70%, at least

75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence homology to the nucleic acid sequence set forth in SEQ ID NO:10. Suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide of the UDP-glycosyltransferase fusion enzyme having an amino acid sequence that is at least 80% sequence identity to the amino acid sequence set forth in SEQ ID NO:9. More suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide of the UDP-glycosyltransferase fusion enzyme having an amino acid sequence that has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to the amino acid sequence set forth in SEQ ID NO:9. The isolated nucleic acid thus includes those nucleotide sequences encoding functional fragments of SEQ ID NO:10, functional variants of SEQ ID NO:9, or other homologous polypeptides that have, for example, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to SEQ ID NO:9. As known by those skilled in the art, the nucleic acid sequence encoding the UDP-glycosyltransferase can be codon optimized for expression in a suitable host organism such as, for example, bacteria and yeast.

[0101] The EUGT11-SUS1 ("EUS") fusion enzyme can have a polypeptide sequence with at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% and even 100% identical to the amino acid sequence set forth in SEQ ID NO:11. Suitably, the amino acid sequence of the EUGT11-SUS1 fusion enzyme has at least 80% identity to SEQ ID NO:11. More suitably, the amino acid sequence of the EUGT11-SUS1 fusion enzyme has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:11.

[0102] The isolated nucleic acid can include a nucleotide sequence encoding a polypeptide of the EUGT11-SUS1 fusion enzyme having a nucleic acid sequence that has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence homology to the nucleic acid sequence set forth in SEQ ID NO:12. Suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide of the EUGT11-SUS1 fusion enzyme having an amino acid sequence that is at least 80% sequence identity to the amino acid sequence set forth in SEQ ID NO:11. More suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide of the EUGT11-SUS1 fusion enzyme having an amino acid sequence that has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to the amino acid sequence set forth in SEQ ID NO:11. The isolated nucleic acid thus includes those nucleotide sequences encoding functional fragments of SEQ ID NO:11, functional variants of SEQ ID NO:11, or other homologous polypeptides that have, for example, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to SEQ ID NO:11. As known by those skilled in the art, the nucleic acid sequence encoding the EUGT11-SUS1 can be codon optimized for expression in a suitable host organism such as, for example, bacteria and yeast.

Orally Consumable Products

[0103] In another aspect, the present disclosure is directed to an orally consumable product having a sweetening amount of rebaudioside V, selected from the group consisting of a beverage product and a consumable product. In another aspect, the present disclosure is directed to an orally consumable product having a sweetening amount of rebaudioside W, selected from the group consisting of a beverage product and a consumable product. In another aspect, the present disclosure is directed to an orally consumable product having a sweetening amount of rebaudioside KA, selected from the group consisting of a beverage product and a consumable product. In another aspect, the present disclosure is directed to an orally consumable product having a sweetening amount of rebaudioside G, selected from the group consisting of a beverage product and a consumable product. In another aspect, the present disclosure is directed to an orally consumable product having a sweetening amount of rebaudioside M, selected from the group consisting of a beverage product and a consumable product.

[0104] The orally consumable product can have a sweetness intensity equivalent to about 1% (w/v-%) to about 4% (w/v-%) sucrose solution.

[0105] The orally consumable product can have from about 5 ppm to about 100 ppm rebaudioside V. The orally consumable product can have from about 5 ppm to about 100 ppm rebaudioside W. The orally consumable product can have from about 5 ppm to about 100 ppm rebaudioside KA. The orally consumable product can have from about 5 ppm to about 100 ppm rebaudioside G. The orally consumable product can have from about 5 ppm to about 100 ppm rebaudioside M.

[0106] The rebaudioside V can be the only sweetener in the orally consumable product. The rebaudioside W can be the only sweetener in the orally consumable product. The rebaudioside KA can be the only sweetener in the orally consumable product. The rebaudioside G can be the only sweetener in the orally consumable product. The rebaudioside M can be the only sweetener in the orally consumable product.

[0107] The orally consumable product can also have at least one additional sweetener. The at least one additional sweetener can be a natural high intensity sweetener, for example. The additional sweetener can be selected from a stevia extract, a steviol glycoside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside D2, rebaudioside E, rebaudioside F, dulcoside A, rubusoside, steviolbioside, sucrose, high fructose corn syrup, fructose, glucose, xylose, arabinose, rhamnose, erythritol, xylitol, mannitol, sorbitol, inositol, AceK, aspartame, neotame, sucralose, saccharine, naringin dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside, mogroside IV, siamenoside I, mogroside V, monatin, thaumatin, monellin, brazzein, L-alanine, glycine, Lo Han Guo, hernandulcin, phyllodulcin, trilobtain, and combinations thereof.

[0108] The orally consumable product can also have at least one additive. The additive can be, for example, a carbohydrate, a polyol, an amino acid or salt thereof, a polyamino acid or salt thereof, a sugar acid or salt thereof, a nucleotide, an organic acid, an inorganic acid, an organic salt, an organic acid salt, an organic base salt, an inorganic salt, a bitter compound, a flavorant, a flavoring ingredient, an astringent compound, a protein, a protein hydrolysate, a surfactant, an emulsifier, a flavonoid, an alcohol, a polymer, and combinations thereof.

[0109] In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of rebaudioside V. In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of rebaudioside W. In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of rebaudioside KA. In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of rebaudioside G. In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of rebaudioside M.

[0110] The beverage product can be, for example, a carbonated beverage product and a non-carbonated beverage product. The beverage product can also be, for example, a soft drink, a fountain beverage, a frozen beverage; a ready-to-drink beverage; a frozen and ready-to-drink beverage, coffee, tea, a dairy beverage, a powdered soft drink, a liquid concentrate, flavored water, enhanced water, fruit juice, a fruit juice flavored drink, a sport drink, and an energy drink.

[0111] In some embodiments, a beverage product of the present disclosure can include one or more beverage ingredients such as, for example, acidulants, fruit juices and/or vegetable juices, pulp, etc., flavorings, coloring, preservatives, vitamins, minerals, electrolytes, erythritol, tagatose, glycerine, and carbon dioxide. Such beverage products may be provided in any suitable form, such as a beverage concentrate and a carbonated, ready-to-drink beverage.

[0112] In certain embodiments, beverage products of the present disclosure can have any of numerous different specific formulations or constitutions. The formulation of a beverage product of the present disclosure can vary to a certain extent, depending upon such factors as the product's intended market segment, its desired nutritional characteristics, flavor profile, and the like. For example, in certain embodiments, it can generally be an option to add further ingredients to the formulation of a particular beverage product. For example, additional (i.e., more and/or other) sweeteners can be added, flavorings, electrolytes, vitamins, fruit juices or other fruit products, tastents, masking agents and the like, flavor enhancers, and/or carbonation typically may be added to any such formulations to vary the taste, mouthfeel, nutritional characteristics, etc. In embodiments, the beverage product can be a cola beverage that contains water, about 5 ppm to about 100 ppm rebaudioside V, an acidulant, and flavoring.

In embodiments, the beverage product can be a cola beverage that contains water, about 5 ppm to about 100 ppm rebaudioside W, an acidulant, and flavoring. In embodiments, the beverage product can be a cola beverage that contains water, about 5 ppm to about 100 ppm rebaudioside M, an acidulant, and flavoring. Exemplary flavorings can be, for example, cola flavoring, citrus flavoring, and spice flavorings. In some embodiments, carbonation in the form of carbon dioxide can be added for effervescence. In other embodiments, preservatives can be added, depending upon the other ingredients, production technique, desired shelf life, etc. In certain embodiments, caffeine can be added. In some embodiments, the beverage product can be a cola-flavored carbonated beverage, characteristically containing carbonated water, sweetener, kola nut extract and/or other flavoring, caramel coloring, one or more acids, and optionally other ingredients.

[0113] Suitable amounts of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G present in the beverage product can be, for example, from about 5 ppm to about 100 ppm. In some embodiments, low concentrations of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G, for example, less than 100 ppm, and has an equivalent sweetness to sucrose solutions having concentrations between 10,000 ppm to 30,000 ppm. The final concentration that ranges from about 5 ppm to about 100 ppm, from about 5 ppm to about 95 ppm, from about 5 ppm to about 90 ppm, from about 5 ppm to about 85 ppm, from about 5 ppm to about 80 ppm, from about 5 ppm to about 75 ppm, from about 5 ppm to about 70 ppm, from about 5 ppm to about 65 ppm, from about 5 ppm to about 60 ppm, from about 5 ppm to about 55 ppm, from about 5 ppm to about 50 ppm, from about 5 ppm to about 45 ppm, from about 5 ppm to about 40 ppm, from about 5 ppm to about 35 ppm, from about 5 ppm to about 30 ppm, from about 5 ppm to about 25 ppm, from about 5 ppm to about 20 ppm, from about 5 ppm to about 15 ppm, or from about 5 ppm to about 10 ppm. Alternatively, rebaudioside V or rebaudioside W can be present in beverage products of the present disclosure at a final concentration that ranges from about 5 ppm to about 100 ppm, from about 10 ppm to about 100 ppm, from about 15 ppm to about 100 ppm, from about 20 ppm to about 100 ppm, from about 25 ppm to about 100 ppm, from about 30 ppm to about 100 ppm, from about 35 ppm to about 100 ppm, from about 40 ppm to about 100 ppm, from about 45 ppm to about 100 ppm, from about 50 ppm to about 100 ppm, from about 55 ppm to about 100 ppm, from about 60 ppm to about 100 ppm, from about 65 ppm to about 100 ppm, from about 70 ppm to about 100 ppm, from about 75 ppm to about 100 ppm, from about 80 ppm to about 100 ppm, from about 85 ppm to about 100 ppm, from about 90 ppm to about 100 ppm, or from about 95 ppm to about 100 ppm.

[0114] In another aspect, the present disclosure is directed to a consumable comprising a sweetening amount of rebaudioside V. In another aspect, the present disclosure is directed to a consumable comprising a sweetening amount of rebaudioside W. In another aspect, the present disclosure is directed to a consumable comprising a sweetening amount of rebaudioside KA. In another aspect, the present disclosure is directed to a consumable comprising a sweetening amount of rebaudioside G. In another aspect, the present disclosure is directed to a consumable comprising a sweetening amount of rebaudioside M. The consumable can be, for example, a food product, a nutraceutical, a pharmaceutical, a dietary supplement, a dental hygienic composition, an edible gel composition, a cosmetic product and a tabletop flavoring.

[0115] As used herein, "dietary supplement(s)" refers to compounds intended to supplement the diet and provide nutrients, such as vitamins, minerals, fiber, fatty acids, amino acids, etc. that may be missing or may not be consumed in sufficient quantities in a diet. Any suitable dietary supplement known in the art may be used. Examples of suitable dietary supplements can be, for example, nutrients, vitamins, minerals, fiber, fatty acids, herbs, botanicals, amino acids, and metabolites.

[0116] As used herein, "nutraceutical(s)" refers to compounds, which includes any food or part of a food that may provide medicinal or health benefits, including the prevention and/or treatment of disease or disorder (e.g., fatigue, insomnia, effects of aging, memory loss, mood disorders, cardiovascular disease and high levels of cholesterol in the blood, diabetes, osteoporosis, inflammation, autoimmune disorders, etc.). Any suitable nutraceutical known in the art may be used. In some embodiments, nutraceuticals can be used as supplements to food and beverages and as pharmaceutical formulations for enteral or parenteral applications which may be solid formulations, such as capsules or tablets, or liquid formulations, such as solutions or suspensions.

[0117] In some embodiments, dietary supplements and nutraceuticals can further contain protective hydrocolloids (such as gums, proteins, modified starches), binders, film-forming agents, encapsulating agents/materials, wall/shell materials, matrix compounds, coatings, emulsifiers, surface active agents, solubilizing agents (oils, fats, waxes, lecithins, etc.), adsorbents, carriers, fillers, co-compounds, dispersing agents, wetting agents, processing aids (solvents), flowing agents, taste-masking agents, weighting agents, jellyfying agents, gel-forming agents, antioxidants and antimicrobials.

[0118] As used herein, a "gel" refers to a colloidal system in which a network of particles spans the volume of a liquid medium. Although gels mainly are composed of liquids, and thus exhibit densities similar to liquids, gels have the structural coherence of solids due to the network of particles that spans the liquid medium. For this reason, gels generally appear to be solid, jelly-like materials. Gels can be used in a number of applications. For example, gels can be used in foods, paints, and adhesives. Gels that can be eaten are referred to as "edible gel compositions." Edible gel compositions typically are eaten as snacks, as desserts, as a part of staple foods, or along with staple foods. Examples of suitable edible gel compositions can be, for example, gel desserts, puddings, jams, jellies, pastes, trifles, aspics, marshmallows, gummy candies, and the like. In some embodiments, edible gel mixes generally are powdered or granular solids to which a fluid may be added to form an edible gel composition. Examples of suitable fluids can be, for example, water, dairy fluids, dairy analogue fluids, juices, alcohol, alcoholic beverages, and combinations thereof. Examples of suitable dairy fluids can be, for example, milk, cultured milk, cream, fluid whey, and mixtures thereof. Examples of suitable dairy analogue fluids can be, for example, soy milk and non-dairy coffee whitener.

[0119] As used herein, the term "gelling ingredient" refers to any material that can form a colloidal system within a liquid medium. Examples of suitable gelling ingredients can be, for example, gelatin, alginate, carageenan, gum, pectin, konjac, agar, food acid, rennet, starch, starch derivatives, and combinations thereof. It is well known to those in the art that the amount of gelling ingredient used in an edible gel mix or an edible gel composition can vary considerably depending on a number of factors such as, for example, the particular gelling ingredient used, the particular fluid base used, and the desired properties of the gel.

[0120] Gel mixes and gel compositions of the present disclosure can be prepared by any suitable method known in the art. In some embodiments, edible gel mixes and edible gel compositions of the present disclosure can be prepared using other ingredients in addition to the gelling agent. Examples of other suitable ingredients can be, for example, a food acid, a salt of a food acid, a buffering system, a bulking agent, a sequestrant, a crosslinking agent, one or more flavors, one or more colors, and combinations thereof.

[0121] Any suitable pharmaceutical composition known in the art may be used. In certain embodiments, a pharmaceutical composition of the present disclosure can contain from about 5 ppm to about 100 ppm of rebaudioside V, and one or more pharmaceutically acceptable excipients. In certain embodiments, a pharmaceutical composition of the present disclosure can contain from about 5 ppm to about 100 ppm of rebaudioside W, and one or more pharmaceutically acceptable excipients. In certain embodiments, a pharmaceutical composition of the present disclosure can contain from about 5 ppm to about 100 ppm of rebaudioside KA, and one or more pharmaceutically acceptable excipients. In certain embodiments, a pharmaceutical composition of the present disclosure can contain from about 5 ppm to about 100 ppm of rebaudioside G, and one or more pharmaceutically acceptable excipients. In certain embodiments, a pharmaceutical composition of the present disclosure can contain from about 5 ppm to about 100 ppm of rebaudioside M, and one or more pharmaceutically acceptable excipients. In some embodiments, pharmaceutical compositions of the present disclosure can be used to formulate pharmaceutical drugs containing one or more active agents that exert a biological effect. Accordingly, in some embodiments, pharmaceutical compositions of the present disclosure can contain one or more active agents that exert a biological effect. Suitable active agents are well known in the art (e.g., The Physician's Desk Reference). Such compositions can be prepared according to procedures well known in the art, for example, as described in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., USA.

[0122] Rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G can be used with any suitable dental and oral hygiene compositions known in the art. Examples of suitable dental and oral hygiene compositions can be, for example, toothpastes, tooth polishes, dental floss, mouthwashes, mouth rinses, dentrifices, mouth sprays, mouth refreshers, plaque rinses, dental pain relievers, and the like.

[0123] Suitable amounts of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G present in the consumable can be, for example, from about 5 parts per million (ppm) to about 100 parts per million (ppm). In some embodiments, low concentrations of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G, for example, less than 100 ppm, has an equivalent sweetness to sucrose solutions having concentrations between 10,000 ppm to 30,000 ppm. The final concentration that ranges from about 5 ppm to about 100 ppm, from about 5 ppm to about 95 ppm, from about 5 ppm to about 90 ppm, from about 5 ppm to about 85 ppm, from about 5 ppm to about 80 ppm, from about 5 ppm to about 75 ppm, from about 5 ppm to about 70 ppm, from about 5 ppm to about 65 ppm, from about 5 ppm to about 60 ppm, from about 5 ppm to about 55 ppm, from about 5 ppm to about 50 ppm, from about 5 ppm to about 45 ppm, from about 5 ppm to about 40 ppm, from about 5 ppm to about 35 ppm, from about 5 ppm to about 30 ppm, from about 5 ppm to about 25 ppm, from about 5 ppm to about 20 ppm, from about 5 ppm to about 15 ppm, or from about 5 ppm to about 10 ppm. Alternatively, rebaudioside V or rebaudioside W can be present in consumable products of the present disclosure at a final concentration that ranges from about 5 ppm to about 100 ppm, from about 10 ppm to about 100 ppm, from about 15 ppm to about 100 ppm, from about 20 ppm to about 100 ppm, from about 25 ppm to about 100 ppm, from about 30 ppm to about 100 ppm, from about 35 ppm to about 100 ppm, from about 40 ppm to about 100 ppm, from about 45 ppm to about 100 ppm, from about 50 ppm to about 100 ppm, from about 55 ppm to about 100 ppm, from about 60 ppm to about 100 ppm, from about 65 ppm to about 100 ppm, from about 70 ppm to about 100 ppm, from about 75 ppm to about 100 ppm, from about 80 ppm to about 100 ppm, from about 85 ppm to about 100 ppm, from about 90 ppm to about 100 ppm, or from about 95 ppm to about 100 ppm.

[0124] In certain embodiments, from about 5 ppm to about 100 ppm of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is present in food product compositions. As used herein, "food product composition(s)" refers to any solid or liquid ingestible material that can, but need not, have a nutritional value and be intended for consumption by humans and animals.

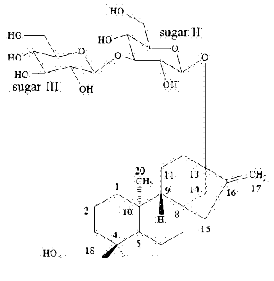
[0125] Examples of suitable food product compositions can be, for example, confectionary compositions, such as candies, mints, fruit flavored drops, cocoa products, chocolates, and the like; condiments, such as ketchup, mustard, mayonnaise, and the like; chewing gums; cereal compositions; baked goods, such as breads, cakes, pies, cookies, and the like; dairy products, such as milk, cheese, cream, ice cream, sour cream, yogurt, sherbet, and the like; tabletop sweetener compositions; soups; stews; convenience foods; meats, such as ham, bacon, sausages, jerky, and the like; gelatins and gelatin-like products such as jams, jellies, preserves, and the like; fruits; vegetables; egg products; icings; syrups including molasses; snacks; nut meats and nut products; and animal feed.

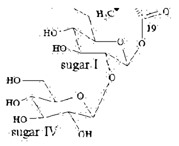
[0126] Food product compositions can also be herbs, spices and seasonings, natural and synthetic flavors, and flavor enhancers, such as monosodium glutamate. In some embodiments, food product compositions can be, for example, prepared packaged products, such as dietetic sweeteners, liquid sweeteners, granulated flavor mixes, pet foods, livestock feed, tobacco, and materials for baking applications, such as powdered baking mixes for the preparation of breads, cookies, cakes, pancakes, donuts and the like. In other embodiments, food product compositions can also be diet and low-calorie food and beverages containing little or no sucrose.

[0127] In certain embodiments that may be combined with any of the preceding embodiments, the rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is the only sweetener, and the product has a sweetness intensity equivalent to about 1% to about 4% (w/v-%) sucrose solution. In certain embodiments that can be combined with any of the preceding embodiments, the consumable products and beverage products can further include an additional sweetener, where the product has a sweetness intensity equivalent to about 1% to about 10% (w/v-%) sucrose solution. In certain embodiments that can be combined with any of the preceding embodiments, every sweetening ingredient in the product is a high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, every sweetening ingredient in the product can a natural high intensity sweetener. In certain embodiments that can be combined with any of the preceding embodiments, the additional sweetener contains one or more sweeteners selected from a stevia extract, a steviol glycoside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside D2, rebaudioside F, dulcoside A, rubusoside, steviolbioside, sucrose, high fructose corn syrup, fructose, glucose, xylose, arabinose, rhamnose, erythritol, xylitol, mannitol, sorbitol, inositol, AceK, aspartame, neotame, sucralose, saccharine, naringin dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside mogroside IV, siamenoside I, mogroside V, monatin, thaumatin, monellin, brazzein, L-alanine, glycine, Lo Han Guo, hernandulcin, phyllodulcin, trilobtain, and combinations thereof. In certain embodiments that can be combined with any of the preceding embodiments, the consumable products and beverage products can further include one or more additives selected from a carbohydrate, a polyol, an amino acid or salt thereof, a poly-amino acid or salt thereof, a sugar acid or salt thereof, a nucleotide, an organic acid, an inorganic acid, an organic salt, an organic acid salt, an organic base salt, an inorganic salt, a bitter compound, a flavorant, a flavoring ingredient, an astringent compound, a protein, a protein hydrolysate, a surfactant, an emulsifier, a flavonoid, an alcohol, a polymer, and combinations thereof. In certain embodiments that can be combined with any of the preceding embodiments, the rebaudioside D2 has a purity of about 50% to about 100% by weight before it is added into the product.

Sweetener

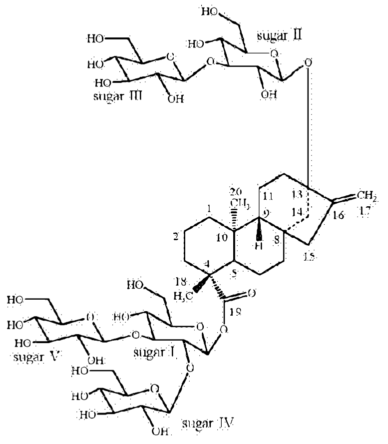
[0128] In another aspect, the present disclosure is directed to a sweetener consisting of a chemical structure:





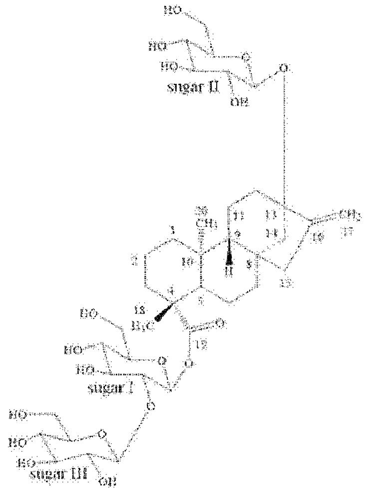
Reb V

[0129] In another aspect, the present disclosure is directed to a sweetener consisting of a chemical structure:



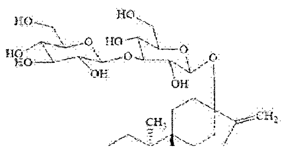
Reb W

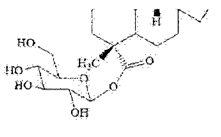
[0130] In another aspect, the present disclosure is directed to a sweetener consisting of a chemical structure:



Reb KA

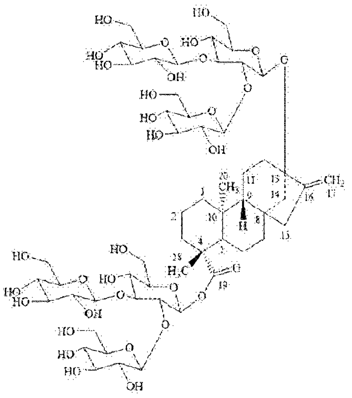
[0131] In another aspect, the present disclosure is directed to a sweetener consisting of a chemical structure:





Reb G

[0132] In another aspect, the present disclosure is directed to a sweetener consisting of a chemical structure:



Reb M

[0133] In certain embodiments, the sweetener can further include at least one of a filler, a bulking agent and an anticaking agent. Suitable fillers, bulking agents and anticaking agents are known in the art.

[0134] In certain embodiments, rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G sweetener can be included and/or added at a final concentration that is sufficient to sweeten and/or enhance the sweetness of the consumable products and beverage products. The "final concentration" of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G refers to the concentration of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G present in the final consumable products and beverage products (i.e., after all ingredients and/or compounds have been added to produce the consumable products and beverage products). Accordingly, in certain embodiments, rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is included and/or added to a compound or ingredient used to prepare the consumable products and beverage products. The rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G may be present in a single compound or ingredient, or multiple compounds and ingredients. In other embodiments, rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is included and/or added to the consumable products and beverage products. In certain preferred embodiments, the rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is included and/or added at a final concentration that ranges from about 5 ppm to about 100 ppm, from about 5 ppm to about 95 ppm, from about 5 ppm to about 90 ppm, from about 5 ppm to about 85 ppm, from about 5 ppm to about 80 ppm, from about 5 ppm to about 75 ppm, from about 5 ppm to about 70 ppm, from about 5 ppm to about 65 ppm, from about 5 ppm to about 60 ppm, from about 5 ppm to about 55 ppm, from about 5 ppm to about 50 ppm, from about 5 ppm to about 45 ppm, from about 5 ppm to about 40 ppm, from about 5 ppm to about 35 ppm, from about 5 ppm to about 30 ppm, from about 5 ppm to about 25 ppm, from about 5 ppm to about 20 ppm, from about 5 ppm to about 15 ppm, or from about 5 ppm to about 10 ppm. Alternatively, the rebaudioside V or rebaudioside W is included and/or added at a final concentration that ranges from about 5 ppm to about 100 ppm, from about 10 ppm to about 100 ppm, from about 15 ppm to about 100 ppm, from about 20 ppm to about 100 ppm, from about 25 ppm to about 100 ppm, from about 30 ppm to about 100 ppm, from about 35 ppm to about 100 ppm, from about 40 ppm to about 100 ppm, from about 45 ppm to about 100 ppm, from about 50 ppm to about 100 ppm, from about 55 ppm to about 100 ppm, from about 60 ppm to about 100 ppm, from about 65 ppm to about 100 ppm, from about 70 ppm to about 100 ppm, from about 75 ppm to about 100 ppm, from about 80 ppm to about 100 ppm, from about 85 ppm to about 100 ppm, from about 90 ppm to about 100 ppm, or from about 95 ppm to about 100 ppm. For example, rebaudioside V or rebaudioside W may be included and/or added at a final concentration of about 5 ppm, about 10 ppm, about 15 ppm, about 20 ppm, about 25 ppm, about 30 ppm, about 35 ppm, about 40 ppm, about 45 ppm, about 50 ppm, about 55 ppm, about 60 ppm, about 65 ppm, about 70 ppm, about 75 ppm, about 80 ppm, about 85 ppm, about 90 ppm, about 95 ppm, or about 100 ppm, including any range in between these values.

[0135] In certain embodiments, rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G is the only sweetener included and/or added to the consumable products and the beverage products. In such embodiments, the consumable products and the beverage products have a sweetness intensity equivalent to about 1% to about 4% (w/v-%) sucrose solution, about 1% to about 3% (w/v-%) sucrose solution, or about 1% to about 2% (w/v-%) sucrose solution. Alternatively, the consumable products and the beverage products have a sweetness intensity equivalent to about 1% to about 4% (w/v-%) sucrose solution, about 2% to about 4% (w/v-%) sucrose solution, about 3% to about 4% (w/v-%) sucrose solution, or about 4%. For example, the consumable products and the beverage products may have a sweetness intensity equivalent to about 1%, about 2%, about 3%, or about 4% (w/v-%) sucrose solution, including any range in between these values.

[0136] The consumable products and beverage products of the present disclosure can include a mixture of rebaudioside V, rebaudioside W, rebaudioside KA, rebaudioside M, or rebaudioside G and one or more sweeteners of the present disclosure in a ratio sufficient to achieve a desirable sweetness intensity, nutritional characteristic, taste profile, mouthfeel, or other organoleptic factor.

[0137] The disclosure will be more fully understood upon consideration of the following non-limiting Examples.

EXAMPLES

EXAMPLE 1

[0138] In this Example, full-length DNA fragments of all candidate UGT genes were synthesized.

[0139] Specifically, the cDNAs were codon optimized for *E. coli* expression (Genscript, Piscataway, NJ). The synthesized DNA was cloned into a bacterial expression vector pETite N-His SUMO Kan Vector (Lucigen). For the nucleotide sequence encoding the UDP-glycosyltransferase fusion enzymes (UGT76G1-AtSUS1 and EUGT11-AtSUSI), a GSG-linker (encoded by the nucleotide sequence: ggttctggt) was inserted in frame between a nucleotide sequence encoding the uridine diphospho glycosyltransferase domain and the nucleotide sequence encoding the sucrose synthase 1 from *A. thaliana* (AtSUS1). Table 2 summarizes the protein and sequence identifier numbers.

Table 2. Sequence Identification Numbers.

Name	SEQ ID NO	Description
UGT76G1	SEQ ID NO: 1	Amino acid
UGT76G1	SEQ ID NO: 2	Nucleic acid
EUGT11	SEQ ID NO: 3	Amino acid
EUGT11	SEQ ID NO: 4	Nucleic acid
HV1	SEQ ID NO: 5	Amino acid
HV1	SEQ ID NO: 6	Nucleic acid
AtSUS1	SEQ ID NO: 7	Amino acid
AtSUS1	SEQ ID NO: 8	Nucleic acid
GS fusion enzyme	SEQ ID NO: 9	Amino acid
GS fusion enzyme	SEQ ID NO: 10	Nucleic acid
EUS fusion enzyme	SEQ ID NO: 11	Amino acid
EUS fusion enzyme	SEQ ID NO: 12	Nucleic acid

[0140] Each expression construct was transformed into *E. coli* BL21 (DE3), which was subsequently grown in LB media containing 50 μg/mL kanamycin at 37 °C until reaching an OD_{600} of 0.8-1.0. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was further grown at 16 °C for 22 hr. Cells were harvested by centrifugation (3,000 x g; 10 min; 4 °C). The cell pellets were collected and were either used immediately or stored at -80 °C.

[0141] The cell pellets were re-suspended in lysis buffer (50 mM potassium phosphate buffer, pH 7.2, 25 μg/ml lysozyme, 5 μg/ml DNase I, 20 mM imidazole, 500 mM NaCl, 10% glycerol, and 0.4% TRITON X-100). The cells were disrupted by sonication at 4 °C, and the cell debris was clarified by centrifugation (18,000 x g; 30 min). Supernatant was loaded to a equilibrated (equilibration buffer: 50 mM potassium phosphate buffer, pH 7.2, 20 mM imidazole, 500 mM NaCl, 10% glycerol) Ni-NTA (Qiagen) affinity column. After loading of protein sample, the column was washed with equilibration buffer to remove unbound contaminant proteins. The His-tagged UGT recombinant polypeptides were eluted by equilibration buffer containing 250mM imidazole. Purified HV1 (61.4kD), UGT76G1 (65.4kD), AtSUS1 (106.3kD), EUGT11 (62kD), UGT76G1-SUS1 (GS) (157.25kD) and EUGT11-AtSUS1 (155kD) fusion proteins were shown in FIG. 2.

EXAMPLE 2

[0142] In this Example, candidate UGT recombinant polypeptides were assayed for glycosyltranferase activity by using tested steviol glycosides as the substrate.

[0143] Typically, the recombinant polypeptide (10 µg) was tested in a 200 µl in vitro reaction system. The reaction system contains 50 mM potassium phosphate buffer, pH 7.2, 3 mM $MgCl_2$, 1 mg/ml steviol glycoside substrate, 1 mM UDP-glucose. The reaction was performed at 30 °C and terminated by adding 200 µL 1-butanol. The samples were extracted three times with 200 µL 1-butanol. The pooled fraction was dried and dissolved in 70 µL 80% methanol for high-performance liquid chromatography (HPLC) analysis. Rubusoside (99%, Blue California, CA), purified Reb G (98.8%), Reb KA (98.4%) and Reb V (80%) was used as substrate in *in vitro* reactions.

[0144] The UGT catalyzed glycosylation reaction was be coupled to a UDP-glucose generating reaction catalyzed by a sucrose synthase (such as AtSUS1). In this method, the UDP-glucose was generated from sucrose and UDP, such that the addition of extra UDP-glucose can be omitted. In the assay, recombinant AtSUS1 was added in UGT reaction system and UDP-glucose can be regenerated from UDP. AtSUS1 sequence (Bieniawska et al., Plant J. 2007, 49: 810-828) was synthesized and inserted into a bacterial expression vector. The recombinant AtSUS1 protein was expressed and purified by affinity chromatography.

[0145] HPLC analysis was performed using a Dionex UPLC ultimate 3000 system (Sunnyvale, CA), including a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Phenomenex Luna NH_2, Luna C18 or Synergi Hydro-RP column with guard column was used for the characterization of steviol glycosides. Acetonitrile in water or in Na_3PO_4 buffer was used for isocratic elution in HPLC analysis. The detection wavelength was 210nm.

EXAMPLE 3

[0146] In this Example, the recombinant HV1 polypeptides were analyzed for transferring a sugar moiety to rubusoside to produce rebaudioside KA ("Minor diterpene glycosides from the leaves of Stevia rebaudiana". Journal of Natural Products (2014), 77(5), 1231-1235) in all reaction conditions with or without AtSUS1.

[0147] As shown in FIG. 3, the recombinant HV1 polypeptides transferred a sugar moiety to rubusoside to produce Reb KA in all reaction conditions with or without AtSUS1. Rubusoside was completely converted to Reb KA and Reb E by the recombinant HV1 in a UGT-SUS coupling reaction system (G, I). However, only partial rubososide was converted to Reb KA after 24 hours (H) by the recombinant HV1 polypeptide alone without being coupled to AtSUS1, indicating AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system. In the HV1-AtSUS1 coupling reaction system, produced Reb KA can be continually converted to Reb E.

EXAMPLE 4

[0148] In this Example, HV1 activity was analyzed using Reb E as a substrate.

[0149] Reb E substrate (0.5mg/ml) was incubated with the recombinant HV1 polypeptide (20 µg) and AtSUS1 (20 µg) in a UGT-SUS coupling reaction system (200 µL) under conditions similar to those used in the examples above. As shown in FIG. 4, Reb Z was produced by the combination of the recombinant HV1 polypeptide and AtSUS1. These results indicated that HV1 can transfer a glucose moiety to Reb E to form RZ. FIG. 4 shows rebaudioside Z ("Reb Z") can be produced from rebaudioside E ("Reb E") catalyzed by a recombinant HV1 polypeptide and a recombinant AtSUS1 in a HV1-AtSUS1 coupling reaction system. HV1 can transfer a glucose to Reb E to produce Reb Z, mixture of Reb Z1 and Reb Z2 in the ratio between 60:40 to 70:30 (US Provisional Application No. 61/898,571, assigned to Conagen Inc.).

EXAMPLE 5

[0150] In this Example, to confirm the conversion of Reb KA to Reb E, purified Reb KA substrate was incubated with recombinant HV1 with or without AtSUS1. As shown in FIG. 5, Reb E was produced by the recombinant HV1 polypeptide in both reaction conditions. However, AtSUS1 polypeptide in a UGT-SUS coupling reaction system can enhance the reaction efficiency. All Reb KA substrate can be completely converted to Reb E in the UGT-SUS coupling system (D).

EXAMPLE 6

[0151] In this Example, EUGT11 activity was analyzed using rubusoside as a substrate.

[0152] As shown in FIG. 6, EUGT11 can transfer a sugar moiety to rubusoside to produce Reb KA and stevioside in all reaction conditions with or without AtSUS1. AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system. In the HV1-AtSUS1 coupling reaction system, Reb E can be continually converted by EUGT11. EUS fusion protein exhibited higher activity under same reaction condition. All produced Reb KA and stevioside was completely converted to Reb E by EUS at 48hr. Reb E can be continually converted to Reb D2.

EXAMPLE 7

[0153] In this Example, EUGT11 activity was analyzed using Reb KA as a substrate.

[0154] EUGT11 is a UGT with a 1,2-19-O-glucose glycosylation activity to produce related steviol glycoside (PCT Published Application WO2013/022989, assigned to Evolva SA). For example, EUGT11 can catalyze the reaction to produce Reb E from stevioside. EUGT11 also has a 1,6-13-O-glucose glycosylation activity that can transfer a glucose molecule to rebaudioside E to form rebaudioside D2 (US Patent Application Serial No. 14/269,435, assigned to Conagen, Inc.). In the experiments, we found EUGT11 can transfer a glucose residue to Reb KA to form Reb E. As shown in FIG. 7, EUGT11 can transfer a sugar moiety to Reb KA to produce Reb E in all reaction conditions with (E, H) or without AtSUS1 (D, G). AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system (E, H). In the EUGT11-AtSUS1 coupling reaction system (E, H) and EUS fusion reaction system (F, I), all Reb KA was completely converted and the produced Reb E can be continually converted to Reb D2.

EXAMPLE 8

[0155] In this Example, UGT76G1 activity was analyzed using rubusoside as a substrate.

[0156] UGT76G1 has 1,3-13-O-glucose glycosylation activity that can transfer a glucose molecule to stevioside to form rebaudioside A and to Reb E to form rebaudioside D. In the example, we found UGT76G1 can transfer a glucose residue to rubusoside to form rebaudioside G.

[0157] As shown in FIG. 8, UGT76G1 can transfer a sugar moiety to rubusoside to produce Reb G in all reaction conditions with (D, G) or without AtSUS1 (C, F). AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system. GS fusion protein exhibited higher activity under same reaction condition (E, H). All rubusoside was completely converted to Reb G by GS at 12hr (E).

EXAMPLE 9

[0158] In this Example, UGT76G1 activity was analyzed using rebaudioside KA as a substrate.

[0159] To further identify the enzymatic activity of UGT76G1, an *in vitro* assay was performed using rebaudioside KA as substrate. Surprisingly, a novel steviol glycoside (rebaudioside V "Reb V") was produced in an early time point. At later time points, Reb V produced in the reaction was converted to another novel steviol glycoside (rebaudioside W "RebW").

[0160] As shown in FIG. 9, UGT76G1 can transfer a sugar moiety to Reb KA to produce Reb V in all reaction conditions with (F, I) or without AtSUS1 (E, H). AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system (F, I). In the UGT76G1-AtSUS1 coupling reaction system (I) and GS fusion reaction system (J), produced Reb V was completely converted to Reb W at 12hr.

EXAMPLE 10

[0161] In this Example, UGT76G1 activity was analyzed using Reb V as a substrate.

[0162] Purified Reb V as substrate was introduced into the reaction system. As shown in FIG. 10C, Reb V was surprisingly completely converted to Reb W by the UGT76G1 recombinant polypeptide in UGT-SUS1 coupling system at 6hr.

EXAMPLE 11

[0163] In this Example, HV1 activity was analyzed using Reb G as a substrate.

[0164] As shown in FIG. 11, the recombinant HV1 polypeptides transferred a sugar moiety to rebaudioside G to produce Reb V in all reaction conditions with or without AtSUS1. Reb G was completely converted to Reb V by the recombinant HV1 in a UGT-SUS coupling reaction system (E, G). However, only partial Reb G was converted to Reb V after 24 hours (F) by the recombinant HV1 polypeptide alone without being coupled to AtSUS1, indicating AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system.

EXAMPLE 12

[0165] In this Example, EUGT11 activity was analyzed using Reb G as a substrate.

[0166] As shown in FIG. 12, the recombinant EUGT11 polypeptides transferred a sugar moiety to rebaudioside G to produce Reb V in all reaction conditions with (F, I) or without AtSUS1 (E, H). More Reb G was converted to Reb V by the recombinant EUGT11 in a UGT-SUS coupling reaction system (F, I). However, only partial Reb G was converted to Reb V by the recombinant EUGT11 polypeptide alone without being coupled to AtSUS1 (E, H), indicating AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system. EUS fusion protein exhibited higher activity under same reaction condition (G, J). All Reb G in the reaction system was completely converted to Reb V by EUS at 24hr (J).

EXAMPLE 13

[0167] In this Example, HV1 combined with UGT76G1 activities were analyzed using rubusoside as a substrate.

[0168] Rebusoside substrate was incubated with the recombinant HV1 polypeptide, UGT76G1, and AtSUS1 in a UGT-SUS coupling reaction system under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 13, Reb V and Reb W was produced by the combination of the recombinant HV1 polypeptide, UGT76G1, and AtSUS1. Thus, the recombinant HV1 polypeptide, which showed a 1,2-19-O-glucose and 1,2-13-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-13-O-glucose and 1,3-19-O-glucose glycosylation activity) for the complex, multi-step biosynthesis of steviol glycosides. If HV1 recombinant protein was combined with GS fusion protein in the reaction system, Reb V and Reb W was also produced by these UGT enzymes, indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 14

[0169] In this Example, EUGT11 combined with UGT76G1 activities were analyzed using rubusoside as a substrate.

[0170] Rebusoside substrate was incubated with the recombinant EUGT11 polypeptide, UGT76G1, and AtSUS1 in a UGT-SUS coupling reaction system under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 14, Reb W was produced by the combination of the recombinant EUGT11 polypeptide, UGT76G1, and AtSUS1. Thus, the recombinant EUGT11 polypeptide, which showed a 1, 2-19-O-glucose and 1, 2-13-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-13-O-glucose and 1,3-19-O-Glucose glycosylation activity) for the complex, multi-step biosynthesis of steviol glycosides. If EUGT11 recombinant protein was combined with GS fusion protein in the reaction system, Reb W was also produced by these UGT enzymes, indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 15

[0171] In this Example, HV1 combined with UGT76G1 activities were analyzed using Reb G as a substrate.

[0172] Reb G substrate was incubated with the recombinant HV1 polypeptide, UGT76G1, and AtSUS1 in a UGT-SUS coupling reaction system under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 15, Reb V and Reb W was produced by the combination of the recombinant HV1 polypeptide, UGT76G1, and AtSUS1. After 12 hours, all rubusoside substrate was converted to Reb V, and after 36 hours, all produced Reb V was converted to Reb W. Thus, the recombinant HV1 polypeptide, which showed a 1,2-19-O-glucose and 1,2-13-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-13-O-glucose and 1,3-19-O-Glucose glycosylation activity) for the complex, multi-step biosynthesis of steviol glycosides. If HV1 recombinant protein was combined with GS fusion protein in the reaction system, Reb V and Reb W was also produced by these UGT enzymes, indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 16

[0173] In this Example, EUGT11 combined with UGT76G1 activities were analyzed using Reb G as a substrate.

[0174] Reb G substrate was incubated with the recombinant EUGT11 polypeptide, UGT76G1, and AtSUS1 in a UGT-SUS coupling reaction system under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 16, Reb W was produced by the combination of the recombinant EUGT11 polypeptide, UGT76G1, and AtSUS1. Thus, the recombinant EUGT11 polypeptide, which showed a 1, 2-19-O-glucose and 1, 2-13-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-13-0-glucose and 1,3-19-O-Glucose glycosylation activity) for the complex, multi-step biosynthesis of steviol glycosides. If EUGT11 recombinant protein was combined with GS fusion protein in the reaction system, Reb W was also produced by these UGT enzymes, indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 17

[0175] In this Example, UGT76G1 and GS fusion enzyme activity was analyzed using Reb D as a substrate.

[0176] Reb D substrate was incubated with the recombinant UGT76G1 under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 22, Reb M was produced by the UGT76G1 with (FIG. 22D and G) or without AtSUS1 (FIG. 22C and F) in the reactions. Thus, the recombinant UGT76G1 polypeptide, which showed a 1, 3-19-O-glucose glycosylation activity, can be used in biosynthesis of rebaudioside M. Reb D was completely converted to Reb M by the recombinant UGT76G1 in a UGT-SUS coupling reaction system (FIG. 22G). However, only partial Reb D was converted to Reb M after 6 hours (F) by the recombinant UGT76G1 polypeptide alone without being coupled to AtSUS1, indicating AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system. GS fusion protein exhibited similar activity as UGT76G1-AtSUS1 coupling reaction under same reaction condition (E, H). All Reb D was completely converted to Reb M by GS at 6hr (H), indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 18

[0177] In this Example, UGT76G1 and GS fusion enzyme activity was analyzed using Reb E as substrate.

[0178] Reb E substrate was incubated with the recombinant UGT76G1 or GS fusion enzyme under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 23, Reb D was produced by the UGT76G1 with (FIG. 23E, H and K) or without AtSUS1 (FIG. 22D, G and J) and GS fusion enzyme (FIG. 23F, I and L) in the reactions. Furthermore, Reb M was formed from Reb D produced in the reactions. Thus, the recombinant UGT76G1 polypeptide, which showed a 1,3-13-O-glucose and 1,3-19-O-glucose glycosylation activity, can be used in the biosynthesis of rebaudioside D and rebaudioside M. Reb E was completely converted to Reb M by the recombinant UGT76G1 in a UGT-SUS coupling reaction system after 24hr (FIG. 23K). However, only Reb D was converted from Reb E completely after 24 hours (J) by the recombinant UGT76G1 polypeptide alone without being coupled to AtSUS1, indicating AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system through continuing UDPG production. GS fusion protein exhibited similar activity as UGT76G1-AtSUS1 coupling reaction under same reaction condition (FIG. 23F, I and L), indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 19

[0179] In this Example, HV1 combined with UGT76G1 activities were analyzed using stevioside as a substrate.

[0180] Stevioside substrate was incubated with the recombinant HV1 polypeptide and UGT76G1 or GS fusion enzyme under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 24, Reb A was produced by the combination of the recombinant HV1 polypeptide and UGT76G1 in all reactions. Furthermore, Reb D and Reb M were detected in the reactions using the combination of recombinant HV1 polypeptide, UGT76G1 polypeptide and AtSUS1 (FIG. 24E, H and K) or the combination of recombinant GS fusion enzyme and HV1 polypeptide (FIG. 24F, I and L). The recombinant HV1 polypeptide, which showed a 1, 2-19-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-13-O-glucose and 1,3-19-O-glucose glycosylation activity) for the complex, multi-step biosynthesis of rebaudioside D and rebaudioside M. The results also showed that AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system through continuing UDPG production (FIG. 24E, H and K). GS fusion protein exhibited similar activity as UGT76G1-AtSUS1 coupling reaction under same reaction condition (FIG. 24F, I and L), indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 20

[0181] In this Example, HV1 combined with UGT76G1 activities were analyzed using Reb A as a substrate.

[0182] Reb A substrate was incubated with the recombinant HV1 polypeptide and UGT76G1 or GS fusion enzyme under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in FIG. 25, Reb D was produced by the combination of the recombinant HV1 polypeptide and UGT76G1 in all reactions. Furthermore, Reb M was detected in the reactions using the combination of recombinant HV1 polypeptide, UGT76G1 polypeptide and AtSUS1 (FIG. 25D, G and J) or the combination of recombinant GS fusion enzyme and HV1 polypeptide (FIG. 25E, H and K). The recombinant HV1 polypeptide, which showed a 1, 2-19-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1, which showed a 1,3-19-O-glucose glycosylation activity) for the complex, multi-step biosynthesis of rebaudioside D and rebaudioside M. The results also showed that AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system through continuing UDPG production (FIG. 25D, G and J). GS fusion protein exhibited similar activity as UGT76G1-AtSUS1 coupling reaction under same reaction condition (FIG. 25E, H and K), indicating UGT-SUS coupling reaction can be generated by the GS fusion protein.

EXAMPLE 21

[0183] In this Example, the structure of Reb V was analyzed by NMR.

[0184] The material used for the characterization of Reb V was produced by using enzymatic conversion of Reb G and purified by HPLC. HRMS data were generated with a LTQ Orbitrap Discovery HRMS instrument, with its resolution set to 30k. Scanned data from *m/z* 150 to 1500 in positive ion electrospray mode. The needle voltage was set to 4 kV; the other source conditions were sheath gas = 25, aux gas = 0, sweep gas = 5 (all gas flows in arbitrary units), capillary voltage = 30V, capillary temperature = 300°C, and tube lens voltage = 75. Sample was diluted with 2:2:1 acetonitrile:methanol:water (same as infusion eluent) and injected 50 microliters. NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instruments using standard pulse sequences. The ID (^1H and ^{13}C) and 2D (TOCSY, HMQC, and HMBC) NMR spectra were performed in C_5D_5N.

[0185] The molecular formula of Reb V has been deduced as $C_{44}H_{70}O_{23}$ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ions corresponding to $[M+Na]^+$ at *m/z* 989.4198; this composition was supported by the ^{13}C NMR spectral data. The ^1H NMR spectral data of Reb V showed the presence of two methyl singlets at δ 0.97 and 1.40, two olefinic protons as singlets at δ 5.06 and 5.71 of an exocyclic double bond, nine sp3 methylene and two sp3 methine protons between δ 0.74-2.72, characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia.* The basic skeleton of *ent*-kaurane diterpenoids was supported by the COSY and TOCSY studies which showed key correlations: H-1/H-2; H-2/H-3; H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12. The ^1H NMR spectrum of Reb V also showed the presence of four anomeric protons resonating at δ 5.08, 5.38, 5.57, and 6.23; suggesting four sugar units in its structure. Acid hydrolysis of Reb V with 5% H_2SO_4 afforded D-glucose which was identified by direct comparison with authentic sample by TLC. Enzymatic hydrolysis of Reb V furnished an aglycone which was identified as steviol by comparison of ^1H NMR and co-TLC with standard compound. The large coupling constants observed for the four anomeric protons of the glucose moieties at δ 5.08 (d, *J*=7.8 Hz), 5.38 (d, *J*=8.1 Hz), 5.57 (d, *J*=8.0 Hz), and 6.23 (d, *J*=7.8 Hz), suggested their β-orientation as reported for steviol glycosides. The ^1H and ^{13}C NMR values for Reb V were assigned on the basis of TOCSY, HMQC and HMBC data and are given in Table 3.

Table 3. ^1H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for Reb V and Reb G^{a-c}.

Position	Reb V		Reb G	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
1	0.74 m, 1.66 m	41.1	0.78 m, 1.69 m	41.3
2	1.43 m, 2.18 m	20.4	1.44 m, 2.20 m	20.0
3	1.06 m, 2.72 d (12.8)	38.4	1.05 m, 2.70 d (11.6)	38.8
4	---	44.8	---	44.9
5	1.32 m	57.9	1.32 m	57.8
6	1.84 m, 2.20 m	22.7	1.87 m, 2.24 m	22.6
7	1.06 m, 1.70 m	42.2	1.07 m, 1.72 m	42.2
8	---	42.5	---	43.1
9	0.91 d (7.8)	54.5	0.92 d (7.6)	54.4
10	---	40.2	---	40.4
11	1.72 m	21.0	1.75 m	21.2

Position	Reb V		Reb G	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
12	2.18 m, 2.38 m	38.3	2.26 m, 2.38 m	37.7
13	---	87.6	---	86.4
14	1.68 m, 2.43 m	44.8	1.78 m, 2.50 m	44.6
15	1.96 m, 2.24 m	48.9	2.06 m, 2.32 m	48.2
16	---	153.7	---	155.0
17	5.06 s, 5.71 s	105.7	5.00 s, 5.49 s	104.8
18	1.40 s	29.6	1.32 s	28.8
19	---	176.4	---	177.4
20	0.97 s	16.7	1.25 s	16.2
1'	6.23 d (7.8)	94.2	6.16 d (7.6)	96.4
2'	3.98 m	74.5	4.01 m	74.5
3'	4.14 m	79.3	4.09m	79.3
4'	4.36 m	71.6	4.34 m	71.6
5'	4.24 m	79.9	4.22 m	79.9
6'	4.06 m, 4.48 m	62.6	4.04 m, 4.44 dd (3.2, 7.6)	62.6
1"	5.08 d (7.8)	99.6	5.06 d (7.4)	99.9
2"	3.94 m	74.7	3.92 m	74.5
3"	4.04 m	89.3	4.06 m	89.5
4"	4.28 m	71.2	4.23 m	71.0
5"	4.00 m	78.2	4.02 m	78.1
6"	4.24 m, 4.58 m	63.0	4.27 m, 4.56 dd (2.8, 8.4)	63.1
1'"	5.38 d (8.1)	106.4	5.27 d (8.4)	106.5
2'"	4.16 m	76.1	4.14 m	76.0
3'"	4.34 m	79.2	4.37 m	79.3
4'"	4.26 m	72.2	4.28 m	72.2
5'"	3.78 m	78.8	3.89 m	78.8
6'"	4.14 m, 4.44 m	63.2	4.18 m, 4.48 m	63.2
1""	5.57 d (8.0)	105.7		
2""	3.96 m	76.5		
3""	4.32 m	79.6		
4""	4.20 m	72.5		
5""	3.87 m	79.0		

Position	Reb V		Reb G	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
6""	4.12 m, 4.46 m	63.5		
[a] assignments made on the basis of TOCSY, HMQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.				

[0186] Based on the results from NMR spectral data and hydrolysis experiments of Reb V, it was concluded that there are four β-D-glucosyl units in its structure connected to the aglycone steviol. A close comparison of the ^{1}H and ^{13}C NMR values of Reb V with Reb G suggested the presence of a steviol aglycone moiety with a 3-O-β-D-glucobiosyl unit at C-13 in the form of ether linkage and another β-D-glucosyl unit at C-19 position in the form of an ester linkage, leaving the assignment of the fourth β-D-glucosyl moiety (FIG. 17). The downfield shift for both the ^{1}H and ^{13}C chemical shifts at 2-position of sugar I of the β-D-glucosyl moiety supported the presence of β-D-glucosyl unit at this position. The structure was further supported by the key TOCSY and HMBC correlations as shown in FIG. 18. Based on the results of NMR and mass spectral data as well as hydrolysis studies, the structure of Reb V produced by the enzymatic conversion of Reb G was deduced as 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester.

[0187] Acid hydrolysis of Reb V. To a solution of Reb V (5 mg) in MeOH (10 ml) was added 3 ml of 5% H_2SO_4 and the mixture was refluxed for 24 hours. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 25 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1); the sugars were identified as D-glucose.

[0188] Enzymatic hydrolysis of Reb V. Reb V (1 mg) was dissolved in 10 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (50 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50° C for 96 hr. The product precipitated out during the reaction from the hydrolysis of 1 was identified as steviol by comparison of its co-TLC with standard compound and ^{1}H NMR spectral data. A compound named Reb V was confirmed as 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

EXAMPLE 22

[0189] In this Example, the structure of Reb W was analyzed by NMR.

[0190] The material used for the characterization of Reb W was produced by using enzymatic conversion of Reb V and purified by HPLC. HRMS data were generated with a LTQ Orbitrap Discovery HRMS instrument, with its resolution set to 30k. Scanned data from *m/z* 150 to 1500 in positive ion electrospray mode. The needle voltage was set to 4 kV; the other source conditions were sheath gas = 25, aux gas = 0, sweep gas = 5 (all gas flows in arbitrary units), capillary voltage = 30V, capillary temperature = 300C, and tube lens voltage = 75. Sample was diluted with 2:2:1 acetonitrile:methanol:water (same as infusion eluent) and injected 50 microliters. NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instruments using standard pulse sequences. The ID (^{1}H and ^{13}C) and 2D (TOCSY, HMQC, and HMBC) NMR spectra were performed in C_5D_5N.

[0191] The molecular formula of Reb W has been deduced as $C_{50}H_{80}O_{28}$ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ions corresponding to $[M+Na]^+$ at *m/z* 1151.4708; this composition was supported by the ^{13}C NMR spectral data. The ^{1}H NMR spectral data of Reb W showed the presence of two methyl singlets at δ 0.92 and 1.39, two olefinic protons as singlets at δ 5.10 and 5.73 of an exocyclic double bond, nine sp3 methylene and two sp3 methine protons between δ 0.72-2.72, characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia.* The basic skeleton of *ent*-kaurane diterpenoids was supported by the TOCSY studies which showed key correlations: H-1/H-2; H-2/H-3; H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12. The ^{1}H NMR spectrum of Reb W also showed the presence of five anomeric protons resonating at δ 5.10, 5.34, 5.41, 5.81, and 6.14; suggesting five sugar units in its structure. Acid hydrolysis of Reb W with 5% H_2SO_4 afforded D-glucose which was identified by direct comparison with authentic sample by TLC. Enzymatic hydrolysis of Reb W furnished an aglycone which was identified as steviol by comparison of ^{1}H NMR and co-TLC with standard compound. The large coupling constants observed for the five anomeric protons of the glucose moieties at δ 5.10 (d, *J*=7.4 Hz), 5.34 (d, *J*=7.9 Hz), 5.41 (d, *J*=7.9 Hz), 5.89 (d, *J*=7.9 Hz), and 6.14 (d, *J*=7.9 Hz), suggested their β-orientation as reported for steviol glycosides [1-5, 9-13]. The ^{1}H and ^{13}C NMR values for Reb W were assigned on the basis of TOCSY, HMQC and HMBC data and are given in Table 4.

Table 4. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for Reb W and Reb V^{a-b}.

Position	**Reb W**		**Reb V**	
	^{1}H NMR	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**
1	0.72 m, 1.67 m	41.0	0.78 m, 1.69 m	41.1
2	1.42 m, 2.18 m	20.4	1.44 m, 2.20 m	20.4
3	1.06 m, 2.72 d (13.4)	38.6	1.05 m, 2.70 d (11.6)	38.4
4	---	44.8	---	44.8
5	1.34 m	57.9	1.32 m	57.9
6	1.84 m, 2.18 m	22.8	1.87 m, 2.24 m	22.7

Position	Reb W		Reb V	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
7	1.07 m, 1.69 m	42.3	1.07 m, 1.72 m	42.2
δ	---	42.4	---	42.5
9	0.90 d (5.8)	54.5	0.92 d (7.6)	54.5
10	---	40.1	---	40.2
11	1.66 m	21.0	1.75 m	21.0
12	2.20 m, 2.39 m	38.3	2.26 m, 2.38 m	38.3
13	---	87.8	---	87.6
14	1.63 m, 2.06 m	44.8	1.78 m, 2.50 m	44.8
15	2.06 m, 2.04 m	48.8	2.06 m, 2.32 m	48.9
16	---	153.5	---	153.7
17	5.10 s, 5.73 s	105.9	5.00 s, 5.49 s	105.7
18	1.39 s	29.4	1.32 s	29.6
19	---	176.5	---	176.4
20	0.92 s	16.6	1.25 s	16.7
1'	6.14 d (7.9)	94.1	6.16 d (7.6)	94.2
2'	3.98 m	79.6	4.01 m	80.7
3'	4.20 m	88.9	4.09 m	79.3
4'	4.34 m	70.0	4.34 m	71.2
5'	4.24 m	79.4	4.22 m	79.9
6'	4.02 m, 4.39	62.6	4.04 m, 4.44 dd (3.2, 7.6)	62.6
1"	5.10 d (7.4)	99.5	5.06 d (7.4)	99.6
2"	3.90 m	74.7	3.92 m	74.7
3"	4.04 m	89.3	4.06 m	89.3
4"	4.25 m	70.4	4.23 m	70.3
5"	3.98 m	78.6	4.02 m	78.2
6"	4.27 m, 4.54 m	62.9	4.27 m, 4.56 dd (2.8, 8.4)	63.0
1'''	5.34 d (7.9)	106.3	5.27 d (8.4)	106.4
2'''	4.12 m	76.1	4.14 m	76.1
3'''	4.33 m	79.2	4.37 m	79.2
4'''	4.25 m	72.1	4.28 m	72.2
5'''	3.88 m	78.8	3.89 m	78.8
6'''	4.16 m, 4.53 m	63.0	4.18 m, 4.48 m	63.2

Position	Reb W		Reb V	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1''''	5.41 d (7.9)	105.3	5.27 d (8.4)	105.7
2''''	4.12 m	73.4	4.14 m	76.5
3''''	4.28 m	88.9	4.37 m	79.6
4''''	4.20 m	72.1	4.28 m	72.5
5''''	3.78 m	79.0	3.89 m	79.0
6''''	4.08 m, 4.42 m	62.9	4.18 m, 4.48 m	63.5
1'''''	5.81 d (7.9)	104.0		
2'''''	4.09 m	77.2		
3'''''	4.24 m	79.3		
4'''''	4.14 m	72.0		
5'''''	3.76 m	79.2		
6'''''	4.04 m, 4.36 m	62.3		
[a] assignments made on the basis of TOCSY, HMQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.				

[0192] Based on the results from NMR spectral data and hydrolysis experiments of Reb W, it was concluded that there are five β-D-glucosyl units in its structure connected to the aglycone steviol. A close comparison of the ^{1}H and ^{13}C NMR values of Reb W with Reb V suggested the presence of a steviol aglycone moiety with a 3-O-β-D-glucobiosyl unit at C-13 in the form of ether linkage and a 2-O-β-D-glucobiosyl unit at C-19 position in the form of an ester linkage, leaving the assignment of the fifth β-D-glucosyl moiety (FIG. 19). The downfield shift for both the ^{1}H and ^{13}C chemical shifts at 3-position of sugar I of the β-D-glucosyl moiety supported the presence of β-D-glucosyl unit at this position. The structure was further supported by the key TOCSY and HMBC correlations as shown in FIG. 20. Based on the results of NMR and mass spectral data as well as hydrolysis studies, the structure of Reb W produced by the enzymatic conversion of Reb V was deduced as 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester.

[0193] Acid hydrolysis of Reb W. To a solution of Reb W (5 mg) in MeOH (10 ml) was added 3 ml of 5% H_2SO_4 and the mixture was refluxed for 24 hours. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 25 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1); the sugars were identified as D-glucose.

[0194] Enzymatic hydrolysis of Reb W. Reb W (1 mg) was dissolved in 10 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (50 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50° C for 96 hr. The product precipitated out during the reaction and was filtered and then crystallized. The resulting product obtained from the hydrolysis of Reb W was identified as steviol by comparison of its co-TLC with standard compound and ^1H NMR spectral data. A compound named Reb W was confirmed as 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester, on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

[0195] After NMR analysis, the structures of Reb V and Reb W were identified as novel steviol glycosides. The above results further demonstrated that UGT76G1 has not only a 1,3-13-O-glucose glycosylation activity but also 1,3-19-O-glucose glycosylation activity.

EXAMPLE 23

[0196] In this Example, the structure of Reb M was analyzed by NMR.

[0197] The material used for the characterization of Reb M was produced from the enzymatic conversion of Reb D and purified by HPLC. HRMS data were generated with a LTQ Orbitrap Discovery HRMS instrument, with its resolution set to 30k. Scanned data from m/z 150 to 1500 in positive ion electrospray mode. The needle voltage was set to 4 kV; the other source conditions were sheath gas = 25, aux gas = 0, sweep gas = 5 (all gas flows in arbitrary units), capillary voltage = 30V, capillary temperature = 300C, and tube lens voltage = 75. Sample was diluted with 2:2:1 acetonitrile:methanol:water (same as infusion eluent) and injected 50 microliters.

[0198] NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instruments using standard pulse sequences. The ID (^1H and ^{13}C) and 2D (COSY, HMQC, and HMBC) NMR spectra were performed in C_5D_5N.

[0199] The molecular formula of compound Reb M has been deduced as $C_{56}H_{90}O_{33}$ on the basis of its positive high resolution (HR) mass spectrum which showed an $[M+NH_4+CH_3CN]^+$ ion at m/z 1349.5964; this composition was supported by ^{13}C NMR spectral data. The ^1H NMR spectrum of Reb M showed the presence of two methyl singlets at δ 1.35 and 1.42, two olefinic protons as singlets at δ 4.92 and 5.65 of an exocyclic double bond, nine methylene and two methine protons between δ 0.77-2.77 characteristic for the ent-kaurane diterpenoids isolated earlier from the genus Stevia. The basic skeleton of ent-kaurane diterpenoids was supported by COSY (H-1/H-2; H-2/H-3; H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12) and HMBC (H-1/C-2, C-10; H-3/C-1, C-2, C-4, C-5, C-18, C-19; H-5/C-4, C-6, C-7, C-9, C-10, C-18, C-19, C-20; H-9/C-8, C-10, C-11, C-12, C-14, C-15; H-14/C-8, C-9, C-13, C-15, C-16 and H-17/C-13, C-15, C-16) correlations. The ^1H NMR spectrum of Reb M also showed the presence of anomeric protons resonating at δ 5.33, 5.47, 5.50, 5.52, 5.85, and 6.43; suggesting six sugar units in its structure. Enzymatic hydrolysis of Reb M furnished an aglycone which was identified as steviol by comparison of co-TLC with standard compound. Acid hydrolysis of Reb M with 5% H_2SO_4 afforded glucose which was identified by direct comparison with authentic samples by TLC. The ^1H and ^{13}C NMR values for selected protons and carbons in Reb M were assigned on the basis of TOCSY, HMQC and HMBC correlations (Table 5).

[0200] Based on the results from NMR spectral data of Reb M, it was concluded that there are six glucosyl units in its structure (FIG.26). A close comparison of the ^1H and ^{13}C NMR spectrum of Reb M with rebaudioside D suggested that Reb M is also a steviol glycoside which has three glucose residues that are attached at the C-13 hydroxyl as a 2,3-branched glucotriosyl substituent and 2-substituted glucobiosyl moiety in the form of an ester at C-19 leaving the assignment of the additional glucosyl moiety. The key TOCSY and HMBC correlations shown in Figure 27 suggested the placement of the sixth glucosyl moiety at C-3 position of Sugar I. The large coupling constants observed for the six anomeric protons of the glucose moieties at δ 5.33 (d, J=8.4 Hz), 5.47 (d, J=7.8 Hz), 5.50 (d, J=7.4 Hz), 5.52 (d, J=7.4 Hz), 5.85 (d, J=7.4 Hz) and 6.43 (d, J=7.8 Hz), suggested their β-orientation as reported for steviol glycosides. Based on the results of NMR and mass spectral studies and in comparison with the spectral values of rebaudioside M reported from the literature, structure of Reb M produced by enzymatic reaction was assigned as 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester.

Table 5. ^1H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for Reb M produced by enzymatic reaction[a-c].

Position	^1H NMR	^{13}C NMR
1	0.77 t (12.4), 1.78 m	40.7
2	1.35 m, 2.24 m	20.0
3	1.01 m, 2.32 m	38.8
4	---	44.7
5	1.08 d (12.4)	57.8
6	2.23 m, 2.45 q (12.8)	23.9
7	1.44 m, 1.83 m	43.0
8	---	41.6
9	0.93 d (7.4)	54.7
10	---	40.1
11	1.68 m, 1.82 m	20.7
12	1.86 m, 2.28 m	38.8
13	---	88.0

Position	^{1}H NMR	^{13}C NMR
14	2.04 m, 2.77 m	43.7
15	1.91 m, 2.03 m	46.8
16	---	153.8
17	4.92 s, 5.65 s	105.2
18	1.35 s	28.7
19	---	177.4
20	1.42 s	17.2
1'	6.43 d (7.8)	95.4
2'	4.54 m	77.3
3'	4.58 m	89.1
4'	4.22 m	70.5
5'	4.16 m	78.8
6'	4.18 m, 4.35 m	62.1
1"	5.50 d (7.4)	96.7
2"	4.19 m	81.9
3"	5.03 m	88.4
4"	4.12 m	70.8
5"	3.98 m	78.1
6"	4.22 m, 4.36 m	62.9
1"'	5.52 d (7.4)	105.4
2"'	4.24 m	76.0
3"'	4.16 m	78.9
4"'	4.02 m	73.6
5"'	3.78 ddd (2.8, 6.4, 9.4)	78.0
6"'	4.32 m, 4.54 m	64.4
1""	5.47 d (7.8)	104.4
2""	4.00 m	75.9
3""	4.40 m	78.2
4""	4.12 m	71.6
5""	3.96 m	78.4
6""	4.20 m, 4.32 m	62.5
1""'	5.85 d (7.4)	104.7
2""'	4.20 m	75.9
3""'	4.30 m	78.9

Position	^1H NMR	^{13}C NMR
4'''''	4.14 m	73.7
5'''''	3.94 ddd (2.8, 6.4, 9.9)	78.3
6'''''	4.32 m, 4.67 d (10.6)	64.4
1''''''	5.33 d (8.4)	104.6
2''''''	3.98 m	76.2
3''''''	4.43 m	78.5
4''''''	4.16 m	71.7
5''''''	3.88 ddd (2.1, 6.4, 9.4)	78.9
6''''''	4.10 m, 4.35 m	62.5
[a]assignments made on the basis of TOCSY, HSQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.		

[0201] Acid hydrolysis of compound 1: To a solution of produced Reb M (5 mg) in MeOH (10 ml) was added 3 ml of 5% H_2SO_4 and the mixture was refluxed for 24 hours. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 25 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/n-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1); the sugars were identified as D-glucose.

[0202] Enzymatic hydrolysis of compound: produced Reb M (1 mg) was dissolved in 10 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from Aspergillus niger (50 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50°C for 96 hr. The product precipitated out during the reaction from the hydrolysis of 1 was identified as steviol by comparison of its co-TLC with standard compound and ^1H NMR spectral data.

[0203] A compound named rebaudisode M (Reb M) was obtained was produced by bio-convesrion. The complete ^1H and ^{13}C NMR spectral assignments for rebaudioside M (Reb M) were made on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data, which suggested the structure as 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)ester.

EXAMPLE 24

[0204] In this Example, the biosynthesis pathway of steviol glycosides is discussed.

[0205] FIG. 21 is a scheme illustrating the novel pathways of steviol glycoside biosynthesis from rubusoside. As described herein, the recombinant HV1 polypeptide ("HV1") contains a 1,2-O-glucose glycosylation activity which transfers a second glucoside moiety to the C-2' of 19-O-glucose of rubusoside to produce rebaudioside KA ("Reb KA"); the recombinant EUGT11 polypeptide ("EUGT11") contains a 1,2-O-glucose glycosylation activity which transfers a second glucose moiety to the C-2' of 19-O-glucose of rubusoside to produce rebaudioside KA; or transfer a second glucose moiety to the C-2' of 13-O-glucose of rubusoside to produce stevioside; the recombinant UGT76G1 enzyme ("UGT76G1") contains a 1,3-O-glucose glycosylation activity which transfer a second glucose moiety to the C-3' of 13-O-glucose of rubusoside to produce rebaudioside G ("Reb G"). Both of HV1 and EUGT11 transfer a second sugar moiety to the C-2' of 19-O-glucose of rebaudioside G to produce rebaudioside V ("Reb V"), or transfer a second glucose moiety to the C-2' of 13-O-glucose of rebaudioside KA to produce rebaudioside E ("Reb E"). FIG. 21 also shows that a recombinant UGT76G1 enzyme catalyzes the reaction that transfers the third sugar moiety to C-3' of the C-19-O-glucose of rebaudioside V to produce rebaudioside W ("Reb W") and EUGT11 can continually transfer the third glucose moiety to C-6' of the C-13-O-glucose of rebaudioside E to produce rebaudioside D2. HV1 can transfer the third glucose moiety to C-2' of the C-13-O-glucose of rebaudioside E to produce rebaudioside Z1 ("Reb Z1"), and can transfer the third glucose moiety to C-2' of the C-19-O-glucose of rebaudioside E to produce rebaudioside Z2 ("Reb Z2"). Both of HV1 and EUGT11 can catalyze the conversion of stevioside to Reb E and the conversion of rebaudioside A ("Reb A") to rebaudioside D ("Reb D"). UGT76G1 can transfer the third glucose moiety to C-3' of the C-13-O-glucose of rebaudioside E ("Reb E") to form rebaudioside D ("Reb D"). UGT76G1 also catalyze the conversion of stevioside to rebaudioside ("Reb A") and the conversion of rebaudioside D ("Reb D") to rebaudioside M ("Reb M").

SEQUENCE LISTING

[0206]

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Lin, Ying

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Phe Ile Leu Asp Asn Asp Pro Gln Asp Glu Arg Ile Ser Asn Leu Pro
65                  70                  75                  80

Thr His Gly Pro Leu Ala Gly Met Arg Ile Pro Ile Ile Asn Glu His
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    130                 135                 140

Met Thr Ser Ser Leu Phe Asn Phe His Ala His Val Ser Leu Pro Gln

145                 150                 155                 160

Phe Asp Glu Leu Gly Tyr Leu Asp Pro Asp Asp Lys Thr Arg Leu Glu
                165                 170                 175

Glu Gln Ala Ser Gly Phe Pro Met Leu Lys Val Lys Asp Ile Lys Ser
            180                 185                 190

Ala Tyr Ser Asn Trp Gln Ile Leu Lys Glu Ile Leu Gly Lys Met Ile
        195                 200                 205

Lys Gln Thr Lys Ala Ser Ser Gly Val Ile Trp Asn Ser Phe Lys Glu
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Ser Phe Leu Ile Pro Leu Pro Lys His Leu Thr Ala Ser Ser Ser Ser
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Ser Phe Leu Trp Val Val Arg Pro Gly Phe Val Lys Gly Ser Thr Trp
305                 310                 315                 320

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                325                 330                 335

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Gly Val Pro Met Ile Phe Ser Asp Phe Gly Leu Asp Gln Pro Leu Asn
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Asp Glu Glu Gly Glu Tyr Ile Arg Gln Asn Ala Arg Val Leu Lys Gln
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<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 2

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ccatttcagg gccatattaa tccgatcctc caattagcaa acgtcctcta ctccaaggga       120

ttttcaataa caatcttcca tactaacttt aacaagccta aaacgagtaa ttatcctcac       180

tttacattca ggttcattct agacaacgac cctcaggatg agcgtatctc aaatttacct       240
```

acgcatggcc ccttggcagg tatgcgaata ccaataatca atgagcatgg agccgatgaa 300

ctccgtcgcg agttagagct tctcatgctc gcaagtgagg aagacgagga agtttcgtgc 360

ctaataactg atgcgctttg gtacttcgcc caatcagtcg cagactcact gaatctacgc 420

cgtttggtcc ttatgacaag ttcattattc aactttcacg cacatgtatc actgccgcaa 480

tttgacgagt tgggttacct ggacccggat gacaaaacgc gattggagga acaagcgtcg 540

ggcttcccca tgctgaaagt caaagatatt aagagcgctt atagtaattg gcaaattctg 600

aaagaaattc tcggaaaaat gataaagcaa accaaagcgt cctctggagt aatctggaac 660

tccttcaagg agttagagga atctgaactt gaaacggtca tcagagaaat ccccgctccc 720

tcgttcttaa ttccactacc caagcacctt actgcaagta gcagttccct cctagatcat 780

gaccgaaccg tgtttcagtg gctggatcag caacccccgt cgtcagttct atatgtaagc 840

tttgggagta cttcggaagt ggatgaaaag gacttcttag agattgcgcg agggctcgtg 900

gatagcaaac agagcttcct gtgggtagtg agaccgggat tcgttaaggg ctcgacgtgg 960

gtcgagccgt tgccagatgg ttttctaggg gagagaggga gaatcgtgaa atgggttcca 1020

cagcaagagg ttttggctca cggagctata ggggcctttt ggacccactc tggttggaat 1080

tctactcttg aaagtgtctg tgaaggcgtt ccaatgatat tttctgattt tgggcttgac 1140

cagcctctaa acgctcgcta tatgtctgat gtgttgaagg ttggcgtgta cctggagaat 1200

ggttgggaaa gggggggaaat tgccaacgcc atacgccggg taatggtgga cgaggaaggt 1260

gagtacatac gtcagaacgc tcgggtttta aaacaaaaag cggacgtcag ccttatgaag 1320

ggaggtagct cctatgaatc cctagaatcc ttggtaagct atatatcttc gttataa 1377

<210> 3
<211> 462
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 3
Met Asp Ser Gly Tyr Ser Ser Ser Tyr Ala Ala Ala Ala Gly Met His
1 5 10 15

Val Val Ile Cys Pro Trp Leu Ala Phe Gly His Leu Leu Pro Cys Leu
20 25 30

Asp Leu Ala Gln Arg Leu Ala Ser Arg Gly His Arg Val Ser Phe Val
35 40 45

Ser Thr Pro Arg Asn Ile Ser Arg Leu Pro Pro Val Arg Pro Ala Leu
50 55 60

Ala Pro Leu Val Ala Phe Val Ala Leu Pro Leu Pro Arg Val Glu Gly
65 70 75 80

Leu Pro Asp Gly Ala Glu Ser Thr Asn Asp Val Pro His Asp Arg Pro

```
Leu Pro Asp Gly Ala Glu Ser Thr Asn Asp Val Pro His Asp Arg Pro
                85                  90                  95

Asp Met Val Glu Leu His Arg Arg Ala Phe Asp Gly Leu Ala Ala Pro
            100                 105                 110

Phe Ser Glu Phe Leu Gly Thr Ala Cys Ala Asp Trp Val Ile Val Asp
        115                 120                 125

Val Phe His His Trp Ala Ala Ala Ala Ala Leu Glu His Lys Val Pro
    130                 135                 140

Cys Ala Met Met Leu Leu Gly Ser Ala His Met Ile Ala Ser Ile Ala
145                 150                 155                 160

Asp Arg Arg Leu Glu Arg Ala Glu Thr Glu Ser Pro Ala Ala Ala Gly
                165                 170                 175

Gln Gly Arg Pro Ala Ala Ala Pro Thr Phe Glu Val Ala Arg Met Lys
            180                 185                 190

Leu Ile Arg Thr Lys Gly Ser Ser Gly Met Ser Leu Ala Glu Arg Phe
        195                 200                 205

Ser Leu Thr Leu Ser Arg Ser Ser Leu Val Val Gly Arg Ser Cys Val
    210                 215                 220

Glu Phe Glu Pro Glu Thr Val Pro Leu Leu Ser Thr Leu Arg Gly Lys
225                 230                 235                 240

Pro Ile Thr Phe Leu Gly Leu Met Pro Pro Leu His Glu Gly Arg Arg
                245                 250                 255

Glu Asp Gly Glu Asp Ala Thr Val Arg Trp Leu Asp Ala Gln Pro Ala
            260                 265                 270

Lys Ser Val Val Tyr Val Ala Leu Gly Ser Glu Val Pro Leu Gly Val
        275                 280                 285

Glu Lys Val His Glu Leu Ala Leu Gly Leu Glu Leu Ala Gly Thr Arg
    290                 295                 300

Phe Leu Trp Ala Leu Arg Lys Pro Thr Gly Val Ser Asp Ala Asp Leu
305                 310                 315                 320

Leu Pro Ala Gly Phe Glu Glu Arg Thr Arg Gly Arg Gly Val Val Ala
                325                 330                 335

Thr Arg Trp Val Pro Gln Met Ser Ile Leu Ala His Ala Ala Val Gly
            340                 345                 350

Ala Phe Leu Thr His Cys Gly Trp Asn Ser Thr Ile Glu Gly Leu Met
        355                 360                 365

Phe Gly His Pro Leu Ile Met Leu Pro Ile Phe Gly Asp Gln Gly Pro
    370                 375                 380
```

```
Asn Ala Arg Leu Ile Glu Ala Lys Asn Ala Gly Leu Gln Val Ala Arg
385                 390                 395                 400

Asn Asp Gly Asp Gly Ser Phe Asp Arg Glu Gly Val Ala Ala Ala Ile
                405                 410                 415

Arg Ala Val Ala Val Glu Glu Glu Ser Ser Lys Val Phe Gln Ala Lys
            420                 425                 430

Ala Lys Lys Leu Gln Glu Ile Val Ala Asp Met Ala Cys His Glu Arg
        435                 440                 445

Tyr Ile Asp Gly Phe Ile Gln Gln Leu Arg Ser Tyr Lys Asp
    450                 455                 460
```

<210> 4
<211> 1389
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 4

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ccgtggctgg cttttggtca cctgctgccg tgcctggatc tggcacagcg tctggcttca     120

cgcggccatc gtgtcagctt cgtgtctacc ccgcgcaata tttcgcgtct gccgccggtt     180

cgtccggcac tggctccgct ggttgcattt gtcgctctgc cgctgccgcg cgtggaaggt     240

ctgccggatg gtgcggaaag taccaacgac gtgccgcatg atcgcccgga catggttgaa     300

ctgcaccgtc gtgcattcga tggtctggca gcaccgtttt ccgaatttct gggtacggcg     360

tgcgccgatt gggtgatcgt tgacgtcttt catcactggg cggcggcggc ggcgctggaa     420

cataaagttc cgtgtgcaat gatgctgctg ggctcagctc acatgattgc gtcgatcgca     480

gaccgtcgcc tggaacgtgc agaaaccgaa agtccggctg cggccggcca gggtcgcccg     540

gcagctgcgc cgaccttcga agtggcccgc atgaaactga ttcgtacgaa aggcagctct     600

ggtatgagcc tggcagaacg ctttagtctg accctgtccc gtagttccct ggtggttggt     660

cgcagttgcg ttgaatttga accggaaacc gtcccgctgc tgtccacgct gcgtggtaaa     720

ccgatcacct ttctgggtct gatgccgccg ctgcatgaag gccgtcgcga agatggtgaa     780

gacgcaacgg tgcgttggct ggatgcacag ccggctaaaa gcgtcgtgta tgtcgccctg     840

ggctctgaag tgccgctggg tgtggaaaaa gttcacgaac tggcactggg cctggaactg     900

gctggcaccc gcttcctgtg ggcactgcgt aaaccgacgg gtgtgagcga tgcggacctg     960

ctgccggccg gttttgaaga acgtacccgc ggccgtggtg ttgtcgcaac gcgttgggtc    1020

ccgcaaatga gcattctggc gcatgccgca gtgggcgcct ttctgaccca ctgtggttgg    1080

aacagcacga tcgaaggcct gatgtttggt cacccgctga ttatgctgcc gatcttcggc    1140

gatcagggtc cgaacgcacg tctgattgaa gcgaaaaatg ccggcctgca agttgcgcgc    1200

aacgatggcg acggttcttt cgaccgtgag ggtgtggctg cggccattcg cgcagtggct    1260
```

```
gttgaagaag aatcatcgaa agtttttcag gcgaaagcca aaaaactgca agaaatcgtc      1320

gcggatatgg cctgccacga acgctacatt gatggtttca ttcagcaact gcgctcctac      1380

aaagactaa                                                              1389
```

<210> 5

<211> 459

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 5

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Met Asp Gly Asn Ser Ser Ser Ser Pro Leu His Val Val Ile Cys Pro
1               5                   10                  15

Trp Leu Ala Leu Gly His Leu Leu Pro Cys Leu Asp Ile Ala Glu Arg
            20                  25                  30

Leu Ala Ser Arg Gly His Arg Val Ser Phe Val Ser Thr Pro Arg Asn
        35                  40                  45

Ile Ala Arg Leu Pro Pro Leu Arg Pro Ala Val Ala Pro Leu Val Asp
    50                  55                  60

Phe Val Ala Leu Pro Leu Pro His Val Asp Gly Leu Pro Glu Gly Ala
65                  70                  75                  80

Glu Ser Thr Asn Asp Val Pro Tyr Asp Lys Phe Glu Leu His Arg Lys
                85                  90                  95

Ala Phe Asp Gly Leu Ala Ala Pro Phe Ser Glu Phe Leu Arg Ala Ala
            100                 105                 110

Cys Ala Glu Gly Ala Gly Ser Arg Pro Asp Trp Leu Ile Val Asp Thr
        115                 120                 125

Phe His His Trp Ala Ala Ala Ala Ala Val Glu Asn Lys Val Pro Cys
    130                 135                 140

Val Met Leu Leu Leu Gly Ala Ala Thr Val Ile Ala Gly Phe Ala Arg
145                 150                 155                 160

Gly Val Ser Glu His Ala Ala Ala Ala Val Gly Lys Glu Arg Pro Ala
                165                 170                 175

Ala Glu Ala Pro Ser Phe Glu Thr Glu Arg Arg Lys Leu Met Thr Thr
            180                 185                 190

Gln Asn Ala Ser Gly Met Thr Val Ala Glu Arg Tyr Phe Leu Thr Leu

        195                 200                 205
```

```
Met Arg Ser Asp Leu Val Ala Ile Arg Ser Cys Ala Glu Trp Glu Pro
    210                 215                 220

Glu Ser Val Ala Ala Leu Thr Thr Leu Ala Gly Lys Pro Val Val Pro
225                 230                 235                 240

Leu Gly Leu Leu Pro Pro Ser Pro Glu Gly Gly Arg Gly Val Ser Lys
                245                 250                 255

Glu Asp Ala Ala Val Arg Trp Leu Asp Ala Gln Pro Ala Lys Ser Val
            260                 265                 270

Val Tyr Val Ala Leu Gly Ser Glu Val Pro Leu Arg Ala Glu Gln Val
        275                 280                 285

His Glu Leu Ala Leu Gly Leu Glu Leu Ser Gly Ala Arg Phe Leu Trp
    290                 295                 300

Ala Leu Arg Lys Pro Thr Asp Ala Pro Asp Ala Ala Val Leu Pro Pro
305                 310                 315                 320

Gly Phe Glu Glu Arg Thr Arg Gly Arg Gly Leu Val Val Thr Gly Trp
                325                 330                 335

Val Pro Gln Ile Gly Val Leu Ala His Gly Ala Val Ala Ala Phe Leu
            340                 345                 350

Thr His Cys Gly Trp Asn Ser Thr Ile Glu Gly Leu Leu Phe Gly His
        355                 360                 365

Pro Leu Ile Met Leu Pro Ile Ser Ser Asp Gln Gly Pro Asn Ala Arg
    370                 375                 380

Leu Met Glu Gly Arg Lys Val Gly Met Gln Val Pro Arg Asp Glu Ser
385                 390                 395                 400

Asp Gly Ser Phe Arg Arg Glu Asp Val Ala Ala Thr Val Arg Ala Val
                405                 410                 415

Ala Val Glu Glu Asp Gly Arg Arg Val Phe Thr Ala Asn Ala Lys Lys
            420                 425                 430

Met Gln Glu Ile Val Ala Asp Gly Ala Cys His Glu Arg Cys Ile Asp
        435                 440                 445

Gly Phe Ile Gln Gln Leu Arg Ser Tyr Lys Ala
    450                 455
```

<210> 6
<211> 1380
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic

<400> 6

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atggatggta actcctcctc ctcgccgctg catgtggtca tttgtccgtg gctggctctg      60

ggtcacctgc tgccgtgtct ggatattgct gaacgtctgg cgtcacgcgg ccatcgtgtc     120

agttttgtgt ccaccccgcg caacattgcc cgtctgccgc cgctgcgtcc ggctgttgca     180

ccgctggttg atttcgtcgc actgccgctg ccgcatgttg acggtctgcc ggagggtgcg     240

gaatcgacca atgatgtgcc gtatgacaaa tttgaactgc accgtaaggc gttcgatggt     300

ctggcggccc cgtttagcga atttctgcgt gcagcttgcg cagaaggtgc aggttctcgc     360

ccggattggc tgattgtgga cacctttcat cactgggcgg cggcggcggc ggtggaaaac     420

aaagtgccgt gtgttatgct gctgctgggt gcagcaacgg tgatcgctgg tttcgcgcgt     480

ggtgttagcg aacatgcggc ggcggcggtg ggtaaagaac gtccggctgc ggaagccccg     540

agttttgaaa ccgaacgtcg caagctgatg accacgcaga atgcctccgg catgaccgtg     600

gcagaacgct atttcctgac gctgatgcgt agcgatctgg ttgccatccg ctcttgcgca     660

gaatgggaac cggaaagcgt ggcagcactg accacgctgg caggtaaacc ggtggttccg     720

ctgggtctgc tgccgccgag tccggaaggc ggtcgtggcg tttccaaaga agatgctgcg     780

gtccgttggc tggacgcaca gccggcaaag tcagtcgtgt acgtcgcact gggttcggaa     840

gtgccgctgc gtgcggaaca agttcacgaa ctggcactgg gcctggaact gagcggtgct     900

cgctttctgt gggcgctgcg taaaccgacc gatgcaccgg acgccgcagt gctgccgccg     960

ggtttcgaag aacgtacccg cggccgtggt ctggttgtca cgggttgggt gccgcagatt    1020

ggcgttctgg ctcatggtgc ggtggctgcg tttctgaccc actgtggctg gaactctacg    1080

atcgaaggcc tgctgttcgg tcatccgctg attatgctgc cgatcagctc tgatcagggt    1140

ccgaatgcgc gcctgatgga aggccgtaaa gtcggtatgc aagtgccgcg tgatgaatca    1200

gacggctcgt ttcgtcgcga agatgttgcc gcaaccgtcc gcgccgtggc agttgaagaa    1260

gacggtcgtc gcgtcttcac ggctaacgcg aaaaagatgc aagaaattgt ggccgatggc    1320

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```

<210> 7
<211> 808
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 7

```
Met Ala Asn Ala Glu Arg Met Ile Thr Arg Val His Ser Gln Arg Glu
1               5                   10                  15


Arg Leu Asn Glu Thr Leu Val Ser Glu Arg Asn Glu Val Leu Ala Leu
            20                  25                  30


Leu Ser Arg Val Glu Ala Lys Gly Lys Gly Ile Leu Gln Gln Asn Gln
```

```
        35                  40                  45

Ile Ile Ala Glu Phe Glu Ala Leu Pro Glu Gln Thr Arg Lys Lys Leu
    50                  55                  60

Glu Gly Gly Pro Phe Phe Asp Leu Leu Lys Ser Thr Gln Glu Ala Ile
65                  70                  75                  80

Val Leu Pro Pro Trp Val Ala Leu Ala Val Arg Pro Arg Pro Gly Val
                85                  90                  95

Trp Glu Tyr Leu Arg Val Asn Leu His Ala Leu Val Val Glu Glu Leu
            100                 105                 110

Gln Pro Ala Glu Phe Leu His Phe Lys Glu Glu Leu Val Asp Gly Val
        115                 120                 125

Lys Asn Gly Asn Phe Thr Leu Glu Leu Asp Phe Glu Pro Phe Asn Ala
    130                 135                 140

Ser Ile Pro Arg Pro Thr Leu His Lys Tyr Ile Gly Asn Gly Val Asp
145                 150                 155                 160

Phe Leu Asn Arg His Leu Ser Ala Lys Leu Phe His Asp Lys Glu Ser
                165                 170                 175

Leu Leu Pro Leu Leu Lys Phe Leu Arg Leu His Ser His Gln Gly Lys
            180                 185                 190

Asn Leu Met Leu Ser Glu Lys Ile Gln Asn Leu Asn Thr Leu Gln His
        195                 200                 205

Thr Leu Arg Lys Ala Glu Glu Tyr Leu Ala Glu Leu Lys Ser Glu Thr
    210                 215                 220

Leu Tyr Glu Glu Phe Glu Ala Lys Phe Glu Glu Ile Gly Leu Glu Arg
225                 230                 235                 240

Gly Trp Gly Asp Asn Ala Glu Arg Val Leu Asp Met Ile Arg Leu Leu
                245                 250                 255

Leu Asp Leu Leu Glu Ala Pro Asp Pro Cys Thr Leu Glu Thr Phe Leu
            260                 265                 270

Gly Arg Val Pro Met Val Phe Asn Val Val Ile Leu Ser Pro His Gly
        275                 280                 285

Tyr Phe Ala Gln Asp Asn Val Leu Gly Tyr Pro Asp Thr Gly Gly Gln
    290                 295                 300

Val Val Tyr Ile Leu Asp Gln Val Arg Ala Leu Glu Ile Glu Met Leu
305                 310                 315                 320

Gln Arg Ile Lys Gln Gln Gly Leu Asn Ile Lys Pro Arg Ile Leu Ile
                325                 330                 335

Leu Thr Arg Leu Leu Pro Asn Ala Val Glu Thr Thr Cys Glu Glu Arg
```

```
Leu Thr Arg Leu Leu Pro Asp Ala Val Gly Thr Thr Cys Gly Glu Arg
            340                 345                 350

Leu Glu Arg Val Tyr Asp Ser Glu Tyr Cys Asp Ile Leu Arg Val Pro
        355                 360                 365

Phe Arg Thr Glu Lys Gly Ile Val Arg Lys Trp Ile Ser Arg Phe Glu
    370                 375                 380

Val Trp Pro Tyr Leu Glu Thr Tyr Thr Glu Asp Ala Ala Val Glu Leu
385                 390                 395                 400

Ser Lys Glu Leu Asn Gly Lys Pro Asp Leu Ile Ile Gly Asn Tyr Ser
                405                 410                 415

Asp Gly Asn Leu Val Ala Ser Leu Leu Ala His Lys Leu Gly Val Thr
            420                 425                 430

Gln Cys Thr Ile Ala His Ala Leu Glu Lys Thr Lys Tyr Pro Asp Ser
        435                 440                 445

Asp Ile Tyr Trp Lys Lys Leu Asp Asp Lys Tyr His Phe Ser Cys Gln
    450                 455                 460

Phe Thr Ala Asp Ile Phe Ala Met Asn His Thr Asp Phe Ile Ile Thr
465                 470                 475                 480

Ser Thr Phe Gln Glu Ile Ala Gly Ser Lys Glu Thr Val Gly Gln Tyr
                485                 490                 495

Glu Ser His Thr Ala Phe Thr Leu Pro Gly Leu Tyr Arg Val Val His
            500                 505                 510

Gly Ile Asp Val Phe Asp Pro Lys Phe Asn Ile Val Ser Pro Gly Ala
        515                 520                 525

Asp Met Ser Ile Tyr Phe Pro Tyr Thr Glu Glu Lys Arg Arg Leu Thr
    530                 535                 540

Lys Phe His Ser Glu Ile Glu Glu Leu Leu Tyr Ser Asp Val Glu Asn
545                 550                 555                 560

Lys Glu His Leu Cys Val Leu Lys Asp Lys Lys Lys Pro Ile Leu Phe
                565                 570                 575

Thr Met Ala Arg Leu Asp Arg Val Lys Asn Leu Ser Gly Leu Val Glu
            580                 585                 590

Trp Tyr Gly Lys Asn Thr Arg Leu Arg Glu Leu Ala Asn Leu Val Val
        595                 600                 605

Val Gly Gly Asp Arg Arg Lys Glu Ser Lys Asp Asn Glu Glu Lys Ala
    610                 615                 620

Glu Met Lys Lys Met Tyr Asp Leu Ile Glu Glu Tyr Lys Leu Asn Gly
625                 630                 635                 640

Gln Phe Arg Trp Ile Ser Ser Gln Met Asp Arg Val Arg Asn Gly Glu
```

```
                645                 650                 655

Leu Tyr Arg Tyr Ile Cys Asp Thr Lys Gly Ala Phe Val Gln Pro Ala
            660                 665                 670

Leu Tyr Glu Ala Phe Gly Leu Thr Val Val Glu Ala Met Thr Cys Gly
        675                 680                 685

Leu Pro Thr Phe Ala Thr Cys Lys Gly Gly Pro Ala Glu Ile Ile Val
    690                 695                 700

His Gly Lys Ser Gly Phe His Ile Asp Pro Tyr His Gly Asp Gln Ala
705                 710                 715                 720

Ala Asp Thr Leu Ala Asp Phe Phe Thr Lys Cys Lys Glu Asp Pro Ser
                725                 730                 735

His Trp Asp Glu Ile Ser Lys Gly Gly Leu Gln Arg Ile Glu Glu Lys
            740                 745                 750

Tyr Thr Trp Gln Ile Tyr Ser Gln Arg Leu Leu Thr Leu Thr Gly Val
        755                 760                 765

Tyr Gly Phe Trp Lys His Val Ser Asn Leu Asp Arg Leu Glu Ala Arg
    770                 775                 780

Arg Tyr Leu Glu Met Phe Tyr Ala Leu Lys Tyr Arg Pro Leu Ala Gln
785                 790                 795                 800

Ala Val Pro Leu Ala Gln Asp Asp
                805
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<210> 8
<211> 2427
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 8

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aaaggtattt tacaacaaaa ccagatcatt gctgaattcg aagctttgcc tgaacaaacc       180

cggaagaaac ttgaaggtgg tcctttcttt gaccttctca aatccactca ggaagcaatt       240

gtgttgccac catgggttgc tctagctgtg aggccaaggc ctggtgtttg ggaatactta       300

cgagtcaatc tccatgctct tgtcgttgaa gaactccaac ctgctgagtt tcttcatttc       360

aaggaagaac tcgttgatgg agttaagaat ggtaatttca ctcttgagct tgatttcgag       420

ccattcaatg cgtctatccc tcgtccaaca ctccacaaat acattggaaa tggtgttgac       480

ttccttaacc gtcatttatc ggctaagctc ttccatgaca aggagagttt gcttccattg       540

gttaagttgg ttggtcttga caggcaccag gggaagaacc tgatgttgag cgagaagatt       600
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cagaacctca acactctgca acacaccttg aggaaagcag aagagtatct agcagagctt 660

aagtccgaaa cactgtatga agagtttgag gccaagtttg aggagattgg tcttgagagg 720

ggatggggag acaatgcaga gcgtgtcctt gacatgatac gtcttctttt ggaccttctt 780

gaggcgcctg atccttgcac tcttgagact tttcttggaa gagtaccaat ggtgttcaac 840

gttgtgatcc tctctccaca tggttacttt gctcaggaca atgttcttgg ttaccctgac 900

actggtggac aggttgttta cattcttgat caagttcgtg ctctggagat agagatgctt 960

caacgtatta agcaacaagg actcaacatt aaaccaagga ttctcattct aactcgactt 1020

ctacctgatg cggtaggaac tacatgcggt gaacgtctcg agagagttta tgattctgag 1080

tactgtgata ttcttcgtgt gcccttcaga acagagaagg gtattgttcg caaatggatc 1140

tcaaggttcg aagtctggcc atatctagag acttacaccg aggatgctgc ggttgagcta 1200

tcgaaagaat tgaatggcaa gcctgacctt atcattggta actacagtga tggaaatctt 1260

gttgcttctt tattggctca caaacttggt gtcactcagt gtaccattgc tcatgctctt 1320

gagaaaacaa agtacccgga ttctgatatc tactggaaga agcttgacga caagtaccat 1380

ttctcatgcc agttcactgc ggatattttc gcaatgaacc acactgattt catcatcact 1440

agtactttcc aagaaattgc tggaagcaaa gaaactgttg ggcagtatga aagccacaca 1500

gcctttactc ttcccggatt gtatcgagtt gttcacggga ttgatgtgtt tgatcccaag 1560

ttcaacattg tctctcctgg tgctgatatg agcatctact tcccttacac agaggagaag 1620

cgtagattga ctaagttcca ctctgagatc gaggagctcc tctacagcga tgttgagaac 1680

aaagagcact tatgtgtgct caaggacaag aagaagccga ttctcttcac aatggctagg 1740

cttgatcgtg tcaagaactt gtcaggtctt gttgagtggt acgggaagaa caccccgcttg 1800

cgtgagctag ctaacttggt tgttgttgga ggagacagga ggaaagagtc aaaggacaat 1860

gaagagaaag cagagatgaa gaaaatgtat gatctcattg aggaatacaa gctaaacggt 1920

cagttcaggt ggatctcctc tcagatggac cgggtaagga acggtgagct gtaccggtac 1980

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gctgatactc ttgctgattt cttcaccaag tgtaaggagg atccatctca ctgggatgag 2220

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aggctcttga cattgactgg tgtgtatgga ttctggaagc atgtctcgaa ccttgaccgt 2340

cttgaggctc gccgttacct tgaaatgttc tatgcattga agtatcgccc attggctcag 2400

gctgttcctc ttgcacaaga tgattga 2427

<210> 9
<211> 1268
<212> PRT
<213> Artificial Sequence

<220>

<223> Synthetic

<400> 9

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Met Glu Asn Lys Thr Glu Thr Thr Val Arg Arg Arg Arg Arg Ile Ile
1               5                   10                  15

Leu Phe Pro Val Pro Phe Gln Gly His Ile Asn Pro Ile Leu Gln Leu
            20                  25                  30

Ala Asn Val Leu Tyr Ser Lys Gly Phe Ser Ile Thr Ile Phe His Thr
        35                  40                  45

Asn Phe Asn Lys Pro Lys Thr Ser Asn Tyr Pro His Phe Thr Phe Arg
    50                  55                  60

Phe Ile Leu Asp Asn Asp Pro Gln Asp Glu Arg Ile Ser Asn Leu Pro
65                  70                  75                  80

Thr His Gly Pro Leu Ala Gly Met Arg Ile Pro Ile Ile Asn Glu His
                85                  90                  95

Gly Ala Asp Glu Leu Arg Arg Glu Leu Glu Leu Leu Met Leu Ala Ser
            100                 105                 110

Glu Glu Asp Glu Glu Val Ser Cys Leu Ile Thr Asp Ala Leu Trp Tyr
        115                 120                 125

Phe Ala Gln Ser Val Ala Asp Ser Leu Asn Leu Arg Arg Leu Val Leu
    130                 135                 140

Met Thr Ser Ser Leu Phe Asn Phe His Ala His Val Ser Leu Pro Gln
145                 150                 155                 160

Phe Asp Glu Leu Gly Tyr Leu Asp Pro Asp Asp Lys Thr Arg Leu Glu
                165                 170                 175

Glu Gln Ala Ser Gly Phe Pro Met Leu Lys Val Lys Asp Ile Lys Ser
            180                 185                 190

Ala Tyr Ser Asn Trp Gln Ile Leu Lys Glu Ile Leu Gly Lys Met Ile
        195                 200                 205

Lys Gln Thr Lys Ala Ser Ser Gly Val Ile Trp Asn Ser Phe Lys Glu
    210                 215                 220

Leu Glu Glu Ser Glu Leu Glu Thr Val Ile Arg Glu Ile Pro Ala Pro
225                 230                 235                 240

Ser Phe Leu Ile Pro Leu Pro Lys His Leu Thr Ala Ser Ser Ser Ser
                245                 250                 255

Leu Leu Asp His Asp Arg Thr Val Phe Gln Trp Leu Asp Gln Gln Pro

            260                 265                 270

Pro Ser Ser Val Leu Tyr Val Ser Phe Gly Ser Thr Ser Glu Val Asp
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Pro Ser Ser Val Leu Tyr Val Ser Phe Gly Ser Thr Ser Glu Val Asp
        275                 280                 285

Glu Lys Asp Phe Leu Glu Ile Ala Arg Gly Leu Val Asp Ser Lys Gln
    290                 295                 300

Ser Phe Leu Trp Val Val Arg Pro Gly Phe Val Lys Gly Ser Thr Trp
305                 310                 315                 320

Val Glu Pro Leu Pro Asp Gly Phe Leu Gly Glu Arg Gly Arg Ile Val
                325                 330                 335

Lys Trp Val Pro Gln Gln Glu Val Leu Ala His Gly Ala Ile Gly Ala
            340                 345                 350

Phe Trp Thr His Ser Gly Trp Asn Ser Thr Leu Glu Ser Val Cys Glu
        355                 360                 365

Gly Val Pro Met Ile Phe Ser Asp Phe Gly Leu Asp Gln Pro Leu Asn
    370                 375                 380

Ala Arg Tyr Met Ser Asp Val Leu Lys Val Gly Val Tyr Leu Glu Asn
385                 390                 395                 400

Gly Trp Glu Arg Gly Glu Ile Ala Asn Ala Ile Arg Arg Val Met Val
                405                 410                 415

Asp Glu Glu Gly Glu Tyr Ile Arg Gln Asn Ala Arg Val Leu Lys Gln
            420                 425                 430

Lys Ala Asp Val Ser Leu Met Lys Gly Gly Ser Ser Tyr Glu Ser Leu
        435                 440                 445

Glu Ser Leu Val Ser Tyr Ile Ser Ser Leu Gly Ser Gly Ala Asn Ala
    450                 455                 460

Glu Arg Met Ile Thr Arg Val His Ser Gln Arg Glu Arg Leu Asn Glu
465                 470                 475                 480

Thr Leu Val Ser Glu Arg Asn Glu Val Leu Ala Leu Leu Ser Arg Val
                485                 490                 495

Glu Ala Lys Gly Lys Gly Ile Leu Gln Gln Asn Gln Ile Ile Ala Glu
            500                 505                 510

Phe Glu Ala Leu Pro Glu Gln Thr Arg Lys Lys Leu Glu Gly Gly Pro
        515                 520                 525

Phe Phe Asp Leu Leu Lys Ser Thr Gln Glu Ala Ile Val Leu Pro Pro
    530                 535                 540

Trp Val Ala Leu Ala Val Arg Pro Arg Pro Gly Val Trp Glu Tyr Leu
545                 550                 555                 560

Arg Val Asn Leu His Ala Leu Val Val Glu Glu Leu Gln Pro Ala Glu
                565                 570                 575
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Phe Leu His Phe Lys Glu Glu Leu Val Asp Gly Val Lys Asn Gly Asn
            580                 585                 590

Phe Thr Leu Glu Leu Asp Phe Glu Pro Phe Asn Ala Ser Ile Pro Arg
        595                 600                 605

Pro Thr Leu His Lys Tyr Ile Gly Asn Gly Val Asp Phe Leu Asn Arg
    610                 615                 620

His Leu Ser Ala Lys Leu Phe His Asp Lys Glu Ser Leu Leu Pro Leu
625                 630                 635                 640

Leu Lys Phe Leu Arg Leu His Ser His Gln Gly Lys Asn Leu Met Leu
                645                 650                 655

Ser Glu Lys Ile Gln Asn Leu Asn Thr Leu Gln His Thr Leu Arg Lys
            660                 665                 670

Ala Glu Glu Tyr Leu Ala Glu Leu Lys Ser Glu Thr Leu Tyr Glu Glu
        675                 680                 685

Phe Glu Ala Lys Phe Glu Glu Ile Gly Leu Glu Arg Gly Trp Gly Asp
    690                 695                 700

Asn Ala Glu Arg Val Leu Asp Met Ile Arg Leu Leu Leu Asp Leu Leu
705                 710                 715                 720

Glu Ala Pro Asp Pro Cys Thr Leu Glu Thr Phe Leu Gly Arg Val Pro
                725                 730                 735

Met Val Phe Asn Val Val Ile Leu Ser Pro His Gly Tyr Phe Ala Gln
            740                 745                 750

Asp Asn Val Leu Gly Tyr Pro Asp Thr Gly Gly Gln Val Val Tyr Ile
        755                 760                 765

Leu Asp Gln Val Arg Ala Leu Glu Ile Glu Met Leu Gln Arg Ile Lys
    770                 775                 780

Gln Gln Gly Leu Asn Ile Lys Pro Arg Ile Leu Ile Leu Thr Arg Leu
785                 790                 795                 800

Leu Pro Asp Ala Val Gly Thr Thr Cys Gly Glu Arg Leu Glu Arg Val
                805                 810                 815

Tyr Asp Ser Glu Tyr Cys Asp Ile Leu Arg Val Pro Phe Arg Thr Glu
            820                 825                 830

Lys Gly Ile Val Arg Lys Trp Ile Ser Arg Phe Glu Val Trp Pro Tyr
        835                 840                 845

Leu Glu Thr Tyr Thr Glu Asp Ala Ala Val Glu Leu Ser Lys Glu Leu
    850                 855                 860

Asn Gly Lys Pro Asp Leu Ile Ile Gly Asn Tyr Ser Asp Gly Asn Leu
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Val Ala Ser Leu Leu Ala His Lys Leu Gly Val Thr Gln Cys Thr Ile
885 890 895

Ala His Ala Leu Glu Lys Thr Lys Tyr Pro Asp Ser Asp Ile Tyr Trp
900 905 910

Lys Lys Leu Asp Asp Lys Tyr His Phe Ser Cys Gln Phe Thr Ala Asp
915 920 925

Ile Phe Ala Met Asn His Thr Asp Phe Ile Ile Thr Ser Thr Phe Gln
930 935 940

Glu Ile Ala Gly Ser Lys Glu Thr Val Gly Gln Tyr Glu Ser His Thr
945 950 955 960

Ala Phe Thr Leu Pro Gly Leu Tyr Arg Val Val His Gly Ile Asp Val
965 970 975

Phe Asp Pro Lys Phe Asn Ile Val Ser Pro Gly Ala Asp Met Ser Ile
980 985 990

Tyr Phe Pro Tyr Thr Glu Glu Lys Arg Arg Leu Thr Lys Phe His Ser
995 1000 1005

Glu Ile Glu Glu Leu Leu Tyr Ser Asp Val Glu Asn Lys Glu His
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Leu Cys Val Leu Lys Asp Lys Lys Lys Pro Ile Leu Phe Thr Met
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Ala Arg Leu Asp Arg Val Lys Asn Leu Ser Gly Leu Val Glu Trp
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Tyr Gly Lys Asn Thr Arg Leu Arg Glu Leu Ala Asn Leu Val Val
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Val Gly Gly Asp Arg Arg Lys Glu Ser Lys Asp Asn Glu Glu Lys
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Ala Glu Met Lys Lys Met Tyr Asp Leu Ile Glu Glu Tyr Lys Leu
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Asn Gly Gln Phe Arg Trp Ile Ser Ser Gln Met Asp Arg Val Arg
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Asn Gly Glu Leu Tyr Arg Tyr Ile Cys Asp Thr Lys Gly Ala Phe
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Val Gln Pro Ala Leu Tyr Glu Ala Phe Gly Leu Thr Val Val Glu
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Ala Met Thr Cys Gly Leu Pro Thr Phe Ala Thr Cys Lys Gly Gly
1145 1150 1155

Pro Ala Glu Ile Ile Val His Gly Lys Ser Gly Phe His Ile Asp
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Pro Tyr His Gly Asp Gln Ala Ala Asp Thr Leu Ala Asp Phe Phe
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Thr Lys Cys Lys Glu Asp Pro Ser His Trp Asp Glu Ile Ser Lys
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Gly Gly Leu Gln Arg Ile Glu Glu Lys Tyr Thr Trp Gln Ile Tyr
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Ser Gln Arg Leu Leu Thr Leu Thr Gly Val Tyr Gly Phe Trp Lys
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His Val Ser Asn Leu Asp Arg Leu Glu Ala Arg Arg Tyr Leu Glu
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Asp Leu Ala Gln Arg Leu Ala Ser Arg Gly His Arg Val Ser Phe Val
        35                  40                  45


Ser Thr Pro Arg Asn Ile Ser Arg Leu Pro Pro Val Arg Pro Ala Leu
    50                  55                  60


Ala Pro Leu Val Ala Phe Val Ala Leu Pro Leu Pro Arg Val Glu Gly
65                  70                  75                  80


Leu Pro Asp Gly Ala Glu Ser Thr Asn Asp Val Pro His Asp Arg Pro
                85                  90                  95


Asp Met Val Glu Leu His Arg Arg Ala Phe Asp Gly Leu Ala Ala Pro
            100                 105                 110


Phe Ser Glu Phe Leu Gly Thr Ala Cys Ala Asp Trp Val Ile Val Asp
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Val Phe His His Trp Ala Ala Ala Ala Ala Leu Glu His Lys Val Pro
    130                 135                 140


Cys Ala Met Met Leu Leu Gly Ser Ala His Met Ile Ala Ser Ile Ala
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Asp Arg Arg Leu Glu Arg Ala Glu Thr Glu Ser Pro Ala Ala Ala Gly
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Gln Gly Arg Pro Ala Ala Ala Pro Thr Phe Glu Val Ala Arg Met Lys
            180                 185                 190

Leu Ile Arg Thr Lys Gly Ser Ser Gly Met Ser Leu Ala Glu Arg Phe
        195                 200                 205

Ser Leu Thr Leu Ser Arg Ser Ser Leu Val Val Gly Arg Ser Cys Val
    210                 215                 220

Glu Phe Glu Pro Glu Thr Val Pro Leu Leu Ser Thr Leu Arg Gly Lys
225                 230                 235                 240

Pro Ile Thr Phe Leu Gly Leu Met Pro Pro Leu His Glu Gly Arg Arg
                245                 250                 255

Glu Asp Gly Glu Asp Ala Thr Val Arg Trp Leu Asp Ala Gln Pro Ala
            260                 265                 270

Lys Ser Val Val Tyr Val Ala Leu Gly Ser Glu Val Pro Leu Gly Val
        275                 280                 285

Glu Lys Val His Glu Leu Ala Leu Gly Leu Glu Leu Ala Gly Thr Arg
    290                 295                 300

Phe Leu Trp Ala Leu Arg Lys Pro Thr Gly Val Ser Asp Ala Asp Leu
305                 310                 315                 320

Leu Pro Ala Gly Phe Glu Glu Arg Thr Arg Gly Arg Gly Val Val Ala
                325                 330                 335

Thr Arg Trp Val Pro Gln Met Ser Ile Leu Ala His Ala Ala Val Gly
            340                 345                 350

Ala Phe Leu Thr His Cys Gly Trp Asn Ser Thr Ile Glu Gly Leu Met
        355                 360                 365

Phe Gly His Pro Leu Ile Met Leu Pro Ile Phe Gly Asp Gln Gly Pro
    370                 375                 380

Asn Ala Arg Leu Ile Glu Ala Lys Asn Ala Gly Leu Gln Val Ala Arg

385                 390                 395                 400

Asn Asp Gly Asp Gly Ser Phe Asp Arg Glu Gly Val Ala Ala Ala Ile
                405                 410                 415

Arg Ala Val Ala Val Glu Glu Glu Ser Ser Lys Val Phe Gln Ala Lys
            420                 425                 430

Ala Lys Lys Leu Gln Glu Ile Val Ala Asp Met Ala Cys His Glu Arg
        435                 440                 445
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Tyr Ile Asp Gly Phe Ile Gln Gln Leu Arg Ser Tyr Lys Asp Gly Ser
    450                 455                 460

Gly Ala Asn Ala Glu Arg Met Ile Thr Arg Val His Ser Gln Arg Glu
465                 470                 475                 480

Arg Leu Asn Glu Thr Leu Val Ser Glu Arg Asn Glu Val Leu Ala Leu
                485                 490                 495

Leu Ser Arg Val Glu Ala Lys Gly Lys Gly Ile Leu Gln Gln Asn Gln
            500                 505                 510

Ile Ile Ala Glu Phe Glu Ala Leu Pro Glu Gln Thr Arg Lys Lys Leu
        515                 520                 525

Glu Gly Gly Pro Phe Phe Asp Leu Leu Lys Ser Thr Gln Glu Ala Ile
    530                 535                 540

Val Leu Pro Pro Trp Val Ala Leu Ala Val Arg Pro Arg Pro Gly Val
545                 550                 555                 560

Trp Glu Tyr Leu Arg Val Asn Leu His Ala Leu Val Val Glu Glu Leu
                565                 570                 575

Gln Pro Ala Glu Phe Leu His Phe Lys Glu Glu Leu Val Asp Gly Val
            580                 585                 590

Lys Asn Gly Asn Phe Thr Leu Glu Leu Asp Phe Glu Pro Phe Asn Ala
        595                 600                 605

Ser Ile Pro Arg Pro Thr Leu His Lys Tyr Ile Gly Asn Gly Val Asp
    610                 615                 620

Phe Leu Asn Arg His Leu Ser Ala Lys Leu Phe His Asp Lys Glu Ser
625                 630                 635                 640

Leu Leu Pro Leu Leu Lys Phe Leu Arg Leu His Ser His Gln Gly Lys
                645                 650                 655

Asn Leu Met Leu Ser Glu Lys Ile Gln Asn Leu Asn Thr Leu Gln His
            660                 665                 670

Thr Leu Arg Lys Ala Glu Glu Tyr Leu Ala Glu Leu Lys Ser Glu Thr
        675                 680                 685

Leu Tyr Glu Glu Phe Glu Ala Lys Phe Glu Glu Ile Gly Leu Glu Arg
    690                 695                 700

Gly Trp Gly Asp Asn Ala Glu Arg Val Leu Asp Met Ile Arg Leu Leu
705                 710                 715                 720

Leu Asp Leu Leu Glu Ala Pro Asp Pro Cys Thr Leu Glu Thr Phe Leu
                725                 730                 735

Gly Arg Val Pro Met Val Phe Asn Val Val Ile Leu Ser Pro His Gly
            740                 745                 750
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Tyr Phe Ala Gln Asp Asn Val Leu Gly Tyr Pro Asp Thr Gly Gly Gln
        755                 760                 765

Val Val Tyr Ile Leu Asp Gln Val Arg Ala Leu Glu Ile Glu Met Leu
    770                 775                 780

Gln Arg Ile Lys Gln Gln Gly Leu Asn Ile Lys Pro Arg Ile Leu Ile
785                 790                 795                 800

Leu Thr Arg Leu Leu Pro Asp Ala Val Gly Thr Thr Cys Gly Glu Arg
                805                 810                 815

Leu Glu Arg Val Tyr Asp Ser Glu Tyr Cys Asp Ile Leu Arg Val Pro
            820                 825                 830

Phe Arg Thr Glu Lys Gly Ile Val Arg Lys Trp Ile Ser Arg Phe Glu
        835                 840                 845

Val Trp Pro Tyr Leu Glu Thr Tyr Thr Glu Asp Ala Ala Val Glu Leu
    850                 855                 860

Ser Lys Glu Leu Asn Gly Lys Pro Asp Leu Ile Ile Gly Asn Tyr Ser
865                 870                 875                 880

Asp Gly Asn Leu Val Ala Ser Leu Leu Ala His Lys Leu Gly Val Thr
                885                 890                 895

Gln Cys Thr Ile Ala His Ala Leu Glu Lys Thr Lys Tyr Pro Asp Ser
            900                 905                 910

Asp Ile Tyr Trp Lys Lys Leu Asp Asp Lys Tyr His Phe Ser Cys Gln
        915                 920                 925

Phe Thr Ala Asp Ile Phe Ala Met Asn His Thr Asp Phe Ile Ile Thr
    930                 935                 940

Ser Thr Phe Gln Glu Ile Ala Gly Ser Lys Glu Thr Val Gly Gln Tyr
945                 950                 955                 960

Glu Ser His Thr Ala Phe Thr Leu Pro Gly Leu Tyr Arg Val Val His
                965                 970                 975

Gly Ile Asp Val Phe Asp Pro Lys Phe Asn Ile Val Ser Pro Gly Ala
            980                 985                 990

Asp Met Ser Ile Tyr Phe Pro Tyr  Thr Glu Glu Lys Arg  Arg Leu Thr
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Lys Phe  His Ser Glu Ile Glu  Glu Leu Leu Tyr Ser  Asp Val Glu
    1010                 1015                 1020

Asn Lys  Glu His Leu Cys Val  Leu Lys Asp Lys Lys  Lys Pro Ile
    1025                 1030                 1035

Leu Phe  Thr Met Ala Arg Leu  Asp Arg Val Lys Asn  Leu Ser Gly
    1040                 1045                 1050
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Leu Val  Glu Trp Tyr Gly Lys  Asn Thr Arg Leu Arg  Glu Leu Ala
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Asn Glu  Glu Lys Ala Glu Met  Lys Lys Met Tyr Asp  Leu Ile Glu
    1085                 1090                 1095

Glu Tyr  Lys Leu Asn Gly Gln  Phe Arg Trp Ile Ser  Ser Gln Met
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Asp Arg  Val Arg Asn Gly Glu  Leu Tyr Arg Tyr Ile  Cys Asp Thr
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Lys Gly  Ala Phe Val Gln Pro  Ala Leu Tyr Glu Ala  Phe Gly Leu
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Thr Val  Val Glu Ala Met Thr  Cys Gly Leu Pro Thr  Phe Ala Thr
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Cys Lys  Gly Gly Pro Ala Glu  Ile Ile Val His Gly  Lys Ser Gly
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Phe His  Ile Asp Pro Tyr His  Gly Asp Gln Ala Ala  Asp Thr Leu
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Ala Asp  Phe Phe Thr Lys Cys  Lys Glu Asp Pro Ser  His Trp Asp
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Glu Ile  Ser Lys Gly Gly Leu  Gln Arg Ile Glu Glu  Lys Tyr Thr
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Trp Gln  Ile Tyr Ser Gln Arg  Leu Leu Thr Leu Thr  Gly Val Tyr
    1220                 1225                 1230

Gly Phe  Trp Lys His Val Ser  Asn Leu Asp Arg Leu  Glu Ala Arg
    1235                 1240                 1245

Arg Tyr  Leu Glu Met Phe Tyr  Ala Leu Lys Tyr Arg  Pro Leu Ala
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cgtttgaacg aaacgcttgt ttctgagaga aacgaagtcc ttgccttgct ttccagggtt    1500
gaagccaaag gtaaaggtat tttacaacaa aaccagatca ttgctgaatt cgaagctttg    1560
cctgaacaaa cccggaagaa acttgaaggt ggtcctttct ttgaccttct caaatccact    1620
caggaagcaa ttgtgttgcc accatgggtt gctctagctg tgaggccaag gcctggtgtt    1680
tgggaatact tacgagtcaa tctccatgct cttgtcgttg aagaactcca acctgctgag    1740
tttcttcatt tcaaggaaga actcgttgat ggagttaaga atggtaattt cactcttgag    1800
cttgatttcg agccattcaa tgcgtctatc cctcgtccaa cactccacaa atacattgga    1860
aatggtgttg acttccttaa ccgtcattta tcggctaagc tcttccatga caaggagagt    1920
ttgcttccat tgcttaagtt ccttcgtctt cacagccacc agggcaagaa cctgatgttg    1980
agcgagaaga ttcagaacct caacactctg caacacacct tgaggaaagc agaagagtat    2040
ctagcagagc ttaagtccga aacactgtat gaagagtttg aggccaagtt tgaggagatt    2100
ggtcttgaga ggggatgggg agacaatgca gagcgtgtcc ttgacatgat acgtcttctt    2160
ttggaccttc ttgaggcgcc tgatccttgc actcttgaga cttttcttgg aagagtacca    2220
atggtgttca acgttgtgat cctctctcca catggttact ttgctcagga caatgttctt    2280
```

```
ggttaccctg acactggtgg acaggttgtt tacattcttg atcaagttcg tgctctggag     2340

atagagatgc ttcaacgtat taagcaacaa ggactcaaca ttaaaccaag gattctcatt     2400

ctaactcgac ttctacctga tgcggtagga actacatgcg gtgaacgtct cgagagagtt     2460

tatgattctg agtactgtga tattcttcgt gtgcccttca gaacagagaa gggtattgtt     2520

cgcaaatgga tctcaaggtt cgaagtctgg ccatatctag agacttacac cgaggatgct     2580

gcggttgagc tatcgaaaga attgaatggc aagcctgacc ttatcattgg taactacagt     2640

gatggaaatc ttgttgcttc tttattggct cacaaacttg gtgtcactca gtgtaccatt     2700

gctcatgctc ttgagaaaac aaagtacccg gattctgata tctactggaa gaagcttgac     2760

gacaagtacc atttctcatg ccagttcact gcggatattt tcgcaatgaa ccacactgat     2820

ttcatcatca ctagtacttt ccaagaaatt gctggaagca aagaaactgt tgggcagtat     2880

gaaagccaca cagcctttac tcttcccgga ttgtatcgag ttgttcacgg gattgatgtg     2940

tttgatccca agttcaacat tgtctctcct ggtgctgata tgagcatcta cttcccttac     3000

acagaggaga agcgtagatt gactaagttc cactctgaga tcgaggagct cctctacagc     3060

gatgttgaga acaaagagca cttatgtgtg ctcaaggaca agaagaagcc gattctcttc     3120

acaatggcta ggcttgatcg tgtcaagaac ttgtcaggtc ttgttgagtg gtacgggaag     3180

aacacccgct tgcgtgagct agctaacttg gttgttgttg gaggagacag gaggaaagag     3240

tcaaaggaca atgaagagaa agcagagatg aagaaaatgt atgatctcat tgaggaatac     3300

aagctaaacg gtcagttcag gtggatctcc tctcagatgg accgggtaag gaacggtgag     3360

ctgtaccggt acatctgtga caccaagggt gcttttgtcc aacctgcatt atatgaagcc     3420

tttgggttaa ctgttgtgga ggctatgact tgtggtttac cgactttcgc cacttgcaaa     3480

ggtggtccag ctgagatcat tgtgcacggt aaatcgggtt tccacattga cccttaccat     3540

ggtgatcagg ctgctgatac tcttgctgat ttcttcacca agtgtaagga ggatccatct     3600

cactgggatg agatctcaaa aggagggctt cagaggattg aggagaaata cacttggcaa     3660

atctattcac agaggctctt gacattgact ggtgtgtatg gattctggaa gcatgtctcg     3720

aaccttgacc gtcttgaggc tcgccgttac cttgaaatgt tctatgcatt gaagtatcgc     3780

ccattggctc aggctgttcc tcttgcacaa gatgattga                            3819
```

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO2014193888A [0003]
- WO2013022989A [0094] [0154]
- US269435A [0094] [0154]
- US61898571B [0149]

Non-patent literature cited in the description

- **REECK et al.**Cell, 1987, vol. 50, 667- [0027]
- **SAMBROOK, J.FRITSCH, E. F.MANIATIS, T.**Molecular Cloning: A Laboratory ManualCold Spring Harbor Laboratory19890000 [0043]
- **SILHAVY, T. J.BENNAN, M. L.ENQUIST, L. W.**Experiments with Gene FusionsCold Spring Harbor Laboratory19840000 [0043]
- **AUSUBEL, F. M. et al.**Current Protocols in Molecular BiologyGreene Publishing and Wiley-Interscience19870000 [0043]
- Remington's Pharmaceutical SciencesMack Publishing Co. [0121]
- **BIENIAWSKA et al.**Plant J., 2007, vol. 49, 810-828 [0144]
- Minor diterpene glycosides from the leaves of Stevia rebaudianaJournal of Natural Products, 2014, vol. 77, 51231-1235 [0146]

Patentkrav

1. Fremgangsmåde til fremstilling af rebaudiosid KA og/eller rebaudiosid E ud fra rubusosid, hvilken fremgangsmåde omfatter:

tilvejebringelse af en reaktionsblanding, der omfatter:

(A)

(i) rubusosid,

(ii) uridindiphosphat-glucose (UDP-glucose) som et substrat og

(iii) HVI-glycosyltransferasen med aminosyresekvensen ifølge SEQ ID NO: 5; eller

(B)

(i) rubusosid,

(ii) saccharose, UDP og UDP-glucose som substrater og

(ii) HVI-glycosyltransferasen med aminosyresekvensen ifølge SEQ ID NO: 5 med en saccharosesyntase;

inkubation af reaktionsblandingen for således at fremstille rebaudiosid KA, hvor en glucose er kovalent koblet til C2'- af 19-O-glucose af rubusosid for at fremstille rebaudiosid KA og/eller rebaudiosid E, hvor glucose endvidere er kovalent koblet med glycosyltransferasen til C2'- af 13-O-glucosen af rebaudiosid KA; og

opnåelse af rebaudiosid KA ud fra reaktionsblandingen til anvendelse som et sødemiddel.

2. Fremgangsmåde ifølge krav 1, hvor rebaudiosid KA opnås til anvendelse som et eneste sødemiddel.

3. Fremgangsmåde ifølge krav 1 eller krav 2, hvor en anvendt saccharosesyntase er valgt fra gruppen bestående af en *Arabidopsis*-saccharosesyntase 1, en *Arabidopsis*-saccharosesyntase 3 og en *Vigna radiate*-saccharosesyntase.

4. Fremgangsmåde ifølge krav 3, hvor saccharosesyntasen er en *Arabidopsis thaliana*-saccharosesyntase 1.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 4, hvor rebaudiosid KA tilvejebringes i en sødemiddelsammensætning, der indbefatter mindst ét af et fyldstof, et kvældemiddel og et antiklumpningsmiddel.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 4, der endvidere omfatter inkorporering af rebaudiosid KA som et sødemiddel i et produkt til oral indtagelse, der er en drikkevare eller et andet produkt til oral indtagelse.

DRAWINGS

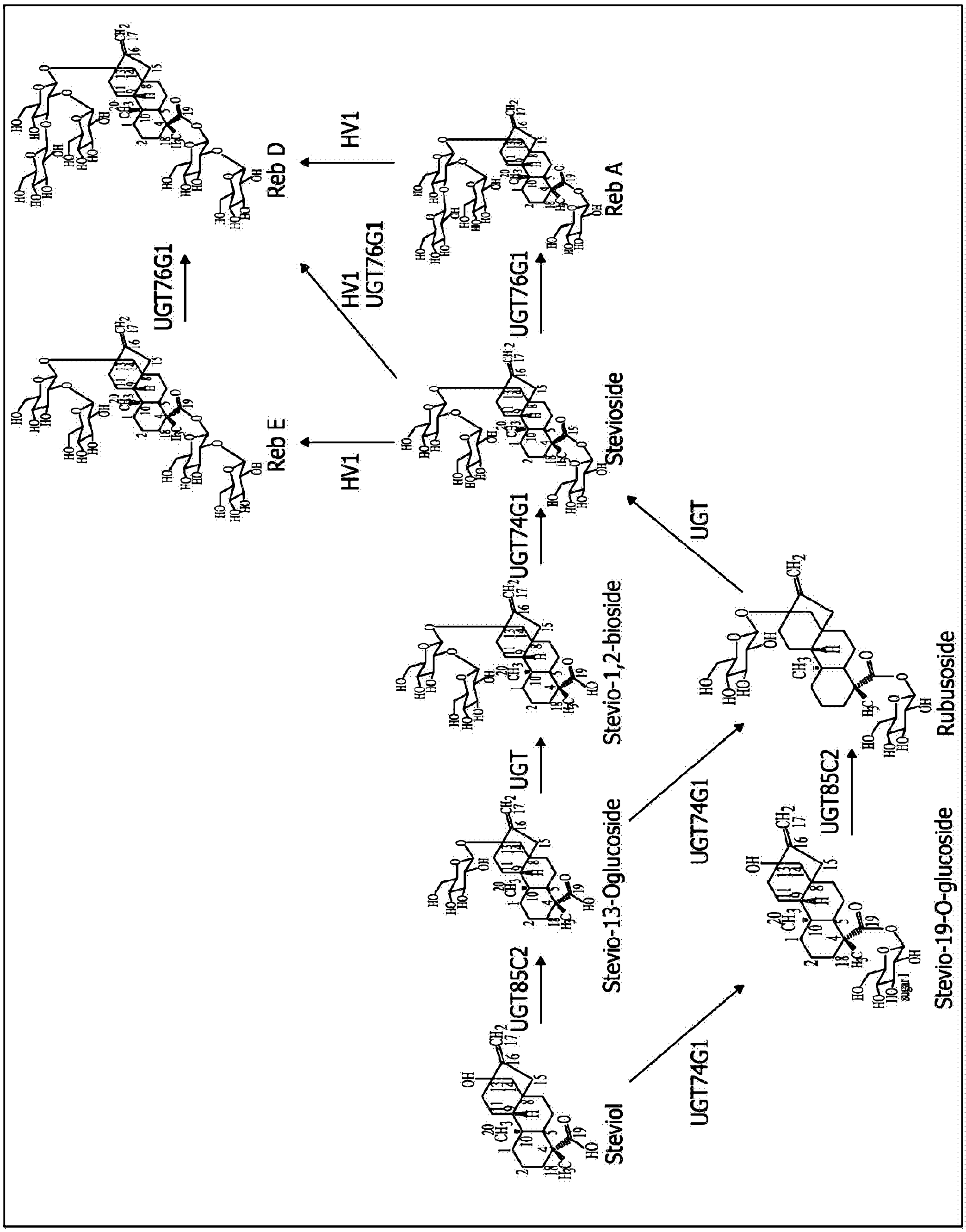


FIG. 1

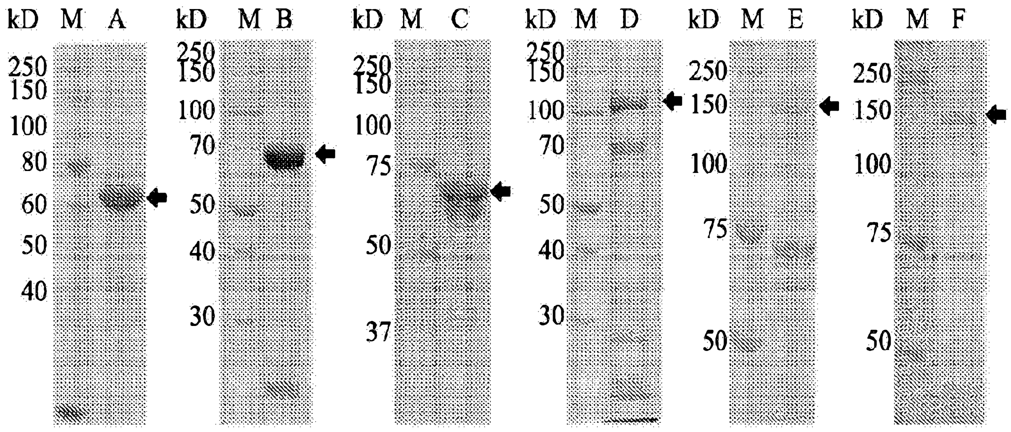

kD M A
250
150
100
80
60
50
40
kD M B
250
150
100
70
50
40
30
kD M C
250
150
100
75
50
37
kD M D
250
150
100
70
50
40
30
kD M E
250
150
100
75
50
kD M F
250
150
100
75
50

FIG. 2

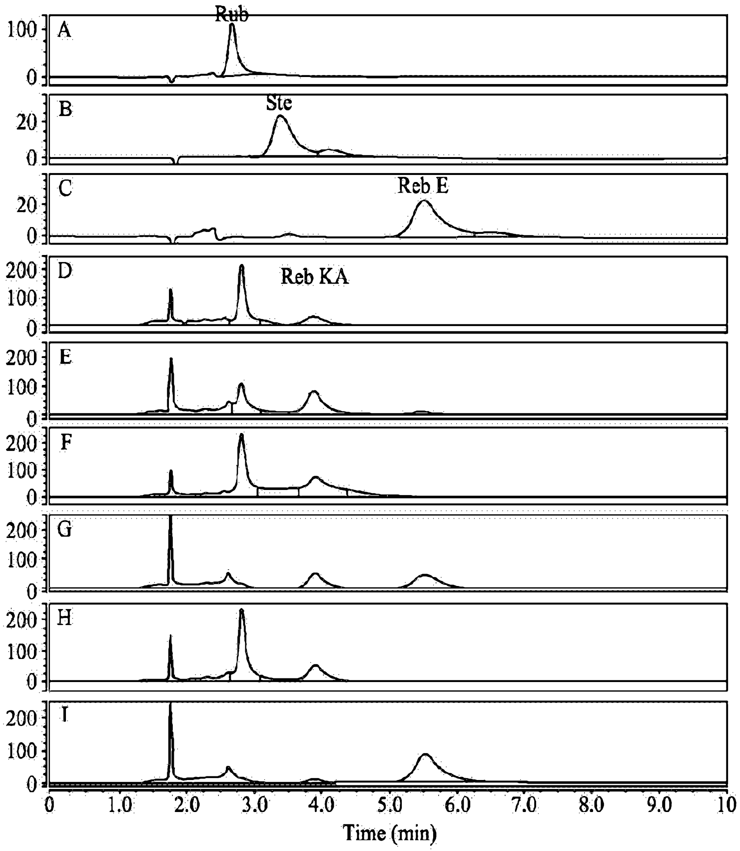

A
Rub
100
0
B
Ste
20
0
C
Reb E
20
0
D
Reb KA
200
100
0
E
F
G
H
I
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 3

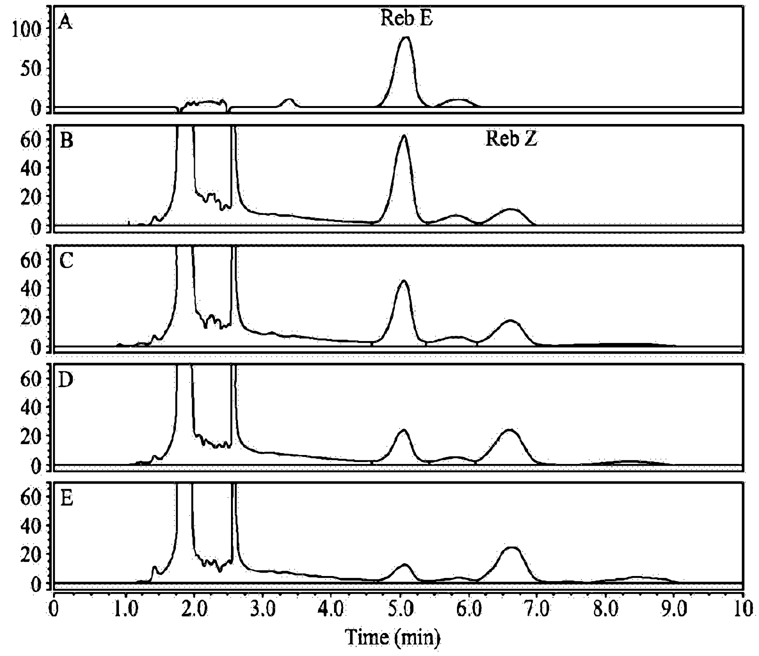

A
Reb E
100
50
0
B
Reb Z
60
40
20
0
C
60
40
20
0
D
60
40
20
0
E
60
40
20
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 4

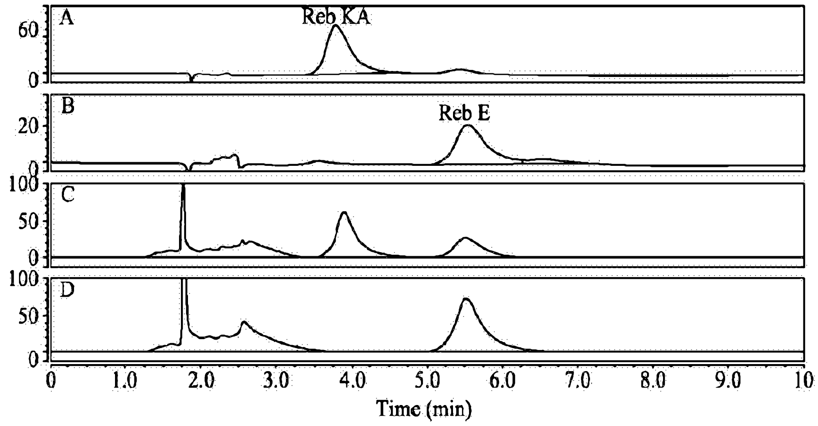

A
Reb KA
60
0
B
Reb E
20
0
C
100
50
0
D
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 5

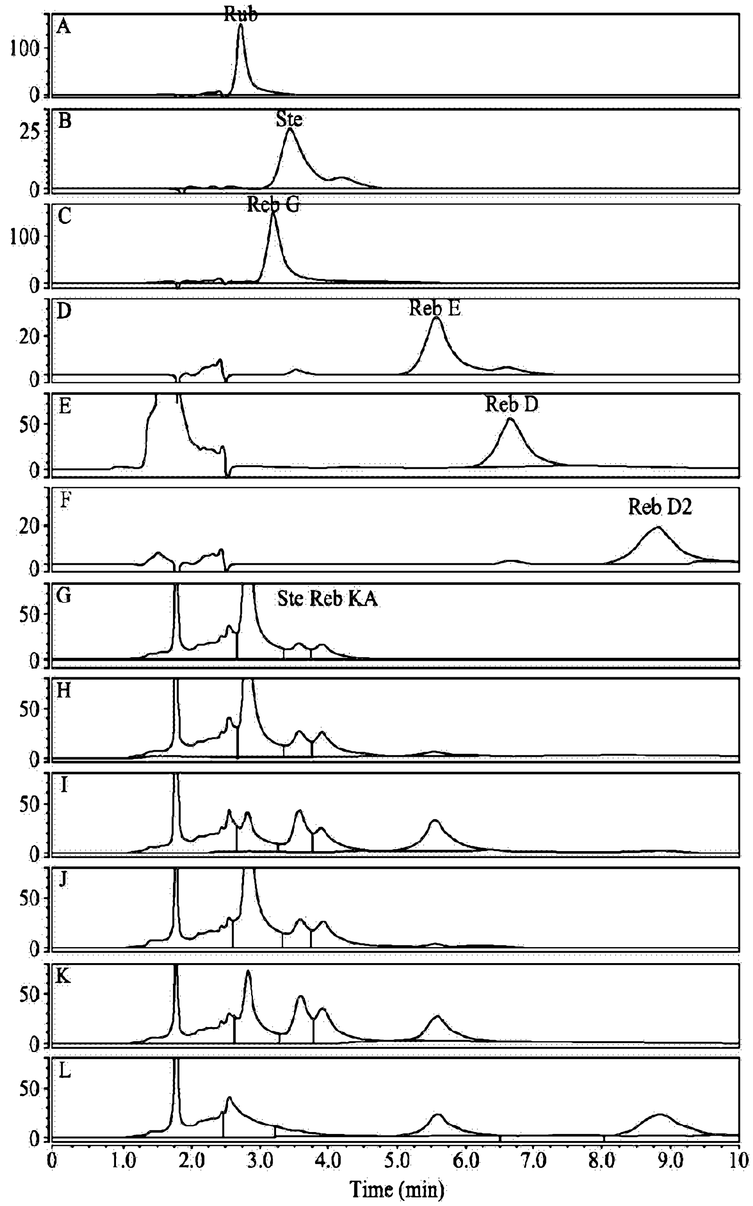

A
Rub
100
0
B
Ste
25
0
C
Reb G
100
0
D
Reb E
20
0
E
Reb D
50
0
F
Reb D2
20
0
G
Ste Reb KA
50
0
H
50
0
I
50
0
J
50
0
K
50
0
L
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 6

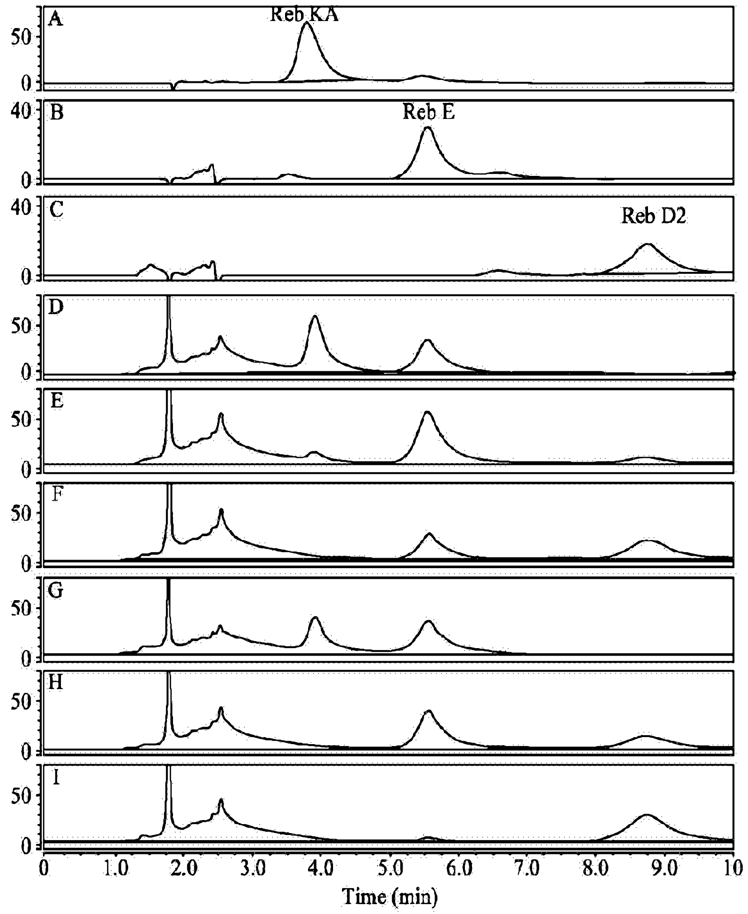

A
Reb KA
B
Reb E
C
Reb D2
D
E
F
G
H
I
50
0
40
0
40
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 7

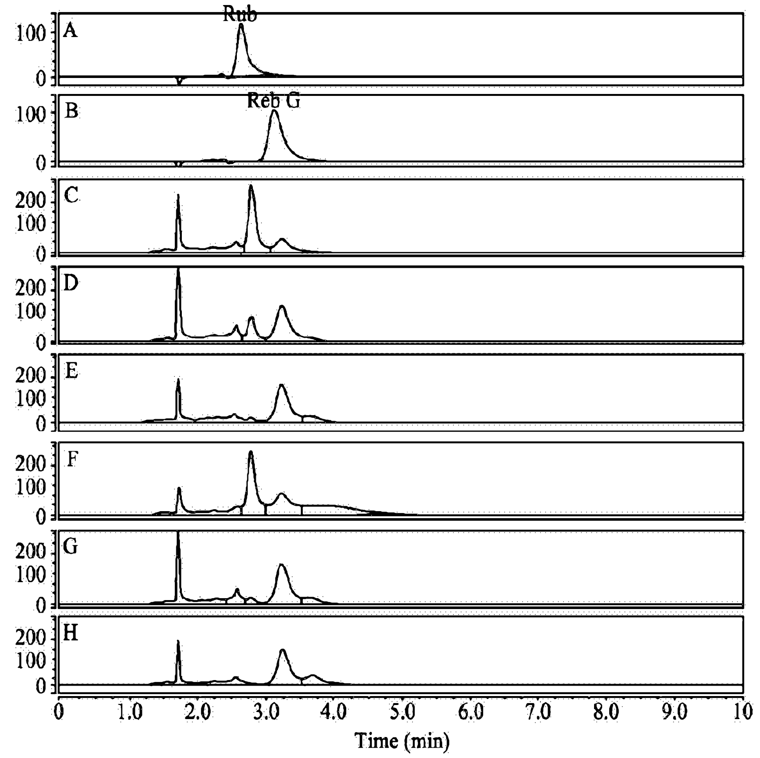

Rub
Reb G
A
B
C
D
E
F
G
H
100
0
200
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 8

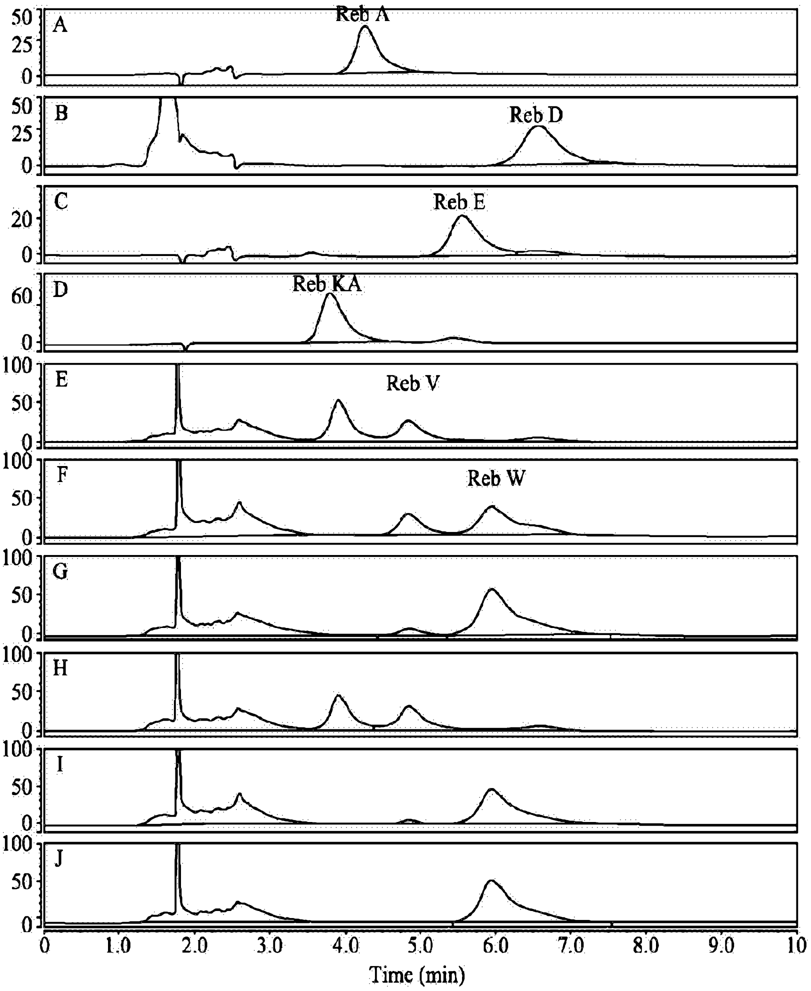

A
Reb A
50
25
0
B
Reb D
50
25
0
C
Reb E
20
0
D
Reb KA
60
0
E
Reb V
100
50
0
F
Reb W
100
50
0
G
100
50
0
H
100
50
0
I
100
50
0
J
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 9

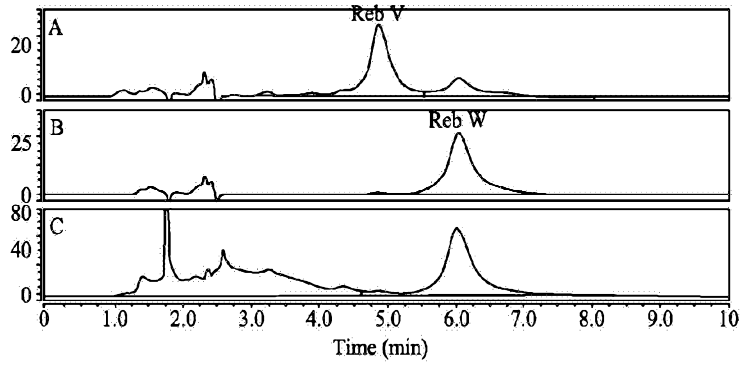

A
Reb V
20
0
B
Reb W
25
0
C
80
40
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 10

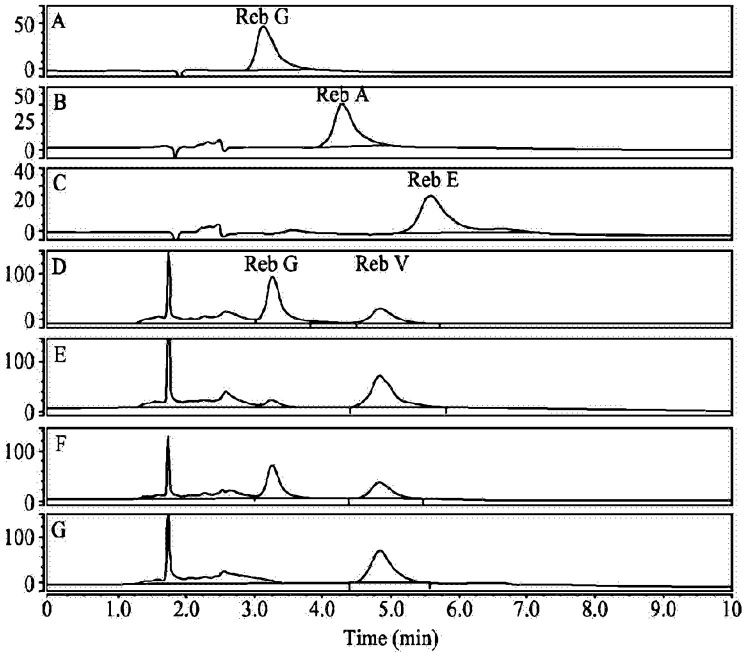

A
Reb G
50
0
B
Reb A
50
25
0
C
Reb E
40
20
0
D
Reb G
Reb V
100
0
E
100
0
F
100
0
G
100
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 11

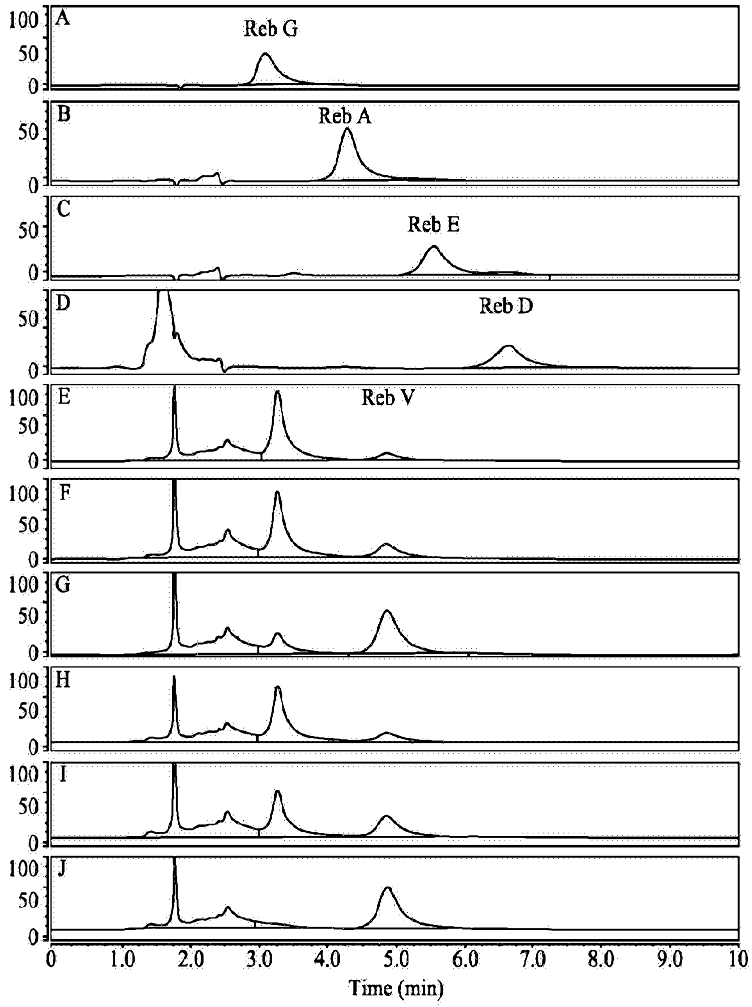

A
Reb G
B
Reb A
C
Reb E
D
Reb D
E
Reb V
F
G
H
I
J
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 12

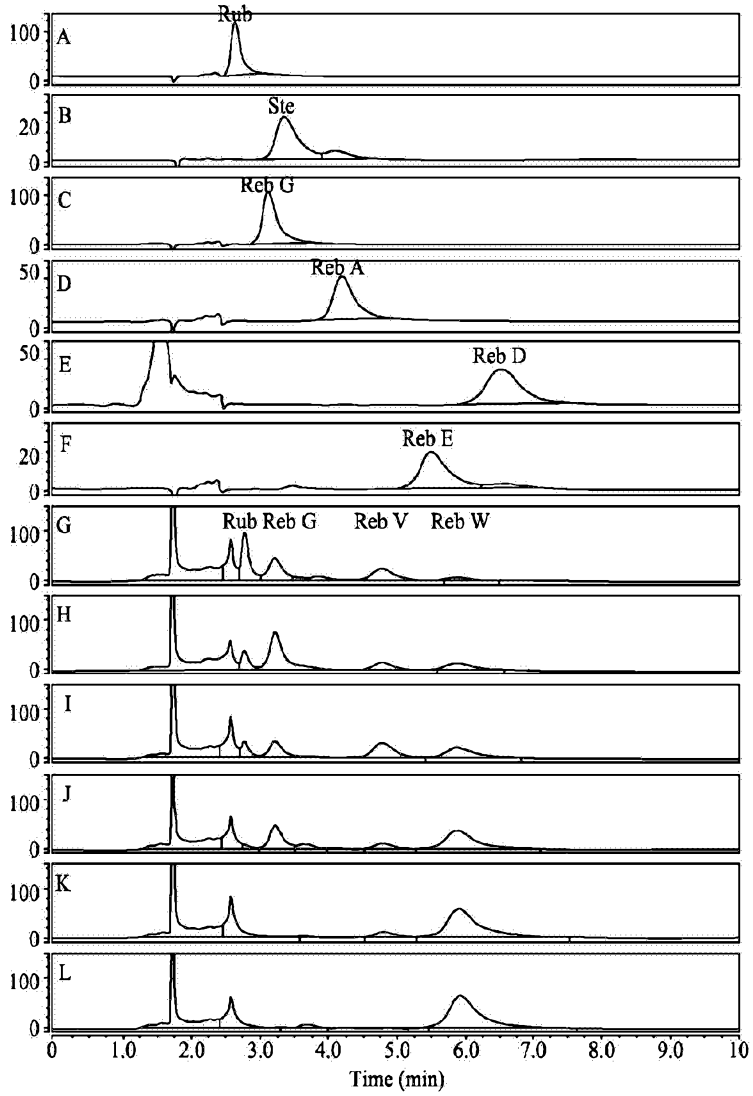

Rub
Ste
Reb G
Reb A
Reb D
Reb E
Rub Reb G
Reb V
Reb W
A
B
C
D
E
F
G
H
I
J
K
L
100
50
20
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 13

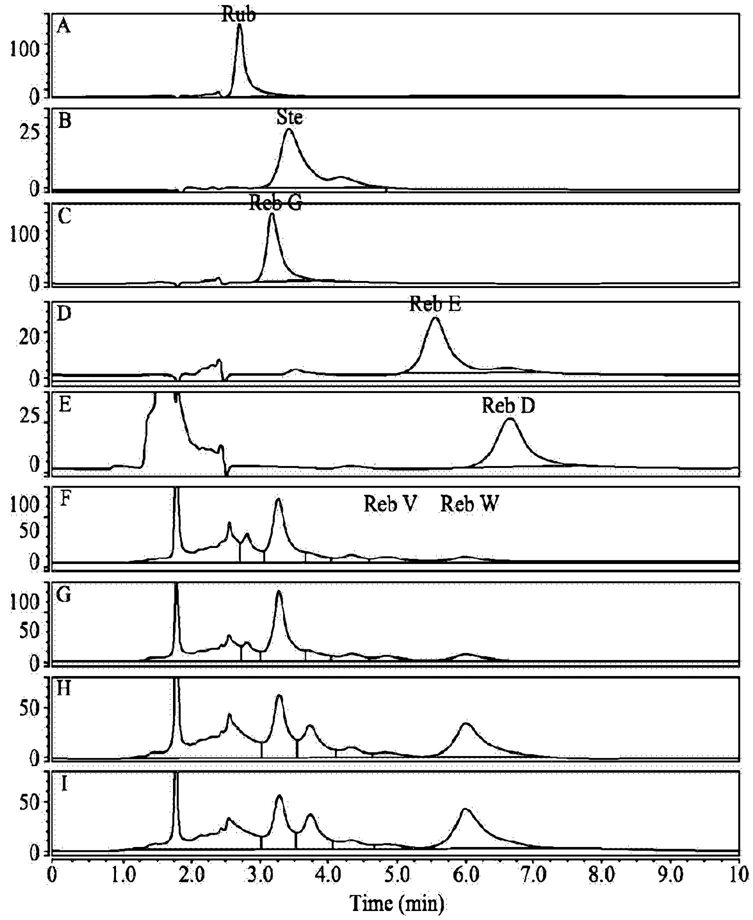

A
Rub
100
0
B
Ste
25
0
C
Reb G
100
0
D
Reb E
20
0
E
Reb D
25
0
F
Reb V
Reb W
100
50
0
G
100
50
0
H
50
0
I
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 14

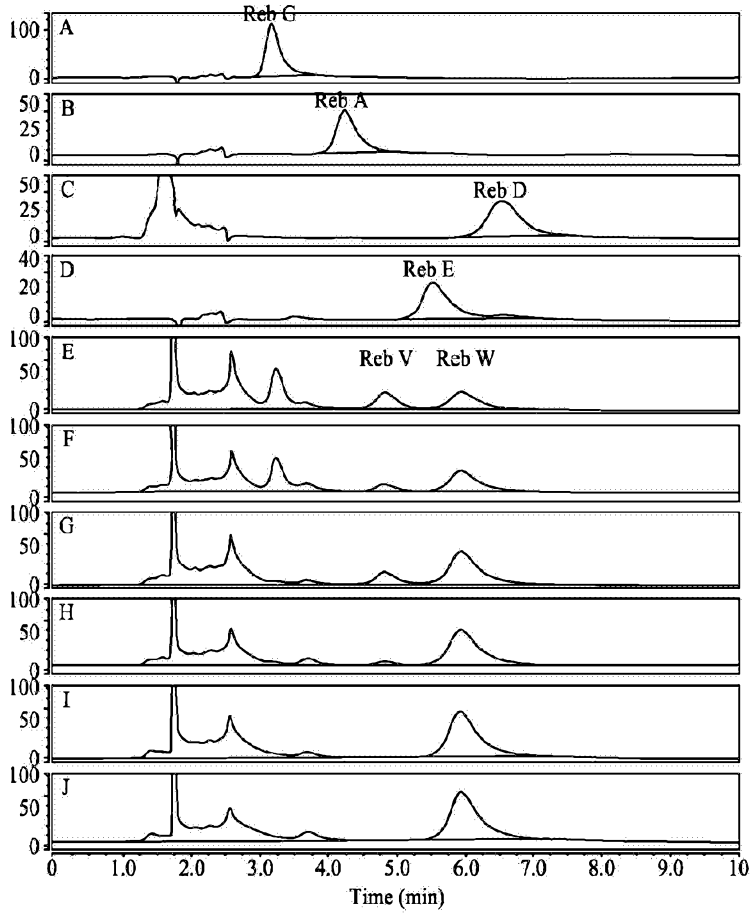

Reb G
Reb A
Reb D
Reb E
Reb V
Reb W
A
B
C
D
E
F
G
H
I
J
100
0
50
25
40
20
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 15

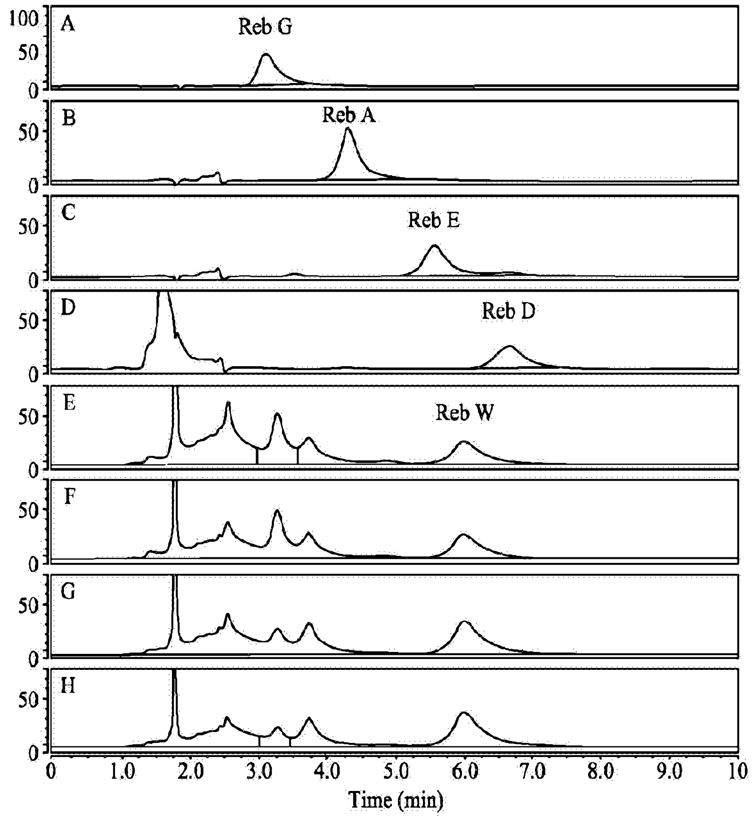
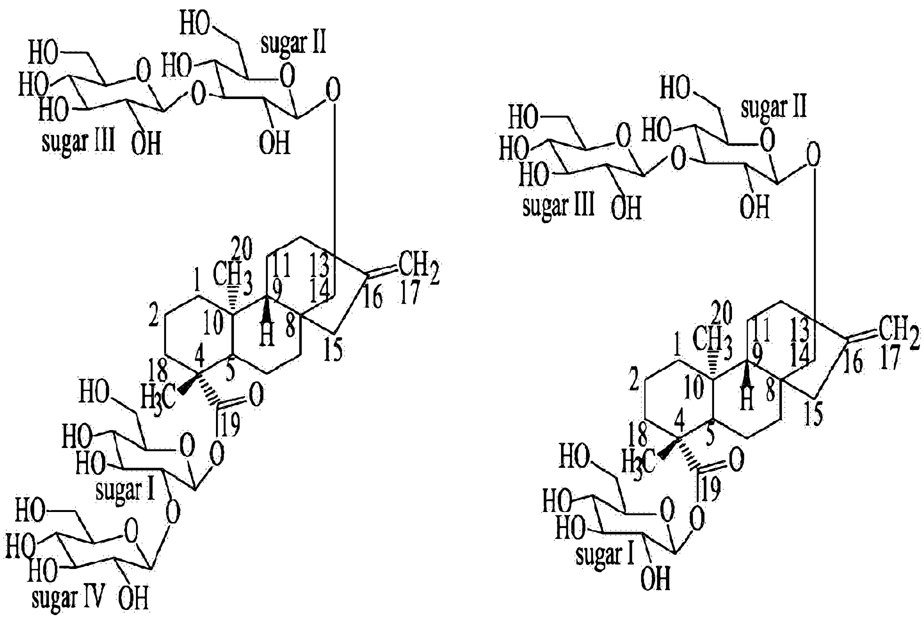

A
Reb G
B
Reb A
C
Reb E
D
Reb D
E
Reb W
F
G
H
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
Time (min)

FIG. 16



Reb V

Reb G

FIG. 17

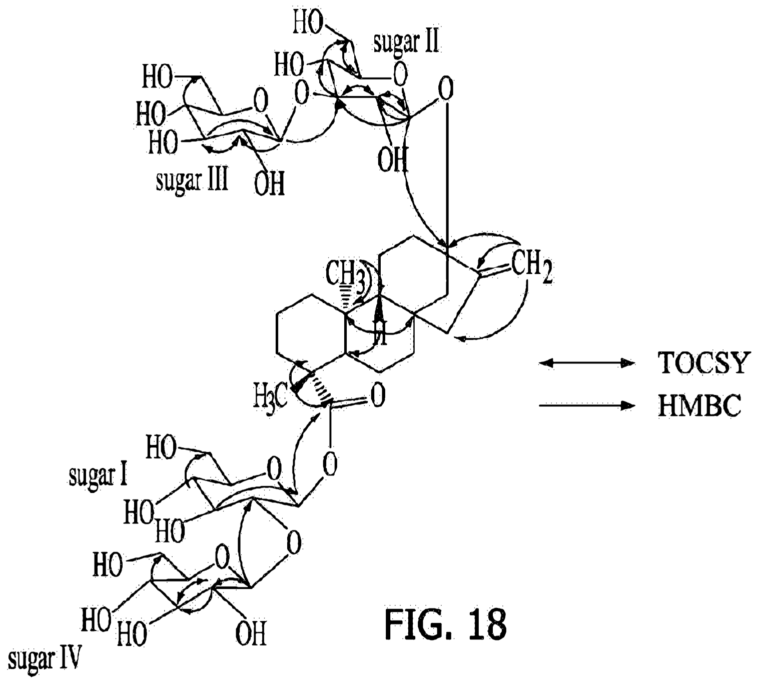
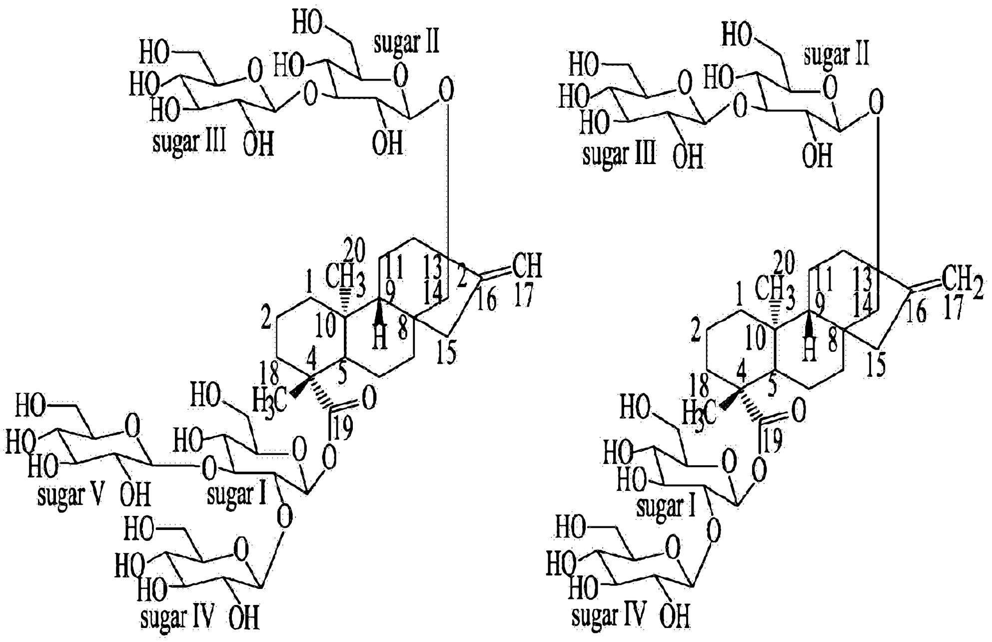


FIG. 18



Reb W

Reb V

FIG. 19

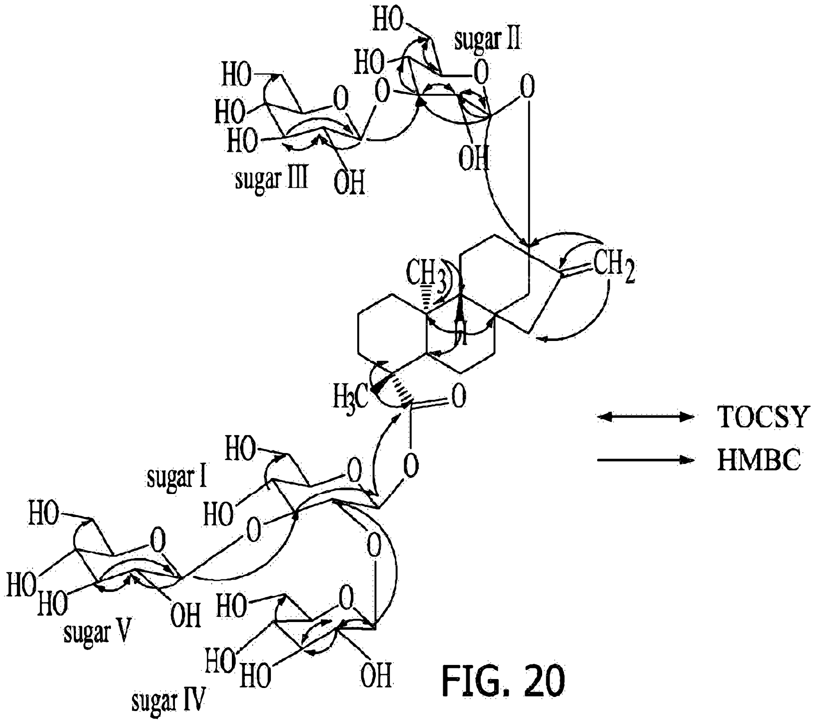


FIG. 20

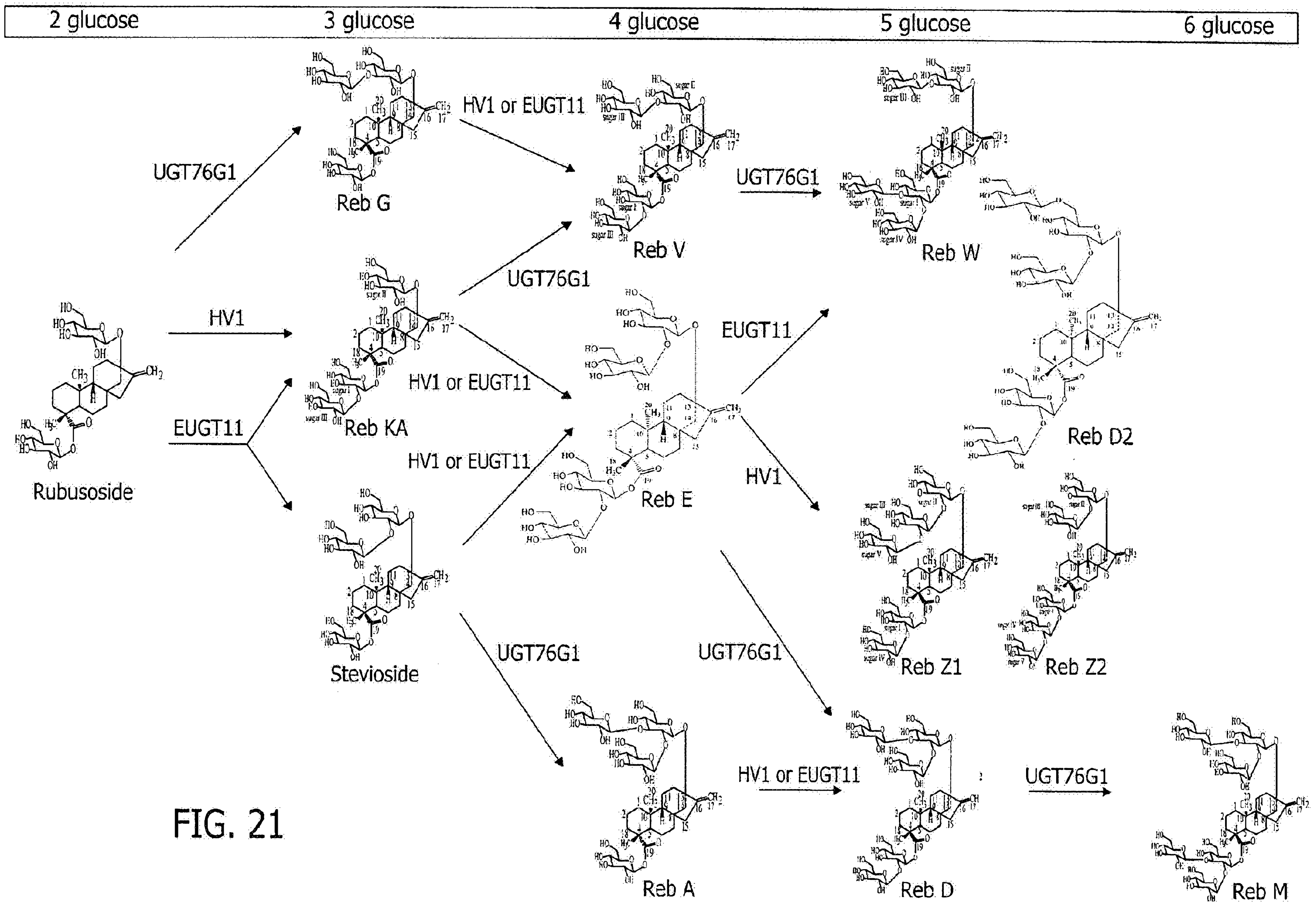

2 glucose
3 glucose
4 glucose
5 glucose
6 glucose
Rubusoside
UGT76G1
HV1
EUGT11
Reb G
Reb KA
Stevioside
HV1 or EUGT11
UGT76G1
HV1 or EUGT11
HV1 or EUGT11
UGT76G1
Reb V
Reb E
Reb A
UGT76G1
EUGT11
HV1
UGT76G1
HV1 or EUGT11
Reb W
Reb D2
Reb Z1
Reb Z2
Reb D
UGT76G1
Reb M

FIG. 21

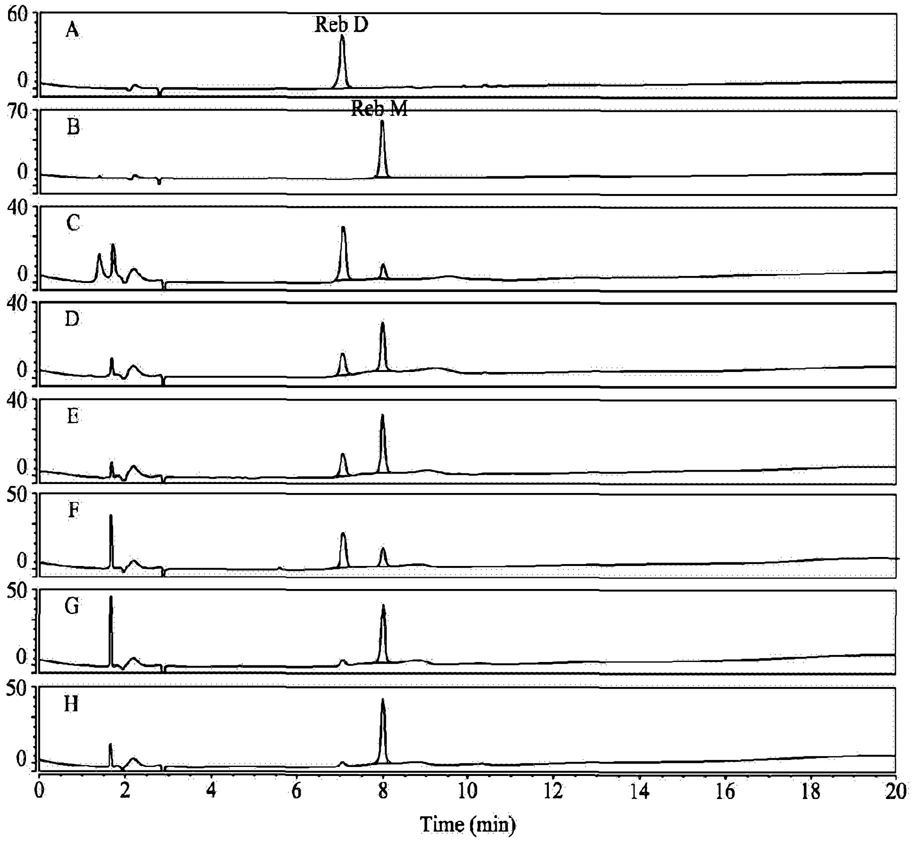

60
0
A
Reb D
70
0
B
Reb M
40
0
C
40
0
D
40
0
E
50
0
F
50
0
G
50
0
H
0
2
4
6
8
10
12
14
16
18
20
Time (min)

FIG. 22

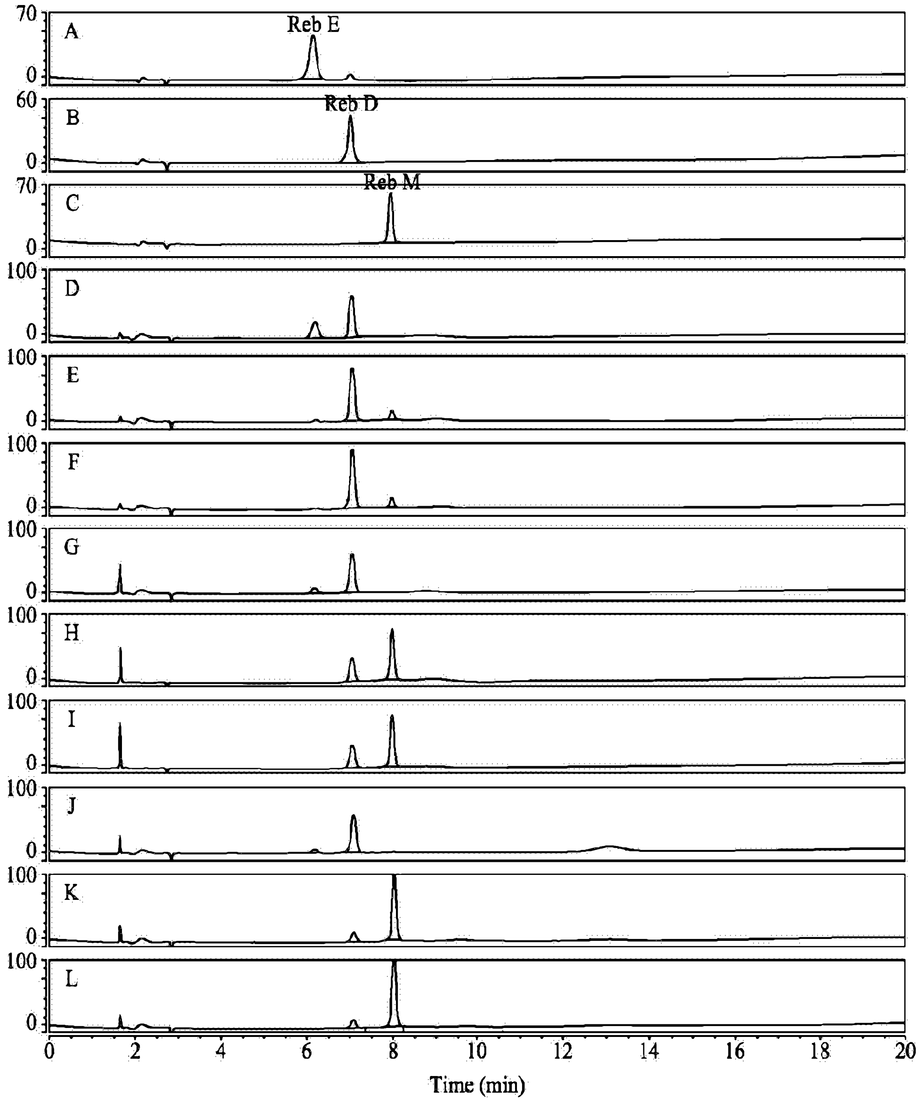

A
Reb E
B
Reb D
C
Reb M
D
E
F
G
H
I
J
K
L
70
60
100
0
2
4
6
8
10
12
14
16
18
20
Time (min)

FIG. 23

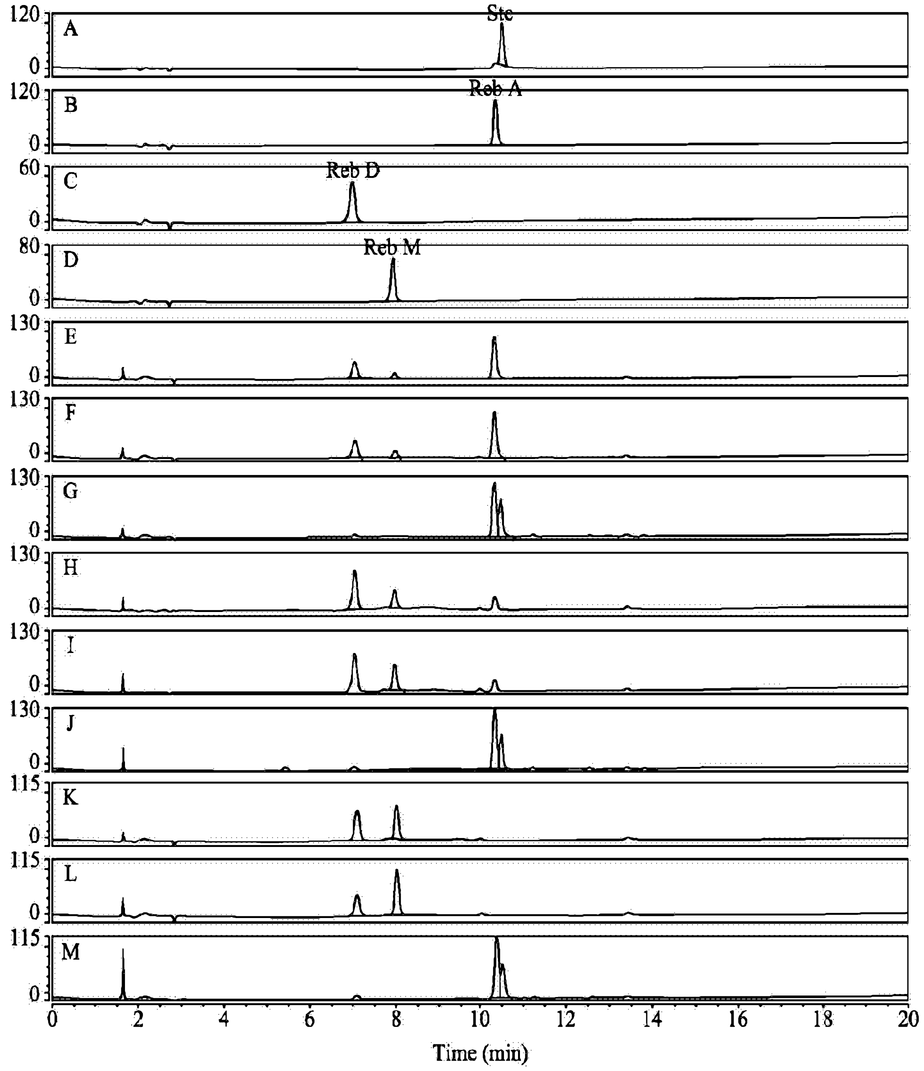

120
0
A
Stc
120
0
B
Reb A
60
0
C
Reb D
80
0
D
Reb M
130
0
E
130
0
F
130
0
G
130
0
H
130
0
I
130
0
J
115
0
K
115
0
L
115
0
M
0
2
4
6
8
10
12
14
16
18
20
Time (min)

FIG. 24

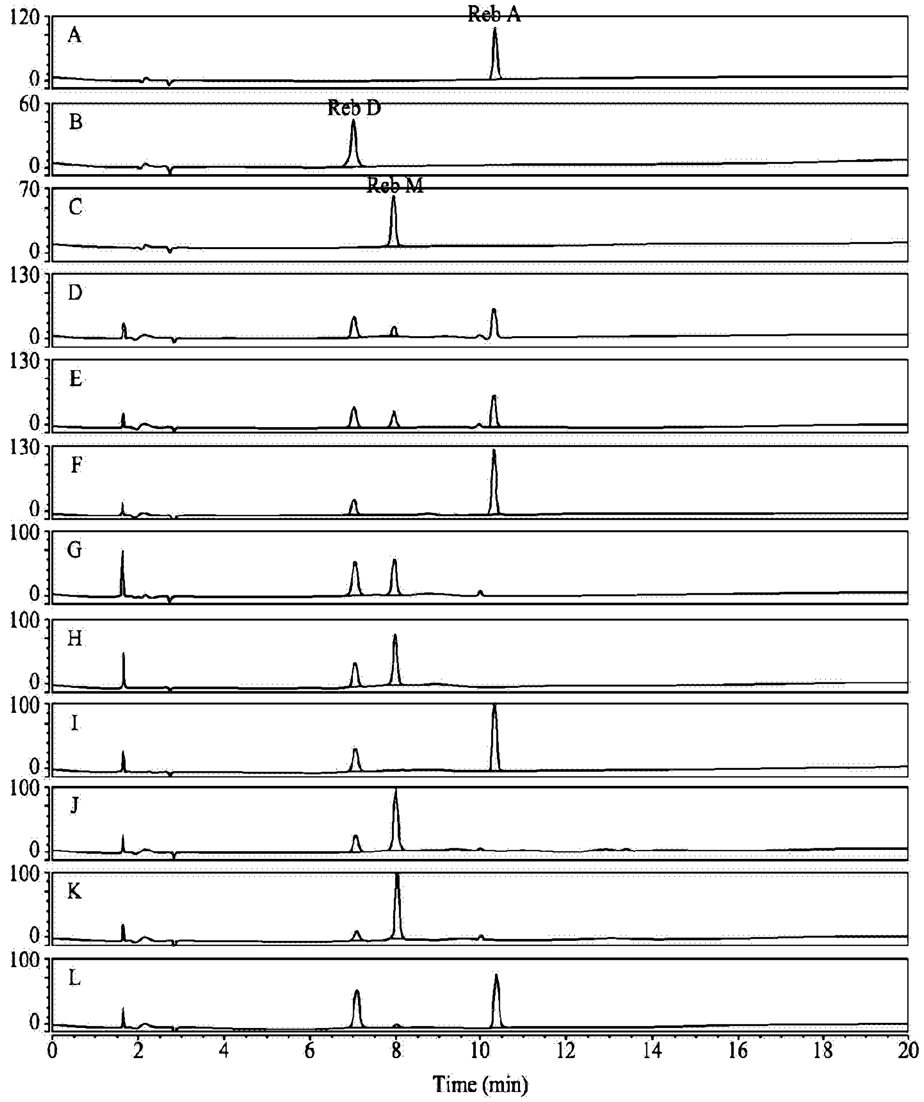
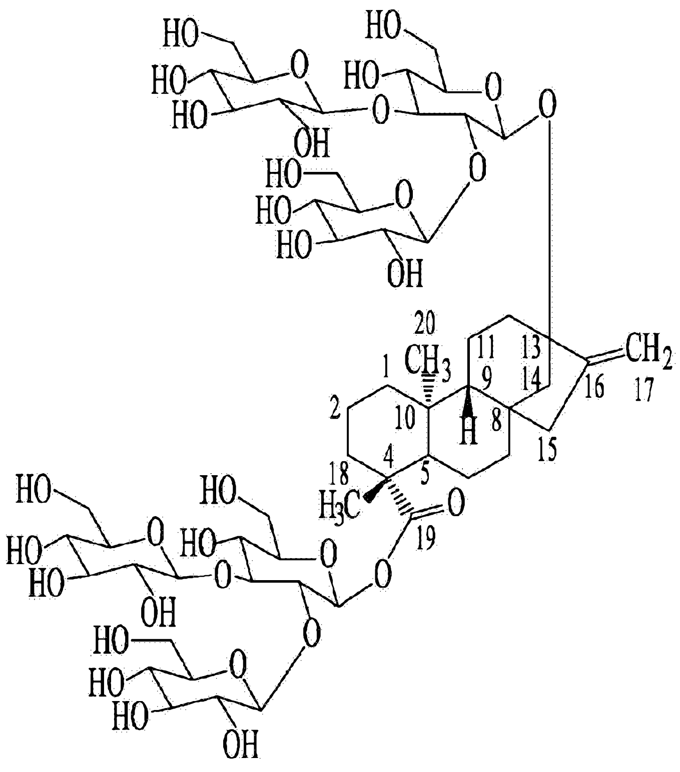

Reb A
Reb D
Reb M
A
B
C
D
E
F
G
H
I
J
K
L
120
60
70
130
130
130
100
100
100
100
100
100
0
0
2
4
6
8
10
12
14
16
18
20
Time (min)

FIG. 25



Reb M

FIG. 26

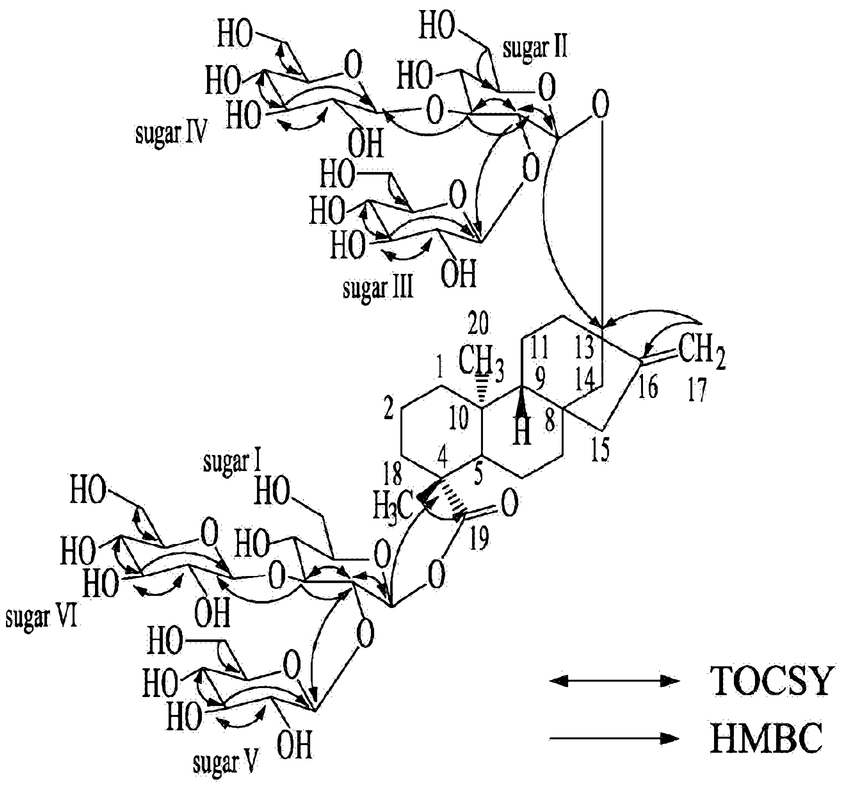

HO
HO
HO
OH
sugar IV
O
HO
HO
sugar II
O
O
O
HO
HO
HO
OH
sugar III
O
20
CH3
1
2
10
9
11
13
14
8
16
CH2
17
15
H
18
4
5
H3C
19
O
sugar I
HO
HO
O
O
HO
HO
HO
OH
sugar VI
O
HO
HO
HO
OH
sugar V
O
TOCSY
HMBC

FIG. 27