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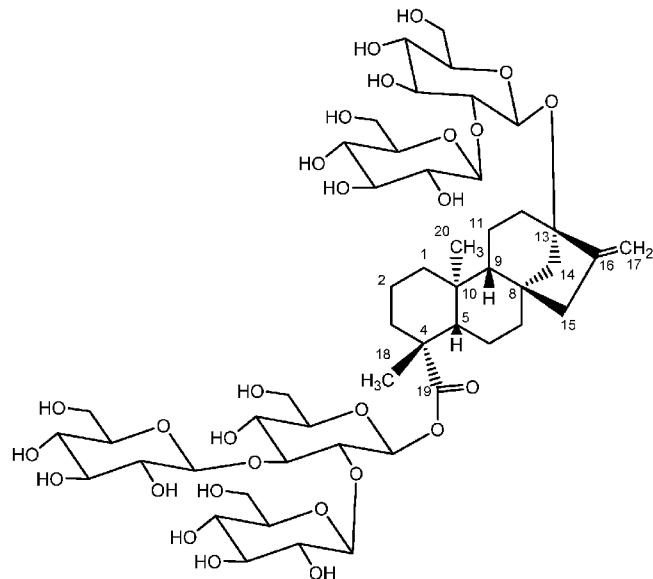


FIG. 1

(57) Abstract: The present invention relates to the production of stevioside rebaudiosides D4, WB1 and WB2 and the production of rebaudioside M from Reb D4.



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**BIOSYNTHETIC PRODUCTION OF STEVIOL GLYCOSIDES AND
PROCESSES THEREFORE**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [001] This application claims priority to U.S. Provisional Application No. 62/408,179, filed on October 14, 2016, and U.S. Provisional Application No. 62/555,809, filed on September 8, 2017, the contents of each of which are incorporated by reference herein in their entirety.

10 **FIELD OF THE INVENTION**

[002] The field of the invention relates to methods and processes useful in the production of specific steviol glycosides. More specifically, the present disclosure provides for the production of a previously unknown rebaudioside, rebaudioside D4 (“Reb D4”) that can then be converted to rebaudioside M (“Reb M”) *via* enzymatic conversion. The present disclosure also provides for production of previously unknown rebaudiosides, rebaudioside WB1 (“Reb WB1”) and rebaudioside WB2 (“Reb WB2”).

BACKGROUND OF THE INVENTION

[003] The present disclosure is focused on the production of novel steviosides 20 Reb D4, Reb WB1 and Reb WB2 and on conversion of Reb D4 to Reb M. In particular, the present disclosure relates to the synthesis of Reb D4 and its consequent use in the production of Reb M.

[004] Steviol glycosides are natural products isolated from *Stevia rebaudiana* 25 leaves, and are widely used as high intensity, low-caloric sweeteners in food, feed and beverages. Naturally occurring steviol glycosides have the same base diterpene structure (steviol) but differ in the number and structure of carbohydrate residue modifications (e.g. glucose, rhamnose, and xylose residues) at the C13 and C19 positions of the steviol backbone. Steviol glycosides with known structures include stevioside, rebaudioside A, rebaudioside B, 30 rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside M and dulcoside A. In terms of commercial utilization rebaudioside M itself has been generally regarded as safe (‘GRAS’ status).

[005] On dry weight basis, stevioside, rebaudioside A, rebaudioside C, and 35 dulcoside A, account for 9.1, 3.8, 0.6, and 0.30 percent of the total weight of the steviol glycosides in wild type *Stevia* leaves, respectively, while the other steviol glucosides, such as Reb M are present in significantly lower amounts. Extracts from *Stevia rebaudiana* plant are

commercially available, where such extracts typically contain stevioside and rebaudioside A as the primary components. The other known steviol glycosides typically are present in the *stevia* extract as minor or trace components. For example, the amount of rebaudioside A in commercial preparations can vary from about 20% to more than 90% of the total steviol

5 glycoside content, while the amount of rebaudioside B is typically 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. In such extracts rebaudioside M is present only in vanishingly small amounts. Interestingly, Rebaudioside E is also one of the least abundant steviol glycosides present in *Stevia rebaudiana* plant varieties, accounting for less than 0.5% of total glycosides.

10 [006] As natural sweeteners, different steviol glycosides have different degrees of sweetness, ‘mouth feel’ and specific after-tastes associated with each rebaudioside species tested. Relative to table sugar (i.e., “sucrose”) the sweetness of steviol glycosides is significantly higher. For example, stevioside is 100-150 times sweeter than sucrose but has a bitter after-taste as noted in taste tests, while rebaudiosides A and E are 250-450 times sweeter
15 than sucrose and the after-taste is much better than stevioside, however, a noticeable aftertaste is still present. Accordingly, the taste profiles of any *stevia* extracts are profoundly affected by the relative content of the steviol glycosides in the extract, which in turn may be affected by the environmental conditions experienced by the underlying plants and the extraction process used. These variations in plant production, weather conditions and extraction conditions can
20 lead to inconsistent compositions of the steviol glycosides in the *stevia* extracts, such that the taste profile varies strongly among different batches of extraction products.

[007] The taste profile of *stevia* extracts also can be affected by plant-derived or environment-derived contaminants (such as pigments, lipids, proteins, phenolics and saccharides) that remain in the product after the extraction process. These contaminants
25 typically have their own off-flavors undesirable for the use of the *stevia* extract as a sweetener in consumer products. In addition, the cost of isolating individual or specific combinations of steviol rebaudiosides that are not abundant in *stevia* extracts is cost and resource prohibitive. Given that there is a limited quality and availability of some specific steviol glycosides, commercial supply can be better addressed by bio-conversion, where natural enzymes, or
30 specific microbes can be modified to carry needed enzymes and use commercially significant fermentation processes to specifically increase the production of glycosides of interest. For example, bio-conversion of stevioside to Reb E has been reported previously (see, e.g., PCT Application Publication Nos. WO/2015/065650 and WO/2015/171555) using enzymes

obtained from modified microbes. Alternatively, other non-biologic synthetic means can be used to develop steviol glycosides of interest.

[008] From a biological perspective all steviol glycosides are formed by a series of glycosylation reactions of steviol, which typically are catalyzed by UDP-glycosyltransferase (UGT) enzymes 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. In these reactions stevioside is often an intermediate in the biosynthesis of various rebaudioside compounds. For example, glycosylation of stevioside at the C-3' at the C-13-O-glucose of stevioside yields rebaudioside A; while glycosylation at the C-2' at the 19-O-glucose position of stevioside yields rebaudioside E.

[009] As described herein, specific and directed glycosylation of rebaudioside E (at the C-19-O-glucose) can produce rebaudioside Reb D4 and further glycosylation of Reb D4 by UGT enzymes produces rebaudioside M. However, until the instant disclosure the synthetic steps for the production of D4 enzymatically had not been reported.

[0010] According to the current disclosure, a practical approach to improve the taste quality of *stevia* extracts is to increase the yield of those rebaudioside compounds that have more desirable taste characteristics in general and to do this *via* a more productive synthetic pathway. Of those steviol glycosides tested many believe that Reb M has the most desirable taste and chemical characteristics for use in food and beverages. As stated above, however, the plant has vanishingly small amounts of this compound present in its leaves and therefore an alternative biosynthetic needs to be developed for the large-scale production of this glycoside as well as to provide alternate sweeteners to the food and beverage industry.

[0011] Accordingly, there is a need for steviol glycosides with better and more consistent taste profiles to be developed as commercial products and for such steviol glycosides to utilize a relative common starting substrate, such as more abundant steviol glycosides as starting molecule, so that such production of desirable glycosides can be commercially as cost effective as possible. The present disclosure provides a method of producing rebaudioside M from a previously unknown steviol glycoside, Reb D4, as well as methods for producing Reb D4, Reb WB1 and Reb WB2.

[0012] Going further, the extraction process from plants, typically employs solid-liquid extraction techniques using solvents like hexane, chloroform, and ethanol for steviol glycoside recovery (Catchpole et al., 2003). However, solvent extraction is itself energy intensive, leads to problems of toxic waste disposal, requires extensive acreage for the plants themselves to be grown and yields a product that requires further purification for minor

constituents to be recovered. Thus, new production methods are also needed to reduce costs of steviol glycoside production and lessen the environmental impact of large scale cultivation and processing (Yao et al., 1994). One such potential solution is the use of fermentation bio-conversion technology that allows the production in certain microbial species that increases the 5 selectivity, abundance and purity of desired steviol glycosides available for commerce.

[0013] In addition to the above, while consumers approve and actively seek natural and biological sources for food, feed, flavor or medicinal components they are also concerned about sourcing, consistent taste profile and environmentally sustainable production. Into this situation the microbial fermentation and production methods of the current disclosure 10 provide Reb M in quantities useful for a variety of industries and research while doing so in a more natural fashion than inorganic synthesis or current plant extraction techniques.

[0014] Accordingly, a need exists for the development of a novel method of producing Reb M economically and conveniently to further enable human and animal consumption.

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SUMMARY OF THE INVENTION

[0015] Aspects of the disclosure relate to steviol glycosides, methods of producing the steviol glycosides, and compositions comprising the steviol glycosides. In some aspects, the present disclosure encompasses methods of producing Reb M from previously unreported 20 steviol glycoside Reb D4.

[0016] In particular, the current disclosure provides for the production of steviol glycoside rebaudioside D4 “Reb D4” which is identified as (13-[(2-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]) and its conversion to Reb M by a specific UDP- 25 glycosyltransferase (See Fig. 1). The present disclosure also provides for the production of Reb WB1 and Reb WB2 as described herein.

[0017] The current methods described herein provide an approach for the synthesis of specific steviol glycosides using synthetic pathways.

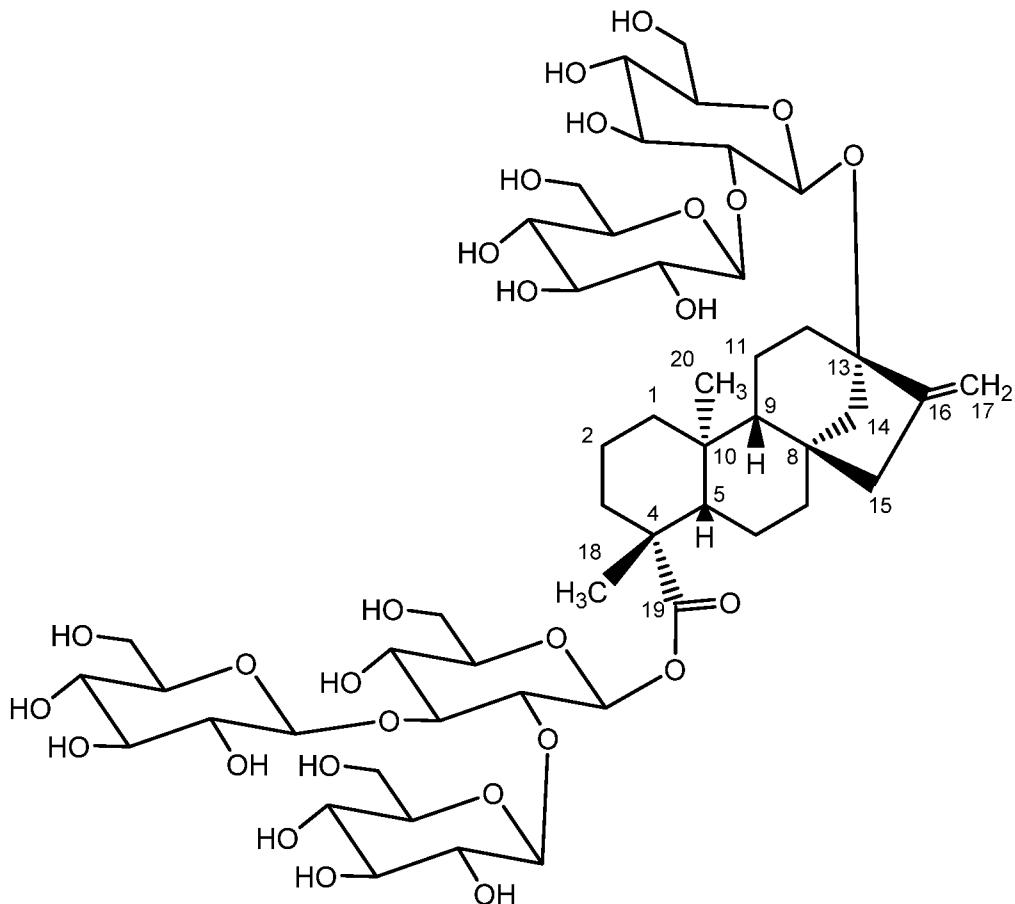
[0018] An alternative embodiment is producing rebaudioside D4 from 30 rebaudioside W utilizing a pathway through RebWB1.

[0019] A further embodiment is producing rebaudioside M from rebaudioside D4.

[0020] In one embodiment of the current disclosure, a method is provided that allows for the production of Reb M using a pathway through Reb WB2, Reb WB1 and Reb D4.

[0021] In an alternative embodiment, beta glucosidase is used to catalyze the enzymatic bioconversion of Reb W to Reb WB1, see FIG. 14.

[0022] In some aspects, the disclosure provides a steviol glycoside Reb D4 having the structure:

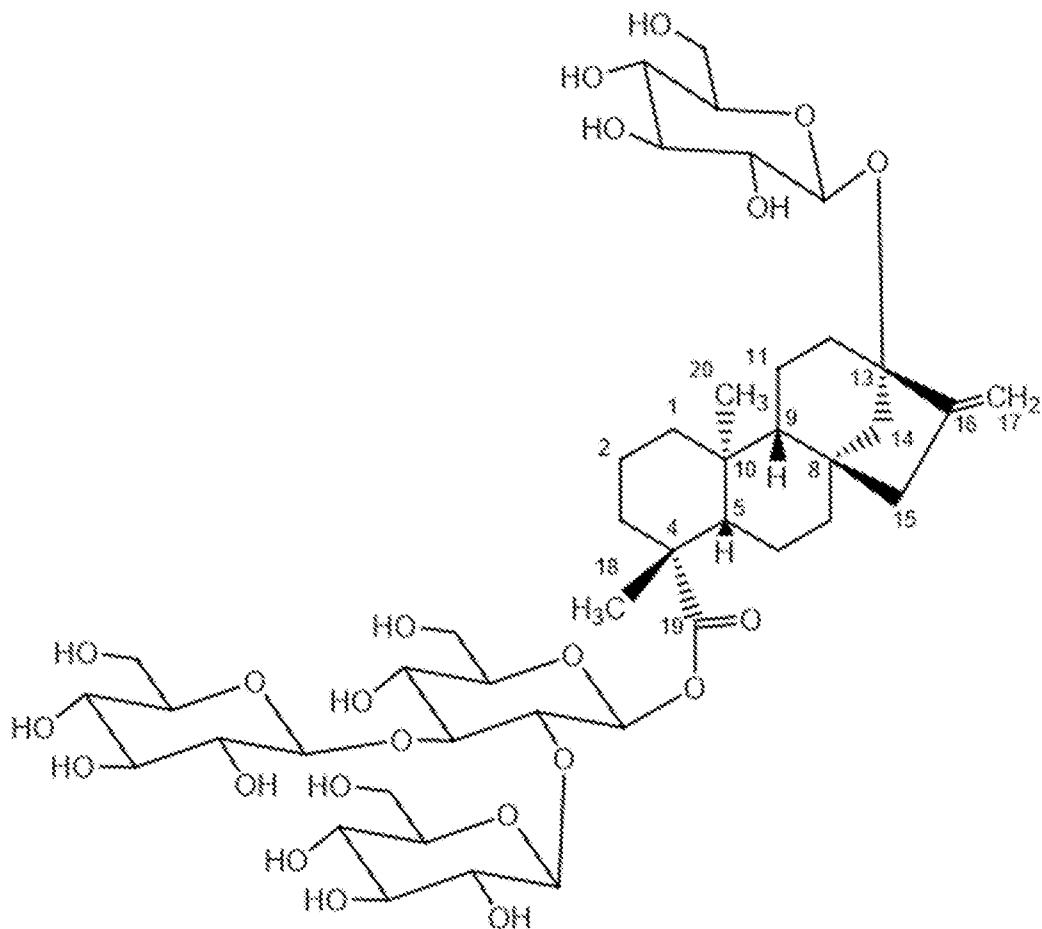


5 [0023] In some embodiments, the disclosure provides a composition comprising Reb D4, optionally wherein said Reb D4 content in the composition is at least 70% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100%) pure. In some embodiments, the disclosure provides a consumable product comprising a sweetening amount of Reb D4. In some embodiments, the consumable product is selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

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[0024] In other aspects, the disclosure provides a composition comprising a mixture of Reb D4 and Reb M. In some embodiments, the disclosure provides a consumable product comprising a sweetening amount of a mixture of Reb D4 and Reb M. In some embodiments, the consumable product is selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

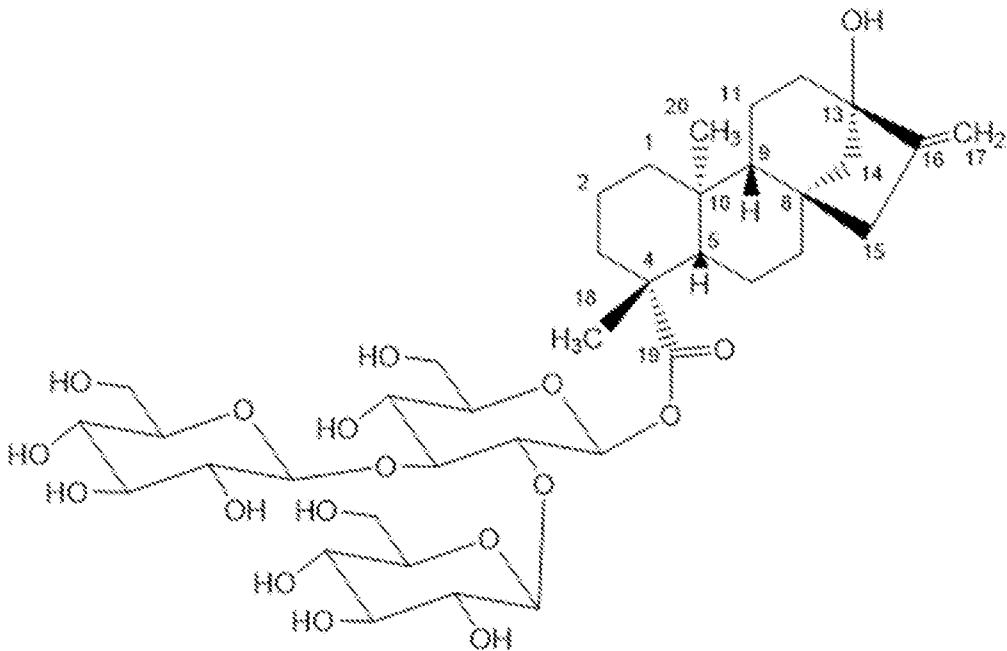
[0025] In yet other aspects, the disclosure provides a steviol glycoside Reb WB1 having the structure:



[0026] In some embodiments, the disclosure provides a composition comprising

5 Reb WB1, optionally wherein said Reb WB1 content in the composition is at least 70% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100%) pure. In some embodiments, the disclosure provides a consumable product comprising a sweetening amount of Reb WB1. In some embodiments, the consumable product is selected from the group consisting of beverages, 10 confectioneries, bakery products, cookies, and chewing gums.

[0027] In yet other aspects, the disclosure provides a steviol glycoside Reb WB2 having the structure:



[0028] In some embodiments, the disclosure provides a composition comprising Reb WB2, optionally wherein said Reb WB2 content in the composition is at least 70% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100%) pure. In some embodiments, the disclosure provides a consumable product comprising a sweetening amount of Reb WB2. In some embodiments, the consumable product is selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

[0029] In some aspects, the disclosure provides a steviol glycoside of interest produced by a transformed cellular system growing within a medium. In some embodiments, said transformed cellular system is selected from the group consisting of: yeast, non-steviol glycoside producing plants, algae and bacteria. In some embodiments, said cellular system is a bacteria and is selected from the group consisting of *Escherichia*; *Salmonella*; *Bacillus*; *Acinetobacter*; *Streptomyces*; *Corynebacterium*; *Methylosinus*; *Methylomonas*; *Rhodococcus*; *Pseudomonas*; *Rhodobacter*; *Synechocystis*; *Saccharomyces*; *Zygosaccharomyces*; *Kluyveromyces*; *Candida*; *Hansenula*; *Debaryomyces*; *Mucor*; *Pichia*; *Torulopsis*; *Aspergillus*; *Arthrobotrys*; *Brevibacteria*; *Microbacterium*; *Arthrobacter*; *Citrobacter*; *Escherichia*; *Klebsiella*; *Pantoea*; *Salmonella* *Corynebacterium*; *Clostridium*; and *Clostridium acetobutylicum*. In some embodiments, said cellular system is *E. Coli*. In some embodiments, said steviol glycoside is Reb D4. In some embodiments, said steviol glycoside is Reb WB1. In some embodiments, said steviol glycoside is Reb WB2. In some embodiments, the source

material is steviol. In some embodiments, said steviol glycoside content is at least 70% pure. In some embodiments, the method of production further comprises: i) purifying a crude product; and, ii) removing solvents under vacuum to provide a concentrated product. In some embodiments, said crude product is purified by column chromatography. In some 5 embodiments, said crude product is purified by acid-base extraction. In some embodiments, said crude product is purified by vacuum distillation. In some embodiments, the method of production further comprises purifying said steviol glycoside using a semi-preparative HPLC. In other aspects, the disclosure provides a consumable product comprising a sweetening amount of the steviol glycoside. In some embodiments, the consumable product is selected 10 from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

[0030] In other aspects, the disclosure provides a CP1 recombinant polypeptide comprising a DNA sequence having at least 80% (e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100%) identity to SEQ ID 15 NO:3. In some embodiments, the amino acid sequence of the CP1 recombinant polypeptide has at least 80% (e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100%) identity to SEQ ID NO:4. In some embodiments, the CP1 recombinant polypeptide has one or more mutations at one or more positions listed in Table 2.

20 [0031] In yet other aspects, the disclosure provides a biosynthetic method of making a steviol glycoside of interest comprising expressing an CP1 enzyme in a transformed cellular system; growing the cellular system in a medium containing a substrate; and producing the steviol glycoside of interest. In some embodiments, the method further comprises incubating a recombinant sucrose synthase with the substrate. In some embodiments, the 25 method further comprises incubating a recombinant UDP glycosyltransferase UGT85C2 with the sucrose synthase, the substrate, and the CP1 recombinant polypeptide. In some embodiments, the method further comprises adding a beta glucosidase enzyme to the reaction mixture. In some embodiments, the sucrose synthase is selected from the group consisting of an *Arabidopsis* sucrose synthase 1, an *Arabidopsis* sucrose synthase 3 and a *Vigna radiata* sucrose synthase. In some embodiments, the sucrose synthase is an *Arabidopsis thaliana* sucrose synthase 1. In some embodiments, the steviol glycosides produced are a mixture of Reb D4 and Reb M. In some embodiments, the method further comprises: i) purifying a crude product; and, ii) removing solvents under vacuum to provide a concentrated product. In some embodiments, said crude product is purified by column chromatography. In some 30

embodiments, said crude product is purified by acid-base extraction. In some embodiments, said crude product is purified by vacuum distillation. In some embodiments, the method further comprises purifying said steviol glycoside using a semi-preparative HPLC. In some embodiments, said steviol glycoside is Reb WB1. In some embodiments, said steviol glycoside is Reb WB2. In some embodiments, said steviol glycoside is Reb D4. In some embodiments, said steviol glycoside is Reb M. In some embodiments, the method further comprises the use of HV1 (SEQ ID NO:9). In some embodiments, the method further comprises the use of UGT76G1 (SEQ ID NO:1).

[0032] In other aspects, the disclosure provides a method of producing rebaudioside M, comprising cultivating a recombinant cell under suitable growth conditions where said recombinant cell exhibits the ability to produce steviol glycosides, the method comprising contacting said recombinant cell with a reaction composition containing stevioside, sucrose synthase and sucrose; wherein said recombinant cell expresses a first UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said 15 stevioside substrate to produce rebaudioside E; wherein said recombinant cell expresses a second UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said Rebaudioside E to produce rebaudioside D4; and, wherein said recombinant cell expresses a third UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said Rebaudioside D4 to produce rebaudioside M. In some embodiments, the 20 method further comprises a sucrose synthase gene or a catalytically active portion thereof being expressed in said recombinant cell. In some embodiments, the method further comprises a sucrose synthase being added to the reaction composition.

[0033] In some aspects, the disclosure provides Reb M produced by a method described in the above paragraph or as otherwise disclosed herein.

[0034] In other aspects, the disclosure provides a recombinant cell that expresses a biosynthetic pathway for producing Reb M (e.g., through conversion of Reb D4 to Reb M or through conversion of Reb E to Reb D4 to Reb M). In some embodiments, the cell expresses one or more of a first UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said stevioside substrate to produce rebaudioside E, a second UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said 25 Reb E to produce rebaudioside D4, and a third UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said Reb D4 to produce rebaudioside M. In some embodiments, the cell is a yeast cell. In some embodiments, the cell is a bacterial cell. In some embodiments, the cell is a plant cell.

[0035] In other aspects, the disclosure provides a method of producing Reb M using the enzymes and substrates described in FIG. 14, or a subset thereof (e.g., starting with Reb W, Reb WB1, or Reb D4 and/or utilizing UGT76G1, CP1 or CR1). In some embodiments, the Reb M is produced using an *in vitro* reaction mixture containing the 5 enzymes and substrates described in FIG. 14, or a subset thereof (e.g., starting with Reb W, Reb WB1, or Reb D4 and/or utilizing UGT76G1, CP1 or CR1). In some embodiments, the Reb M is produced *in vivo* in a cell that expresses the enzymes described in FIG. 14, or a subset thereof (e.g., UGT76G1, CP1 or CR1), wherein the cell is incubated with a substrate described in FIG. 14 (e.g., Reb W, Reb WB1, or Reb D4). In some embodiments, the cell is a 10 yeast cell. In some embodiments, the cell is a bacterial cell. In some embodiments, the cell is a plant cell.

[0036] In terms of product/commercial utility there are several dozen products containing steviol glycosides on the market in the United States and can be used in everything from analgesics to pest repellents as well as in foods and as a dietary supplement. Products 15 containing steviol glycosides can be aerosols, liquids, or granular formulations.

[0037] As for the cellular system in the embodiment, in some embodiments, it is selected from the group consisting of bacteria, yeast, and a combination thereof, or any cellular system that would allow the genetic transformation with the selected genes and thereafter the biosynthetic production of the desired steviol glycosides from steviol. In a most preferred 20 microbial system, *E. coli* are used to produce the desired steviol glycoside compounds.

[0038] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawing and will herein be described in detail. It should be understood, however, that the drawings and detailed description presented herein are not intended to limit the disclosure to the particular 25 embodiment disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present disclosure as defined by the appended claims.

[0039] Other features and advantages of this invention will become apparent in the following detailed description of preferred embodiments of this invention, taken with reference 30 to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] **FIG. 1.** Shows the structure of rebaudioside D4 (13-[(2-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]).

5 [0041] **FIG. 2.** Shows the structures of rebaudioside WB1 and WB2.

[0042] **FIG. 3.** Shows the HPLC profiles of the rebaudioside W hydrolysis products. Rebaudioside W is hydrolyzed by B-glu1 enzyme. A: Standard of rebaudioside W (“W”); B-D: Rebaudioside W was hydrolyzed by recombinant B-glu1 enzyme at 1 hour (B), 6 hours (C) and 24 hours (D).

10 [0043] **FIG. 4.** Shows the HPLC profile of the bioconversion of rebaudioside WB2 (“WB2”) to rebaudioside WB1 (“WB1”) by UGT85C2. Rebaudioside WB2 was incubated with UGT85C2 enzyme at 0 hour (A), 2 hours (B), 6 hours (C) and 18 hours (D).

[0044] **FIG. 5.** Shows the LC-MS analysis of rebaudioside WB1 and WB2.

15 [0045] **FIG. 6.** Shows the HPLC profiles of the bioconversion of rebaudioside WB1 to rebaudioside D4 by HV1 enzyme. A: Standard of rebaudioside WB1 (“WB1”); B-C: Rebaudioside WB1 was converted by HV1 enzyme at 2 hours (B) and 6 hours (C).

[0046] **FIGs. 7A and 7B.** Shows the structure and LC-MS data around the Reb D4 molecule.

20 [0047] **FIG. 8.** Shows the structure of the UDP enzyme UGT71G1. The standard orientation with Histidine located on the left and UDP on the right is shown.

[0048] **FIG. 9.** Shows the structure of the UGT76G1 enzyme, highlighting the alpha helices and beta sheets in the UGT76G1 structure.

[0049] **FIG. 10.** Shows a comparison of the CP1 and UGT76G1 enzymes. The UGT76G1 crystal structure is colored gray, while the CP1 model is colored black.

25 [0050] **FIG. 11.** Shows the UGT76G1 Crystal Structure and its interaction with the CP1 molecule. This crystal structure highlights the absence of beta sheets in the CP1 model. The UGT76G1 crystal structure is colored gray, while the CP1 model is colored black.

[0051] **FIG. 12.** Shows the Rebaudioside D4 in the reaction center of the enzyme CP1. The dark gray molecule located at the bottom of the image is rebaudioside D4.

30 [0052] **FIG. 13.** Shows the *in vitro* production of Reb M from Reb D4 catalyzed by a combination of a recombinant UGT76G1 polypeptide, a recombinant CP1, and a mutant (CR1). A: show the standards of rebaudioside D (“D”) and rebaudioside M (“M”). B: show the standard of rebaudioside D4 (“D4”). Reb M enzymatically produced by UGT76G1 at

30min (C) and 1hr (F), Reb M enzymatically produced by CP1 at 30min (D) and 1hr (G). Reb M enzymatically produced by CR1 at 30min (E) and 1hr (H).

[0053] **FIG. 14.** Shows the Synthetic Pathway for Rebaudioside M biosynthesis pathway from Rebaudioside W.

5 [0054] **FIG. 15** Shows key GHMBC correlations of Reb WB2.

[0055] **FIG. 16** Shows key GHMBC correlations of Reb WB1.

[0056] **FIG. 17** Shows key TOCSY and GHMBC correlations of Reb D4.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS THE INVENTION

10

Explanation of Terms Used Herein:

[0057] Steviol Glycosides are a class of chemical compounds responsible for the sweet taste of the leaves of the South American plant *Stevia rebaudiana* (Asteraceae), and can be used as sweeteners in food, feed and beverages.

15

Definitions:

[0058] **“Cellular system”** is any cells that provide for the expression of ectopic proteins. It included bacteria, yeast, plant cells and animal cells. It includes both prokaryotic and eukaryotic cells. It also includes the *in vitro* expression of proteins based on cellular components, such as ribosomes.

[0059] **“Coding sequence”** is to be given its ordinary and customary meaning to 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.

20 [0060] **“Growing the Cellular System”**. Growing includes providing an appropriate medium that would allow cells to multiply and divide. It also includes providing resources so that cells or cellular components can translate and make recombinant proteins.

25 [0061] **“Protein Expression”**. Protein production can occur after gene expression. It consists of the stages after DNA has been transcribed to messenger RNA (mRNA). The mRNA is then translated into polypeptide chains, which are ultimately folded into proteins.

30 DNA is present in the cells through transfection - a process of deliberately introducing nucleic acids into cells. The term is often used for non-viral methods in eukaryotic cells. It may also refer to other methods and cell types, although other terms are preferred: "transformation" is more often used to describe non-viral DNA transfer in bacteria, non-animal eukaryotic cells,

including plant cells. In animal cells, transfection is the preferred term as transformation is also used to refer to progression to a cancerous state (carcinogenesis) in these cells. Transduction is often used to describe virus-mediated DNA transfer. Transformation, transduction, and viral infection are included under the definition of transfection for this application.

5 [0062] **“Yeast”**. According to the current disclosure a yeast as claimed herein are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. Yeasts are unicellular organisms which evolved from multicellular ancestors but with some species useful for the current disclosure being those that have the ability to develop multicellular characteristics by forming strings of connected budding cells known as pseudo hyphae or false

10 hyphae.

15 [0063] **“UGT Enzymes”**. The names of the UGT enzymes used in the present disclosure are consistent with the nomenclature system adopted by the UGT Nomenclature Committee (Mackenzie et al., *"The UDP glycosyltransferase gene super family: recommended nomenclature updated based on evolutionary divergence,"* PHARMACOGENETICS, 1997, vol. 7, pp. 255-269), which classifies the UGT genes by the combination of a family number, a letter denoting a subfamily, and a number for an individual gene. For example, the name "UGT76G1" refers to a UGT enzyme encoded by a gene belonging to UGT family number 76 (which is of plant origin), subfamily G, and gene number 1.

20 **Structural Terms:**

25 [0064] As used herein, the singular forms "a, an" and "the" include plural references unless the content clearly dictates otherwise.

20 [0065] To the extent that the term "include," "have," or the like is used in the description or the claims, such term is intended to be inclusive in a manner similar to the term "comprise" as "comprise" is interpreted when employed as a transitional word in a claim.

25 [0066] The word "exemplary" is used herein to mean "serving as an example, instance, or illustration." Any embodiment described herein as "exemplary" is not necessarily to be construed as preferred or advantageous over other embodiments.

30 [0067] The term "complementary" is to be given its ordinary and customary meaning to 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, adenine 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

[0068] The terms "nucleic acid" and "nucleotide" are to be given their respective ordinary and customary meanings to 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.

[0069] The term "isolated" is to be given its ordinary and customary meaning to 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.

[0070] The terms "incubating" and "incubation" as used herein means 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.

[0071] 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.

[0072] The terms "polypeptide," "protein," and "peptide" are to be given their respective ordinary and customary meanings to 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.

5 [0073] The terms "polypeptide fragment" and "fragment," when used in reference to a reference polypeptide, are to be given 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
10 reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both.

15 [0074] 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).

20 [0075] 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.

25 [0076] 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;
30 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.

[0077] 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.

[0078] 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 super families 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.

[0079] "Suitable regulatory sequences" is to be given its ordinary and customary meaning to 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.

[0080] "Promoter" is to be given its ordinary and customary meaning to 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 tissues or 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

5 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.

[0081] 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.

10 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.

[0082] The term "expression" as used herein, is to be given its ordinary and customary meaning to 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.

20 [0083] "Transformation" is to be given its ordinary and customary meaning to a person of reasonable skill in the field, and is used without limitation to refer to the transfer of a polynucleotide into a target cell for further expression by that 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

25 chromosomal. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[0084] The terms "transformed," "transgenic," and "recombinant," when used herein in connection with host cells, are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a

30 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.

[0085] The terms "recombinant," "heterologous," and "exogenous," when used herein in connection with polynucleotides, are to be given their ordinary and customary meanings to 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.

[0086] 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.

[0087] The terms "plasmid," "vector," and "cassette" are to be given their respective ordinary and customary meanings to 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.

DETAILED DESCRIPTION

[0088] The present disclosure relates to the production of a steviol glycoside of interest, Reb D4 and then using UGT enzymes to allow Reb D4 glycoside to convert to Reb M. The present disclosure also relates to the production of other steviol glycosides of interest, Reb

5 WB1 and Reb WB2. The subject technology provides recombinant polypeptides with UDP glycosyltransferase activities, such as 1, 2-13-O-glucose glycosylation activity and 1, 3-13-O-glucose glycosylation activity for synthesizing steviol glycosides. The recombinant polypeptide of the subject technology is useful for the biosynthesis of steviol glycoside compounds. In the present disclosure, UDP-glycosyltransferase (UGT) refers to an enzyme 10 that transfers a sugar residue from an activated donor molecule (typically UDP-glucose) to an acceptor molecule. The 1,3-13-O-glucose glycosylation activity refers to an enzymatic activity that transfers a sugar moiety to the C-3' of the 13-O glucose moiety of rebaudioside D4 to produce Reb M (FIG. 14). The subject technology also provides recombinant polypeptides with beta-glucosidase activity for synthesizing steviol glycosides.

15

Synthetic Biology

[0089] Standard recombinant DNA and molecular cloning techniques used here 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 20 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 25 PROTOCOLS IN MOLECULAR BIOLOGY, published by GREENE PUBLISHING AND WILEY-INTERSCIENCE, 1987; (the entirety of each of which is hereby incorporated herein by reference).

[0090] 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 can be used in the practice or testing of the present disclosure, the preferred 30 materials and methods are described below.

[0091] The disclosure will be more fully understood upon consideration of the following non-limiting Examples. It should be understood that these Examples, while indicating preferred embodiments of the subject technology, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the

essential characteristics of the subject technology, and without departing from the spirit and scope thereof, can make various changes and modifications of the subject technology to adapt it to various uses and conditions.

[0092] Glycosylation is often considered a ubiquitous reaction controlling the 5 bioactivity and storage of plant natural products. Glycosylation of small molecules is catalyzed by a superfamily of transferases in most plant species that have been studied to date. These glycosyltransferases (GTs) have been classified into over 60 families. Of these, the family 1 GT enzymes, also known as the UDP glycosyltransferases (UGTs), transfer UDP-activated sugar moieties to specific acceptor molecules. These are the molecules that transfer such sugar 10 moieties in the steviol glycosides to create various rebaudiosides. Each of these UGTs have their own activity profile and preferred structure locations where they transfer their activated sugar moieties.

Production Systems

[0093] Expression of proteins in prokaryotes is most often carried out in a 15 bacterial host cell with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 20 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such vectors are within the scope of the present disclosure.

[0094] In an embodiment, the expression vector includes those genetic elements 25 for expression of the recombinant polypeptide in bacterial cells. The elements for transcription and translation in the bacterial cell can include a promoter, a coding region for the protein complex, and a transcriptional terminator.

[0095] A person of ordinary skill in the art will be aware of the molecular biology 30 techniques available for the preparation of expression vectors. The polynucleotide used for incorporation into the expression vector of the subject technology, as described above, can be prepared by routine techniques such as polymerase chain reaction (PCR).

[0096] A number of molecular biology techniques have been developed to operably link DNA to vectors *via* complementary cohesive termini. In one embodiment,

complementary homopolymer tracts can be added to the nucleic acid molecule to be inserted into the vector DNA. The vector and nucleic acid molecule are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

5 [0097] In an alternative embodiment, synthetic linkers containing one or more restriction sites provide are used to operably link the polynucleotide of the subject technology to the expression vector. In an embodiment, the polynucleotide is generated by restriction endonuclease digestion. In an embodiment, the nucleic acid molecule is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove 10 protruding, 3'-single-stranded termini with their 3'-5'-exonucleaseolytic activities, and fill in recessed 3'-ends with their polymerizing activities, thereby generating blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended 15 DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the product of the reaction is a polynucleotide carrying polymeric linker sequences at its ends. These polynucleotides are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the polynucleotide.

20 [0098] Alternatively, a vector having ligation-independent cloning (LIC) sites can be employed. The required PCR amplified polynucleotide can then be cloned into the LIC vector without restriction digest or ligation (Aslanidis and de Jong, NUCL. ACID. RES. 18 6069-74, (1990), Haun, et al, BIOTECHNIQUES 13, 515-18 (1992), which is incorporated herein by reference to the extent it is consistent herewith).

25 [0099] In an embodiment, in order to isolate and/or modify the polynucleotide of interest for insertion into the chosen plasmid, it is suitable to use PCR. Appropriate primers for use in PCR preparation of the sequence can be designed to isolate the required coding region of the nucleic acid molecule, add restriction endonuclease or LIC sites, place the coding region in the desired reading frame.

30 [00100] In an embodiment, a polynucleotide for incorporation into an expression vector of the subject technology is prepared by the use of PCR using appropriate oligonucleotide primers. The coding region is amplified, whilst the primers themselves become incorporated into the amplified sequence product. In an embodiment, the amplification primers contain restriction endonuclease recognition sites, which allow the amplified sequence product to be cloned into an appropriate vector.

[00101] The expression vectors can be introduced into plant or microbial host cells by conventional transformation or transfection techniques. Transformation of appropriate cells with an expression vector of the subject technology is accomplished by methods known in the art and typically depends on both the type of vector and cell. Suitable techniques include 5 calcium phosphate or calcium chloride co-precipitation, DEAE-dextran mediated transfection, lipofection, chemoporation or electroporation.

[00102] Successfully transformed cells, that is, those cells containing the expression vector, can be identified by techniques well known in the art. For example, cells transfected with an expression vector of the subject technology can be cultured to produce polypeptides 10 described herein. Cells can be examined for the presence of the expression vector DNA by techniques well known in the art.

[00103] The host cells can contain a single copy of the expression vector described previously, or alternatively, multiple copies of the expression vector,

[00104] In some embodiments, the transformed cell is an animal cell, an insect cell, 15 a plant cell, an algal cell, a fungal cell, or a yeast cell. In some embodiments, the cell is a plant cell selected from the group consisting of: canola plant cell, a rapeseed plant cell, a palm plant cell, a sunflower plant cell, a cotton plant cell, a corn plant cell, a peanut plant cell, a flax plant cell, a sesame plant cell, a soybean plant cell, and a petunia plant cell.

[00105] Microbial host cell expression systems and expression vectors containing 20 regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct vectors for expression of the recombinant polypeptide of the subjection technology in a microbial host cell. These vectors could then be introduced into appropriate microorganisms *via* transformation to allow for high level expression of the recombinant polypeptide of the subject technology.

[00106] Vectors or cassettes useful for the transformation of suitable microbial host 25 cells are well known in the art. Typically the vector or cassette contains sequences directing transcription and translation of the relevant polynucleotide, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the polynucleotide which harbors transcriptional initiation controls and a region 3' 30 of the DNA fragment which controls transcriptional termination. It is preferred for both control regions to be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a host.

[00107] Initiation control regions or promoters, which are useful to drive expression of the recombinant polypeptide in the desired microbial host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the subject technology including but not limited to CYCI, HIS3, GALI, GALIO, ADHI, PGK,

5 PH05, GAPDH, ADCI, TRPI, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOXI (useful for expression in *Pichia*); and lac, trp, JPL, IPR, T7, tac, and trc (useful for expression in *Escherichia coli*).

[00108] Termination control regions may also be derived from various genes native to the microbial hosts. A termination site optionally may be included for the microbial hosts
10 described herein.

[00109] In plant cells, the expression vectors of the subject technology can include a coding region operably linked to promoters capable of directing expression of the recombinant polypeptide of the subject technology in the desired tissues at the desired stage of development. For reasons of convenience, the polynucleotides to be expressed may comprise
15 promoter sequences and translation leader sequences derived from the same polynucleotide. 3' non-coding sequences encoding transcription termination signals should also be present. The expression vectors may also comprise one or more introns in order to facilitate polynucleotide expression.

[00110] For plant host cells, any combination of any promoter and any terminator
20 capable of inducing expression of a coding region may be used in the vector sequences of the subject technology. Some suitable examples of promoters and terminators include those from nopaline synthase (nos), octopine synthase (ocs) and cauliflower mosaic virus (CaMV) genes. One type of efficient plant promoter that may be used is a high level plant promoter. Such promoters, in operable linkage with an expression vector of the subject technology should be
25 capable of promoting the expression of the vector. High level plant promoters that may be used in the subject technology include the promoter of the small subunit (s) of the ribulose-1,5-bisphosphate carboxylase for example from soybean (Berry-Lowe et al., J. MOLECULAR AND APP. GEN., 1:483 498 (1982), the entirety of which is hereby incorporated herein to the extent it is consistent herewith), and the promoter of the chlorophyll binding protein. These two
30 promoters are known to be light-induced in plant cells (see, for example, GENETIC ENGINEERING OF PLANTS, AN AGRICULTURAL PERSPECTIVE, A. Cashmore, Plenum, N.Y. (1983), pages 29 38; Coruzzi, G. et al., The Journal of Biological CHEMISTRY, 258: 1399 (1983), and Dunsmuir, P. et al., JOURNAL OF MOLECULAR AND APPLIED GENETICS, 2:285

(1983), each of which is hereby incorporated herein by reference to the extent they are consistent herewith).

Precursor Synthesis to Reb D4

5 [00111] As previously stated steviol glycosides are the chemical compounds responsible for the sweet taste of the leaves of the South American plant *Stevia rebaudiana* (Asteraceae) and in the plant *Rubus chingii* (Rosaceae). These compounds are glycosylated diterpenes. Specifically, their molecules can be viewed as a steviol molecule, with its hydroxyl hydrogen atom replaced by a glucose molecule to form an ester, and a hydroxyl hydrogen with
10 combinations of glucose and rhamnose to form an acetal.

[00112] One method of making the compounds of interest in the current disclosure is to take common or inexpensive precursors such as steviol or rubosuside derived chemically or produced *via* biosynthesis in engineered microbes such as bacteria and/or yeast and to synthesize targeted steviol glycosides through known or inexpensive methods, such as Reb D4.

15 [00113] Aspects of the present disclosure relate to methods involving recombinantly expressing enzymes in a microbial system capable of producing steviol. In general, such enzymes may include: a copalyl diphosphate synthase (CPS), a kaurene synthase (KS) and a geranylgeranyl diphosphate to synthase (GGPPS) enzyme. This should occur in a microbial strain that expresses an endogenous isoprenoid synthesis pathway, such as the non-
20 mevalonate (MEP) pathway or the mevalonic acid pathway (MVA). In some embodiments the cell is a bacterial cell, including *E. coli*, or yeast cell such as a *Saccharomyces* cell, *Pichia* cell, or a *Yarrowia* cell. In some embodiments, the cell is an algal cell or a plant cell.

25 [00114] Thereafter, the precursor is recovered from the fermentation culture for use in chemical synthesis. Typically, this is steviol though it can be kaurene, or a steviol glycoside from the cell culture. In some embodiments, the steviol, kaurene and/or steviol glycosides is recovered from the gas phase while in other embodiments, an organic layer or polymeric resin is added to the cell culture, and the kaurene, steviol and/or steviol glycosides is recovered from the organic layer or polymeric resin. In some embodiments, the steviol glycoside is selected from rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F or dulcoside A. In some embodiments, the terpenoid produced is steviobioside or stevioside. It should also be appreciated that in some embodiments, at least one enzymatic step, such as one or more glycosylation steps, are performed *ex vivo*.

[00115] Part of the invention is the production of the Reb D4 steviol glycoside that is then subject to further enzymatic conversion to Reb M. According to the current disclosure

the biosynthesis for the conversion of microbially produced steviol to a desired steviol glycosides (here Reb D4) occurs when the diterpenoid steviol is converted to stevioside and rebaudioside A using multi-step chemical assembly of sugar moiety into the steviol backbone. More specifically the chemical synthesis consists of following steps: 1) A trimethylsilyl (TMS) 5 protected at C19 COOH group of the steviol is synthesized from the starting precursor steviol. Tri-glucosylation at the C13-OH position of the steviol is performed using protected β -Glc- β -Glc(2 \rightarrow 1)- β -Glc(3 \rightarrow 1) group. This is followed by a deprotection of the TMS and coupling of a protected mono β -Glc-Br moiety. The final deprotection removes all of the protecting groups to produce rebaudioside D4.

10

Biosynthesis of Steviol Glycosides

[00116] As described herein, the recombinant polypeptides of the present technology have UDP-glycosyltransferase activities and are useful for developing biosynthetic methods for preparing steviol glycosides that are not present or typically of low abundance in 15 natural sources, such as rebaudioside D4 and rebaudioside M respectively. The recombinant polypeptides of the present technology have beta-glucosidase or UDP-glycosyltransferase activities, are useful for developing biosynthetic methods for preparing novel steviol glycosides, such as rebaudioside D4 and useful in the production of rebaudioside M.

[00117] The substrate can be any natural or synthetic compound capable of being 20 converted into a steviol glycoside compound in a reaction catalyzed by one or more UDP glycosyltransferases. For example, the substrate can be natural *stevia* extract, steviol, steviol-13-O-glucoside, steviol-19-O-glucoside, steviol-1,2-bioside, rubusoside, stevioside, rebaudioside A, rebaudioside G or rebaudioside E. The substrate can be a pure compound or a mixture of different compounds. Preferably, the substrate includes a compound selected from 25 the group consisting of rubusoside, stevioside, steviol, rebaudioside A, rebaudioside E and combinations thereof.

[00118] The method described herein also provides a coupling reaction system in which the recombinant peptides described herein is allowed to function in combination with one or more additional enzymes to improve the efficiency or modify the outcome of the overall 30 biosynthesis of steviol glycoside compounds. For example, the additional enzyme may regenerate the UDP-glucose needed for the glycosylation reaction by converting the UDP produced from the glycosylation reaction back to UDP-glucose (using, for example, sucrose as a donor of the glucose residue), thus improving the efficiency of the glycosylation reaction.

[00119] In another embodiment, the method of the subject technology further includes incubating a recombinant UDP-glycosyltransferase with the recombinant sucrose synthase, the substrate, and the recombinant polypeptide described herein. The recombinant UDP-glycosyltransferase can catalyze a different glycosylation reaction than the one catalyzed by the recombinant polypeptide of the subject technology.

[00120] Suitable UDP-glycosyltransferase includes any UGT known in the art as capable of catalyzing one or more reactions in the biosynthesis of steviol glycoside compounds, such as UGT85C2, UGT74G1, UGT76G1, or the functional homologs thereof.

[00121] Typically, in the *in vitro* method of the subject technology, UDP-Glucose is included in the buffer at a concentration of from about 0.2 mM to about 5 mM, preferably from about 0.5 mM to about 2 mM, more preferably from about 0.7 mM to about 1.5 mM. In an embodiment, when a recombinant sucrose synthase is included in the reaction, sucrose is also included in the buffer at a concentration of from about 100 mM to about 500 mM, preferably from about 200 mM to about 400 mM, more preferably from about 250 mM to about 350 mM.

[00122] Typically, in the *in vitro* method of the subject technology, the weight ratio of the recombinant polypeptide to the substrate, on a dry weight basis, is from about 1:100 to about 1:5, preferably from about 1:50 to about 1:10, more preferably from about 1:25 to about 1:15.

[00123] Typically, the reaction temperature of the *in vitro* method is from about 20°C to about 40°C, suitably from 25° C to about 37° C, more suitably from 28° C to about 32° C.

[00124] One with skill in the art will recognize that the steviol glycoside composition produced by the method described herein can be further purified and mixed with other steviol glycosides, flavors, or sweeteners to obtain a desired flavor or sweetener composition. For example, a composition enriched with rebaudioside D4 produced as described herein can be mixed with a natural *stevia* extract containing rebaudioside A as the predominant steviol glycoside, or with other synthetic or natural steviol glycoside products to make a desired sweetener composition. Alternatively, a substantially purified steviol glycoside (e.g., rebaudioside D4) obtained from the steviol glycoside composition described herein can be combined with other sweeteners, such as sucrose, maltodextrin, aspartame, sucralose, neotame, acesulfame potassium, and saccharin. The amount of steviol glycoside relative to other sweeteners can be adjusted to obtain a desired taste, as known in the art. The steviol glycoside composition described herein (including rebaudioside D, rebaudioside E,

rebaudioside D4, rebaudioside M or a combination thereof) can be included in food products (such as beverages, soft drinks, ice cream, dairy products, confectioneries, cereals, chewing gum, baked goods, etc.), dietary supplements, medical nutrition, as well as pharmaceutical products.

5 [00125] One with skill in the art will recognize that the steviol glycoside composition produced by the method described herein can be further purified and mixed with other steviol glycosides, flavors, or sweeteners to obtain a desired flavor or sweetener composition. For example, a composition enriched with rebaudioside D4 produced as described herein can be mixed with a natural *stevia* extract containing rebaudioside A as the 10 predominant steviol glycoside, or with other synthetic or natural steviol glycoside products to make a desired sweetener composition. Alternatively, a substantially purified steviol glycoside (e.g., rebaudioside D4) obtained from the steviol glycoside composition described herein can be combined with other sweeteners, such as sucrose, maltodextrin, aspartame, sucralose, neotame, acesulfame potassium, and saccharin. The amount of steviol glycoside relative to 15 other sweeteners can be adjusted to obtain a desired taste, as known in the art. The steviol glycoside composition described herein (including rebaudioside D, rebaudioside E, rebaudioside D4, rebaudioside WB1, rebaudioside WB2, rebaudioside M or a combination thereof) can be included in food products (such as beverages, soft drinks, ice cream, dairy products, confectioneries, cereals, chewing gum, baked goods, etc.), dietary supplements, 20 medical nutrition, as well as pharmaceutical products.

Analysis of Sequence Similarity Using Identity Scoring

25 [00126] As used herein "sequence identity" refers to the extent to which two optimally aligned polynucleotide or peptide sequences are invariant throughout a window of alignment of components, e.g., nucleotides or amino acids. An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence.

30 [00127] As used herein, the term "percent sequence identity" or "percent identity" refers to the percentage of identical nucleotides in a linear polynucleotide sequence of a reference ("query") polynucleotide molecule (or its complementary strand) as compared to a test ("subject") polynucleotide molecule (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide insertions, deletions, or gaps totaling less

than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and preferably by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG® Wisconsin Package® (Accelrys Inc., Burlington, MA). An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more polynucleotide sequences may be to a full-length polynucleotide sequence or a portion thereof, or to a longer polynucleotide sequence. For purposes of this disclosure "percent identity" may also be determined using BLASTX version 2.0 for translated nucleotide sequences and BLASTN version 2.0 for polynucleotide sequences.

[00128] The percent of sequence identity is preferably determined using the "Best Fit" or "Gap" program of the Sequence Analysis Software Package™ (Version 10; Genetics Computer Group, Inc., Madison, WI). "Gap" utilizes the algorithm of Needleman and Wunsch (Needleman and Wunsch, JOURNAL OF MOLECULAR BIOLOGY 48:443-453, 1970) to find the alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. "BestFit" performs an optimal alignment of the best segment of similarity between two sequences and inserts gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (Smith and Waterman, ADVANCES IN APPLIED MATHEMATICS, 2:482-489, 1981, Smith et al., NUCLEIC ACIDS RESEARCH 11:2205-2220, 1983). The percent identity is most preferably determined using the "Best Fit" program.

[00129] Useful methods for determining sequence identity are also disclosed in the Basic Local Alignment Search Tool (BLAST) programs which are publicly available from National Center Biotechnology Information (NCBI) at the National Library of Medicine, National Institute of Health, Bethesda, Md. 20894; see BLAST Manual, Altschul et al., NCBI, NLM, NIH; Altschul et al., J. MOL. BIOL. 215:403-410 (1990); version 2.0 or higher of BLAST programs allows the introduction of gaps (deletions and insertions) into alignments; for peptide sequence BLASTX can be used to determine sequence identity; and, for polynucleotide sequence BLASTN can be used to determine sequence identity.

[00130] As used herein, the term "substantial percent sequence identity" refers to a percent sequence identity of at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity. Thus, one embodiment 5 of the disclosure is a polynucleotide molecule that has at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity with a polynucleotide sequence described herein. Polynucleotide molecules that have the activity of the Blu1 and CP1 genes of the current disclosure are capable of directing the 10 production of a variety of steviol glycosides and have a substantial percent sequence identity to the polynucleotide sequences provided herein and are encompassed within the scope of this disclosure.

Identity and Similarity

[00131] Identity is the fraction of amino acids that are the same between a pair of sequences after an alignment of the sequences (which can be done using only sequence information or structural information or some other information, but usually it is based on sequence information alone), and similarity is the score assigned based on an alignment using some similarity matrix. The similarity index can be any one of the following BLOSUM62, 15 PAM250, or GONNET, or any matrix used by one skilled in the art for the sequence alignment of proteins.

[00132] Identity is the degree of correspondence between two sub-sequences (no gaps between the sequences). An identity of 25% or higher implies similarity of function, while 18- 25% implies similarity of structure or function. Keep in mind that two completely 25 unrelated or random sequences (that are greater than 100 residues) can have higher than 20% identity. Similarity is the degree of resemblance between two sequences when they are compared. This is dependent on their identity.

Consumable Products

[00133] In another aspect, the present disclosure is directed to a consumable 30 product comprising a rebaudioside as described herein, e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M. In some embodiments, the consumable product comprises a sweetening amount of a rebaudioside as described herein, e.g., Reb W1, Reb W2, Reb D4, and/or Reb M. In some embodiments, the consumable

product is selected from the group consisting of a beverage product, 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.

[00134] In some embodiments, the consumable product can have a sweetness

5 intensity equivalent to about 1% (w/v-%) to about 4% (w/v-%) sucrose solution.

[00135] In some embodiments, the rebaudioside as described herein, e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M, is the only sweetener in the orally consumable product.

[00136] In some embodiments, the consumable product can also have at least one

10 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, 15 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, trilobtatin, and combinations thereof.

[00137] In some embodiments, the consumable product can also have at least one

20 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 25 combinations thereof.

[00138] In one aspect, the present disclosure is directed to a beverage product comprising a sweetening amount of a rebaudioside as described herein, e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M.

[00139] The beverage product can be, for example, a carbonated beverage product

30 or 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, or an energy drink.

[00140] 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 5 any suitable form, such as a beverage concentrate or a carbonated, ready-to-drink beverage.

[00141] 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 10 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 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 15 embodiments, the beverage product can be a cola beverage that contains water, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb 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 20 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.

25 [00142] In another aspect, the present disclosure is directed to a consumable product comprising a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M), wherein the consumable product is a food product, a nutraceutical, a pharmaceutical, a dietary supplement, a dental hygienic composition, an edible gel composition, a cosmetic product or a tabletop flavoring. In some 30 embodiments, the rebaudioside is present in a sweetening amount.

[00143] 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.

[00144] 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 5 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 10 may be solid formulations, such as capsules or tablets, or liquid formulations, such as solutions or suspensions.

[00145] 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, 15 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.

[00146] As used herein, a “gel” refers to a colloidal system in which a network of 20 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 25 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 30 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.

[00147] 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, alginic acid, 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.

[00148] 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 cross-linking agent, one or more flavors, one or more colors, and combinations thereof.

[00149] Any suitable pharmaceutical composition known in the art may be used. 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.

[00150] A rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) 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.

[00151] As used herein, “food product” 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.

[00152] Examples of suitable food products 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

5 meats and nut products; and animal feed.

[00153] Food products can also be herbs, spices and seasonings, natural and synthetic flavors, and flavor enhancers, such as monosodium glutamate. In some embodiments, food products can be, for example, prepared packaged products, such as dietetic sweeteners, liquid sweeteners, granulated flavor mixes, pet foods, livestock feed, tobacco, and

10 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 products can also be diet and low-calorie food and beverages containing little or no sucrose.

[00154] In certain embodiments that may be combined with any of the preceding embodiments, the rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or

15 a combination thereof such as Reb D4 and Reb M) is the only sweetener, optionally wherein 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, optionally wherein the product has a sweetness intensity equivalent to

20 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

25 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

30 dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside mogroside IV, siamenoside I, mogroside V, monatin, thaumatin, monellin, brazzein, L-alanine, glycine, Lo Han Guo, hernandulcin, phyllodulcin, trilobtatin, 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 as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) has a purity of about 50% to about 100% by weight before it is added into the product.

10 [00155] In some embodiments, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) is provided in a composition further comprising one or more of a filler, a bulking agent and an anticaking agent. Suitable fillers, bulking agents and anticaking agents are known in the art.

15 [00156] In certain embodiments, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) 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 the rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) present in the final consumable products and beverage 20 products (i.e., after all ingredients and/or compounds have been added to produce the consumable products and beverage products). Accordingly, in certain embodiments, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) is included and/or added to a compound or ingredient used to prepare the consumable products and beverage products. The rebaudioside as described 25 herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) may be present in a single compound or ingredient, or multiple compounds and ingredients. In some embodiments, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) included and/or added to the consumable products and beverage products.

30 [00157] In certain embodiments, a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, Reb M, or a combination thereof such as Reb D4 and Reb M) is the only sweetener included and/or added to the consumable products and the beverage products. In some embodiments, the consumable products and the beverage products comprising the rebaudiosides 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 5 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.

[00158] The consumable products and beverage products of the present disclosure can include a mixture of a rebaudioside as described herein (e.g., Reb W1, Reb W2, Reb D4, 10 Reb M, or a combination thereof such as Reb D4 and Reb M) 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.

15 [00159] As is evident from the foregoing description, certain aspects of the present disclosure are not limited by the particular details of the examples illustrated herein, and it is therefore contemplated that other modifications and applications, or equivalents thereof, will occur to those skilled in the art. It is accordingly intended that the claims shall cover all such 20 modifications and applications that do not depart from the spirit and scope of the present disclosure.

[00160] Moreover, 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 or those described herein can be used in the practice or testing of the present disclosure, the 25 preferred methods and materials are described above.

[00161] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by 30 the appended claims.

EXAMPLE 1: Enzymatic Synthesis of Reb D4

[00162] There are several enzymatic methods of making Reb D4. One of the method starting from Reb W is presented here.

[00163] Previously, we demonstrated the production of Reb W from Reb V (WO2016054540). Here, we found Reb W can be hydrolyzed by beta-glucosidase (B-glu1, SEQ: 5) from *Pichia pastoris* to produce a novel steviol glycoside we called it “Rebaudioside WB1”. The produced Rebaudioside WB1 can be hydrolyzed in turn by B-glu1 to produce Rebaudioside WB2. (see, FIG. 14).

[00164] More specifically, the full-length DNA fragments of B-glu1 (SEQ ID NO: 6) gene was synthesized. Specifically, the cDNA was 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). The nucleotide sequence (SEQ ID NO: 6) encoding the B-glu1 (see, SEQ ID NO:5) was inserted in frame.

[00165] The 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 OD600 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.

[00166] 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). The supernatant was loaded to an 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 B-glu1 recombinant polypeptide was eluted by equilibration buffer containing 250mM imidazole.

[00167] The recombinant B-glu1 (10 µg) was added in a 200 µL *in vitro* reaction system. The reaction system contained 50 mM potassium phosphate buffer, pH 7.2, and 1 mg/ml Rebaudioside W as the substrate. The reaction was performed at 37 °C and terminated by adding 200 µL of 1-butanol. The samples were extracted three times with 200 µL of 1-butanol. The pooled fraction was dried and dissolved in 70 µL of 80% methanol for high-performance liquid chromatography (HPLC) analysis.

[00168] 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. Synergi Hydro-RP column with guard column was used for the characterization of steviol glycosides. Acetonitrile in water was used for 5 elution in HPLC analysis. The detection wavelength was 210nm.

[00169] As shown in FIG. 3, B-glu1 hydrolyzed rebaudioside W substrate to produce rebaudioside WB1 at 1 hour (Fig. 3B). The produced rebaudioside WB1 can be further converted to rebaudioside WB2 at later reaction time points (Fig. 3C and 3D).

[00170] In conclusion, Rebaudioside W was hydrolyzed by B-glu1 to produce 10 WB1, the produced WB1 was further hydrolyzed by B-glu1 to produce WB2.

[00171] The above intermediate rebaudioside WB2 can be converted back to rebaudioside WB1 by incubating with UGT85C2 enzyme (FIG. 4). The recombinant UGT85C2 enzyme (10 μ g) was tested in a 200 μ L *in vitro* reaction system. The reaction system contained 50 mM potassium phosphate buffer, pH 7.2, 3 mM MgCl₂, 0.5 mg/ml 15 rebaudioside WB2 substrate, and 3 mM UDP-glucose. As shown in FIG. 3, rebaudioside WB2 can be converted to rebaudioside WB1 by UGT85C2 (SEQ ID NO: 7, FIG. 4). UGT85C2 enzyme has activity to form steviol-13-monoside from steviol adding a glucose to C-13 of C4 carboxyl. These results indicated that B-glu1 hydrolyzed a glucose from C13 position of rebaudioside WB1 to produce rebaudioside WB2. The predicted structures of rebaudioside 20 WB1 and rebaudioside WB2 are shown in FIG. 2. The structures were confirmed by LC-MS analysis (FIG. 5).

[00172] The above intermediate Reb WB1 can be converted to Reb D4 by incubating with HV1 UGT enzyme (WO/2015/065650). The recombinant HV1 (10 μ g) was tested in a 200 μ L *in vitro* reaction system. The reaction system contained 50 mM potassium 25 phosphate buffer, pH 7.2, 3 mM MgCl₂, 0.5 mg/ml rebaudioside WB1 substrate, and 3 mM UDP-glucose. As shown in FIG. 4, rebaudioside WB1 can be converted to rebaudioside D4 by HV1 completely at 6 hours (FIG. 6 C).

[00173] According to above enzymatic reactions, the structure of D4 was predicted as (13-[(2-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] (FIG. 7 A). The 30 structure of Reb D4 was confirmed by LC-MS (FIG. 7 B). Mass spectral analysis showed the same mass [(M+Na) 1151.47 m/z] as the predicated structure.

EXAMPLE 2: Converting Reb D4 to Reb M by wild type enzyme

[00174] We discovered that the Reb D4 can be further converted to Reb M by UGT76G1. The full-length DNA fragments of UGT76G1 (SEQ ID NO: 2) was synthesized. The cDNA was codon optimized for *E. coli* expression (Genscript). The synthesized DNA was 5 cloned into a bacterial expression vector pETite N-His SUMO Kan Vector (Lucigen). The nucleotide sequence encoding the 76G1 was inserted in frame.

[00175] The 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 OD600 of 0.8-1.0. Protein expression was induced by addition of 0.5 mM IPTG and the 10 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.

[00176] The cell pellets were re-suspended in lysis buffer as described above. The cells were disrupted by sonication at 4 °C, and the cell debris was clarified by centrifugation 15 (18,000 x g; 30 min). The supernatant was loaded to an equilibrated Ni-NTA (Qiagen) affinity column as described above. After loading of protein sample, the column was washed with equilibration buffer to remove unbound contaminant proteins. The His-tagged 76G1 recombinant polypeptide was eluted by equilibration buffer containing 250 mM imidazole.

[00177] The recombinant UGT76G1 (10 µg) was added in a 200 µL *in vitro* 20 reaction system. The reaction system contained 50 mM potassium phosphate buffer, pH 7.2, 1 mM UDPG as co-factor, and 1 mg/ml Reb D4 as the substrate. The reaction was performed at 37 °C and terminated by adding 200 µL of 1-butanol. The samples were extracted three times with 200 µL of 1-butanol. The pooled fraction was dried and dissolved in 70 µL of 80% methanol for high-performance liquid chromatography (HPLC) analysis.

[00178] HPLC analysis was performed using a Dionex UPLC ultimate 3000 25 system, including a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Synergi Hydro-RP column with guard column was used for the characterization of steviol glycosides. Acetonitrile in water was used for elution in HPLC analysis. The detection wavelength was 210nm.

[00179] As shown in FIG. 13, UGT76G1 can convert Reb D4 to Reb M (FIG. 13 C; 30 and F).

EXAMPLE 3: Resolving the reaction centers of UGT enzymes catalyzing**Reb D4 to Reb M**

[00180] To more efficiently determine the chemical processes of the UDP-glucosyl transferase we obtained the crystal structure of a wild type steviol UDP-glucosyl transferase 5 UGT76G1. This enzyme is the first reported enzyme that carry out steviol glycoside bio- conversions. We want to acquire the structural information of the reaction center and substrate binding sites to design enzymes for Reb D4 to Reb M conversion to understand it more completely and thereafter use this knowledge to find more efficiently find or design enzymes that can be useful in the Reb D4 to Reb M bioconversion.

10 [00181] For production of selenomethionine (SeMet)-substituted protein, *Escherichia coli* BL21 (DE3) cells were transformed with the pET-28a-UGT76G1 vector and grown in M9 minimal media supplemented with SeMet (Doublie, 2007) containing 50ug mL⁻¹ kanamycin at 37 °C (250 rpm) until A600nm~0.8. Addition of isopropyl 1-thio-β-D- galactopyranoside (0.8 mM final) induced protein expression with cells grown overnight (16 15 °C). Cell pellets were harvested by centrifugation (10,000 x g; 10 min) and suspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol (β- ME), 10% (v/v) glycerol, and 1% (v/v) Tween-20). Following lysis by sonication, cell debris was removed by centrifugation (30,000 x g; 45 min) and the supernatant passed over a Ni²⁺- nitriloacetic acid (NTA; Qiagen) column equilibrated with wash buffer (lysis buffer minus 20 Tween-20). After loading, the column was washed with 10 column volumes of wash buffer. Bound fusion protein was eluted with elution buffer (wash buffer with 250 mM imidazole) and collected. For further purification, size-exclusion chromatography was performed on a Superdex-200 26/60 HiLoad FPLC column equilibrated with 50 mM Tris, pH 8.0, 25 mM NaCl, 1 mM tris(2-carboxyethyl) phosphine (TCEP). Peak fractions were collected and 25 concentrated using centrifugal concentrators (Amicon) with protein concentration determined using the Bradford assay with bovine serum albumin as the standard. Purified protein was flash-frozen in liquid nitrogen and stored at -80 °C.

[00182] Purified UGT761 was concentrated to 10 mg mL⁻¹ and crystallized using the hanging-drop vapor-diffusion method with a 2 µl drop (1:1 concentrated protein and 30 crystallization condition). Diffraction quality crystals were obtained at 4 °C with 20% (w/v) PEG-4000, 20% 2-propanol (v/v), and 100 mM sodium citrate tribasic dihydrate buffer (pH 5.6). Individual crystals were flash-frozen in liquid nitrogen with the mother liquor containing 25% glycerol as a cryoprotectant. Diffraction data (100 K) was collected at the Argonne National Laboratory Advanced Photon Source 19-ID beamline (λ=0.98 Å). HKL3000

(Otwinowski & Minor, 1997) was used to index, integrate, and scale diffraction data. The structure of SeMet-substituted UGT76G1 was determined by single-wavelength anomalous diffraction (SAD) phasing. SHELX (Sheldrick, 2008) was used to determine SeMet positions and to estimate initial phases from the peak wavelength data set. Refinement of SeMet 5 positions and parameters was performed with MLPHARE (Terwilliger, 2000). Solvent flattening using density modification implemented with ARP/wARP (Morris et al., 2003) was employed to build an initial model. Subsequent iterative rounds of manual model building and refinement, which included translation-libration-screen parameter refinement, used COOT (Emsley et al., 2010) and PHENIX (Adams et al., 2007), respectively. Data collection and 10 refinement data are summarized in Table 1.

Table 1. Summary of crystallographic statistics

| <u>Data Collection</u> | AtGH3.5•AMP•IAA |
|--|--|
| Space group | P2 ₁ |
| Cell dimensions | $a = 91.61 \text{ \AA}$, $b = 143.5 \text{ \AA}$, $c = 102.3 \text{ \AA}$; $\beta = 114.7^\circ$ |
| Wavelength (Å) | 0.979 |
| Resolution (Å) (highest shell) | 42.2 - 2.20 (2.25 - 2.20) |
| Reflections (total/unique) | 254,788 / 108,566 |
| Completeness (highest shell) | 89.5% (77.3%) |
| $\langle I/\sigma \rangle$ (highest shell) | 11.2 (2.0) |
| R_{sym} (highest shell) | 9.3% (51.8%) |
| <u>Refinement</u> | |
| R_{sym} / R_{free} | 20.5% / 24.5% |
| No. of protein atoms | 18,186 |
| No. of waters | 1,002 |
| No. of ligand atoms | 154 |
| R.m.s.d., bond lengths | 0.008 |
| R.m.s.d., bond angles (°) | 1.167 |
| Avg. B-factor (Å ²): protein, water, ligand | 30.7, 28.3, 26.4 |
| Stereochemistry: most favored, allowed, disallowed | 97.3, 2.5, 0.2% |

[00183] The structure of UGT71G1 consists of a N-terminal domain and C-terminal domain with similar Rossmann-type folds and, as predicted, belongs to the GT-B fold (FIG. 8). The standard orientation of the UGT76G1 crystal structure is shown in FIG. 8. The N-terminal domain contains a central seven-stranded parallel β sheet flanked by eight α helices. The 5 domain also contains the catalytic histidine. The C-terminal domain contains a six-stranded β sheet flanked by seven α helices (FIG. 9). The two domains pack very tightly and form a deep cleft with a UDP molecule is bound.

EXAMPLE 4: Rational Design of Mutants

[00184] Based on UGT76G1 structure, we were able to design the circular permutations (PLoS computational Biology, 2012, 8(3) e1002445; BIOINFORMATICS, 2015, (3) and a set of mutations. Circular permutation analysis is a powerful tool to develop useful or valuable enzymes. After test several version of circular permutations, we found one version of circular mutation with very high activity “circular permutation 1” (“CP1”) has the highest 15 activity. According to the current disclosure, we studied the activity of CP1 (SEQ ID NO: 3) enzyme and its ability to assist the conversions of Reb D4 to Reb M.

[00185] The structure of UGT76G1 and CP1 is compared in FIG. 10. Though the majority of the structural features of the UGT76G1 crystal structure are similar as between UGT and CP1 the CP1 has significantly different sequence and structure in the “beta sheets” 20 portion of its structure (FIG. 11).

[00186] In order to predict the catalytic activity of our enzymes, we docked the Reb D4 into the reaction center of CP1. We highlighted the reaction center focusing on the interactions of the enzyme with Reb D4. The docked rebaudioside D4 ligand is in a favorable position relative to the catalytic histidine and the bound UDP (FIG. 12). Based on this docking 25 experiment, we were able to find specific residues worth testing activity *via* mutagenesis studies.

[00187] Based on the CP1 modeling analysis, we selected and tested multiple mutation sites of CP1 to increase enzymatic activity. Finally, we found several mutation sites (Table 2) related to bioconversion of rebaudioside D4 to rebaudioside M. CR1 is a kind of CP1 30 mutant includes at least one mutation site in the Table 2.

Table 2. Summary of mutation sites of CP1.

| Position | Amino acid |
|----------|---------------|
| 3 | W-L |
| 6 | L-A, L-G |
| 90 | T-A; T-G |
| 91 | S-G; S-L |
| 93 | V-A; V-G |
| 181 | S-G |
| 183 | F-V; F-A; F-G |
| 184 | G-A |
| 185 | L-A |
| 350 | G-A |
| 389 | L-V |
| 410 | S-G |
| 418 | H-V |
| 450 | T-A; T-G |
| 451 | K-A |
| 452 | D-A |
| 454 | K-L; K-V |

EXAMPLE 5: Converting Reb D4 to Reb M by use of mutant UGTs.

[00188] In this Example, to confirm the conversion of Reb D4 to rebaudioside M *in vitro*, the UGT76G1, CP1 and enzyme mutants were assayed using Reb D4 as the steviol glycoside substrate. The recombinant polypeptide (10 µg) was tested in a 200 µL *in vitro* reaction system. The reaction system contained 50 mM potassium phosphate buffer, pH 7.2, 3 mM MgCl₂, 1 mg/ml steviol glycoside substrate, and 1 mM UDP-glucose. The reaction was performed at 30 °C and terminated by adding 200 µL of 1-butanol. The samples were extracted three times with 200 µL of 1-butanol. The pooled fraction was dried and dissolved in 70 µL of 80% methanol for high-performance liquid chromatography (HPLC) analysis. Rebaudioside D4 were used as substrate. 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. Synergi Hydro-RP

column with guard column was used for the characterization of steviol glycosides. Acetonitrile in water was used for elution in HPLC analysis. The detection wavelength was 210nm.

[00189] As shown in FIG. 13, UGT76G1, CP1 and CR1 mutant can transfer one glucose molecule to Reb D4 to form Reb M. However, CP1 and CR1 has significantly higher 5 enzymatic activity than UGT76G1 enzyme.

EXAMPLE 6: The structure of Reb WB2 analyzed by NMR.

[00190] The material used for the characterization of Reb WB2 was produced by using enzymatic conversion of Reb W and purified by HPLC. NMR spectra were acquired on 10 Agilent VNMRS 500 MHz instrument instruments using standard pulse sequences. The 1D (¹H and ¹³C) and 2D (TOCSY, ASAPHMQC, GCOSY and GHMBC) NMR spectra were performed in CD3OD.

[00191] The molecular formula of Reb WB2 has been deduced as C₃₈H₆₀O₁₈ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ions 15 corresponding to [M+ Na]⁺ at *m/z* 827.3671; this composition was supported by the NMR spectral data.

[00192] The NMR spectral data of Reb WB2 revealed the basic skeleton of *ent*-kaurane diterpenoids and was further confirmed by the GHMBC, COSY and TOCSY experiments. Carbon multiplicities were confirmed using the APT test. The ¹³C NMR showed 20 3 anomeric carbons (δ 102.8, 101.7, and 92.46) as well as three –CH₂OH signals at δ 62.2, 61.14 and 60.88 confirming the 3 sugar units. Also present were one carbonyl resonance at δ 177.1 and two alkene carbons at δ 152.2 and 104.4. GHMBC correlations from H₂₁ to C₁₉ confirmed the attachment points of the sugars to the diterpenoid core structure. The Chemical shift of C₁₃ at δ 79.4 indicates an oxygen attached to this carbon. The ¹H and ¹³C NMR values 25 for Reb WB2 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 WB2^{a-c}. ^a assignments made on the basis of TOCSY, ASAPHMQC, and GHMBC correlations; ^bChemical shift values are in δ (ppm); ^c Coupling constants are in Hz.

| C# | C13 δ | 1H δ (ppm), multp, J (Hz) |
|----|--------------|---|
| 1 | 40.3 | 1.89m / 0.85 dt, <i>J</i> 12.8, 4.0 |
| 2 | 19.0 | 1.96 m / 1.43 m |
| 3 | 37.2 | 2.39 bd, <i>J</i> 13.7 / 1.02 dd, <i>J</i> 13.6, 4.2 |
| 4 | 43.8 | - |
| 5 | 57.2 | 1.09 dd, <i>J</i> 10.6, 3.8 |
| 6 | 21.4 | 1.87 m |
| 7 | 41.3 | 1.56 m / 1.45 m |
| 8 | 41.3 | - |
| 9 | 53.9 | 0.99 m |
| 10 | 39.2 | - |
| 11 | 39.2 | 1.76 m / 1.47 m |
| 12 | 19.9 | 1.73 m / 1.60 m |
| 13 | 79.4 | - |
| 14 | 46.2 | 2.05 s / 1.30 m |
| 15 | 47.4 | 2.18 m / 2.08 m |
| 16 | 155.5 | - |
| 17 | 102.1 | 4.95 s / 4.78 s |
| 18 | 28.0 | 1.24 s |
| 19 | 176.1 | - |
| 20 | 15.5 | 0.92 s |
| 21 | 92.5 | 5.58 d, <i>J</i> 8.1 |
| 22 | 77.0 | 3.43 m |
| 23 | 68.2 | 3.56 m |
| 24 | 86.6 | 3.86 m |
| 25 | 75.7 | 4.03 t, <i>J</i> 8.5 |
| 26 | 60.9 | 3.83 dd, <i>J</i> 12.7, 2.7 / 3.72 dd, <i>J</i> 12.1, 4.6 |
| 27 | 102.8 | 4.69 d, <i>J</i> 7.8 |
| 28 | 70.2 | 3.30 m |
| 29 | 74.0 | 3.28 m |
| 30 | 76.6 | 3.37 m |
| 31 | 76.8 | 3.38 m |
| 32 | 61.1 | 3.91 m / 3.64 m |
| 33 | 101.7 | 5.01 d, <i>J</i> 7.9 |
| 34 | 71.2 | 3.18 m |
| 35 | 76.7 | 3.36 m |
| 36 | 76.7 | 3.33 m |
| 37 | 74.4 | 3.12 m |
| 38 | 62.1 | 3.89 m / 3.67 m |

[00193] Key GHMBC correlations between H33 to C25, H27 to C24 confirmed the connectivity of the 3 sugar molecules. Based on all the observed 2D correlations and the chemical shift signals the structure of Reb WB2 as that shown in FIG. 15. The structure of Reb WB2 was deduced as 13-hydroxy-ent-kaur-16-en-19-oic acid-[(2-O- β -D-glucopyranosyl-3-O-
5 β -D-glucopyranosyl- β -D-glucopyranosyl) ester.

EXAMPLE 7: The structure of Reb WB1 analyzed by NMR.

[00194] The material used for the characterization of Reb WB1 was produced by using enzymatic conversion of Reb W and purified by HPLC. NMR spectra were acquired on 10 Agilent VNMRS 500 MHz instrument instruments using standard pulse sequences. The 1D (1 H and 13 C) and 2D (TOCSY, ASAPHMQC, GCOSY and GHMBC) NMR spectra were performed in 80% CD3OD – 20% D2O.

[00195] The molecular formula of Reb WB1 has been deduced as C₄₄H₇₀O₂₃ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ions 15 corresponding to [M+ Na]⁺ at *m/z* 989.4206; this composition was supported by the NMR spectral data.

[00196] The NMR spectral data of Reb WB1 revealed the basic skeleton of *ent*-kaurane diterpenoids and was further confirmed by the GHMBC, COSY and TOCSY experiments. Carbon multiplicities were confirmed using the APT test. The 13 C NMR showed 20 4 anomeric carbons (δ 102.6, 101.6 97.6 and 92.61 as well as four –CH₂OH signals at δ 62.0, 61.1, 61.0 and 60.8 confirming the 4 sugar units. Also present were one carbonyl resonance at δ 177.0 and two alkene carbons at δ 152.5 and 104.4. GHMBC correlations from H21 to C19 and H39 to C13 confirmed the attachment points of the sugars to the diterpenoid core structure. The 1 H and 13 C NMR values for Reb WB1 were assigned on the basis of TOCSY, HMQC and 25 HMBD data and are given in Table 4.

Table 4. 1 H and 13 C NMR spectral data (chemical shifts and coupling constants) for Reb WB1^{a-c}. ^a assignments made on the basis of TOCSY, ASAPHMQC, and GHMBC correlations; ^bChemical shift values are in δ (ppm); ^c Coupling constants are in Hz.

| C# | C13 δ | 1H δ (ppm), multp, J (Hz) |
|----|--------------|----------------------------------|
| 1 | 40.2 | 1.86 m / 0.83 m |
| 2 | 19.0 | 1.93 m / 1.41 m |
| 3 | 37.1 | 2.35 d, <i>J</i> 13.8 / 1.00 m |
| 4 | 44.0 | - |

| | | |
|----|-------|--------------------------------------|
| 5 | 51.1 | 1.09 m |
| 6 | 21.5 | 1.89 m |
| 7 | 41.1 | 1.57 m / 1.44 m |
| 8 | 41.7 | - |
| 9 | 53.7 | 0.97 m |
| 10 | 39.2 | - |
| 11 | 19.9 | 1.81 m / 1.62 m |
| 12 | 37.3 | 1.97 m / 1.50 m |
| 13 | 86.7 | - |
| 14 | 43.9 | 2.18 m / 1.54 m |
| 15 | 47.6 | 2.15 m / 2.05 m |
| 16 | 152.5 | - |
| 17 | 104.4 | 5.17 s / 4.88 s |
| 18 | 28.1 | 1.23 s |
| 19 | 177.0 | - |
| 20 | 15.5 | 0.91 s |
| 21 | 92.6 | 5.55d, <i>J</i> 7.9 |
| 22 | 85.9 | 3.72 m |
| 23 | 68.2 | 3.57 m |
| 24 | 85.9 | 3.91 m |
| 25 | 75.9 | 4.05 m |
| 26 | 61.1 | 3.90 m / 3.72 dd, <i>J</i> 12.3, 4.7 |
| 27 | 102.6 | 4.72 d, <i>J</i> 8.0 |
| 28 | 76.4 | 3.42 m |
| 29 | 76.6 | 3.45 m |
| 30 | 76.6 | 3.48 m |
| 31 | 70.0 | 3.34 m |
| 32 | 61.9 | 3.90 m / 3.64 m |
| 33 | 101.6 | 4.96 m |
| 34 | 74.2 | 3.16 m |
| 35 | 76.5 | 3.43 m |
| 36 | 76.1 | 3.23 m |
| 37 | 73.8 | 3.30 m |
| 38 | 60.8 | 3.83 m / 3.65 m |
| 39 | 97.6 | 4.54 d, <i>J</i> 7.8 |
| 40 | 76.8 | 3.50 m |
| 41 | 73.8 | 3.31 m |
| 42 | 76.4 | 3.40 m |
| 43 | 71.0 | 3.21 m |
| 44 | 61.0 | 3.79 m / 3.68 m |

[00197] Other key GHMBC correlations between H33 to C25, H27 to C24 (and vice versa) confirmed the linkages of 3 of the sugar molecules. Based on all the observed 2D

correlations and the chemical shift signals the structure of Reb WB1 as that shown in FIG. 16. The structure of Reb WB1 was deduced as 13- β -D-glucopyranosyloxy ent-kaur-16-en-19-oic acid-[(2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl) ester.

5 **EXAMPLE 8: The structure of Reb D4 analyzed by NMR.**

[00198] The material used for the characterization of Reb D4 was produced by using enzymatic conversion of Reb WB1 and purified by HPLC. NMR spectra were acquired on Agilent VNMRS 500 MHz instrument instruments using standard pulse sequences. The 1D (^1H and ^{13}C) and 2D (TOCSY, ASAPHMQC, GCOSY and GHMBC) NMR spectra were 10 performed in 80%CD3OD and 20% D2O.

[00199] The molecular formula of Reb D4 has been deduced as $\text{C}_{50}\text{H}_{80}\text{O}_{28}$ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ions corresponding to $[\text{M}^+ \text{Na}]^+$ at m/z 1151.4728; this composition was supported by the NMR spectral data.

[00200] The ^1H NMR spectral data of Reb D4 showed the presence of two methyl singlets at δ 1.24 and 0.92, two olefinic protons as singlets at δ 5.20 and 4.86 of an exocyclic double bond. The basic skeleton of *ent*-kaurane diterpenoids was supported by the GHMBC, COSY and TOCSY experiments. Carbon multiplicities were confirmed using the APT test. The ^{13}C NMR showed 5 anomeric carbons (δ 103.5, 102.5, 101.8, 95.6 and 92.8) confirming 20 the 5 sugar units, one carbonyl at δ 177.1 and two alkene carbons at δ 152.2 and 104.4. GHMBC correlations from H40 to C12 and H22 to C19 confirmed the attachment points of the sugars to the diterpenoid core structure The ^1H and ^{13}C NMR values for Reb D4 were assigned on the basis of TOCSY, HMQC and HMBC data and are given in Table 5.

25 **Table 5.** ^1H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for Reb D4 ^a-
c. ^a assignments made on the basis of TOCSY, ASAPHMQC, and GHMBC correlations; ^b Chemical shift values are in δ (ppm); ^c Coupling constants are in Hz.

| C# | C13 δ | ^1H δ (ppm), multp, J (Hz) |
|----|--------------|--|
| 1 | 19.0 | 1.94, m/ 1.44, m |
| 2 | 37.0 | 2.31, m/ 1.04, m |
| 3 | 43.9 | - |
| 4 | 57.0 | 1.07, m |
| 5 | 39.2 | - |
| 6 | 40.0 | 1.83, m/ 0.84, m |
| 7 | 21.9 | 1.89, m |

| | | |
|----|-------|-------------------------|
| 8 | 41.3 | 1.56, m/ 1.40, m |
| 9 | 41.3 | - |
| 10 | 53.6 | 0.97, m |
| 11 | 43.9 | 2.22, m/ 1.50, m |
| 12 | 87.2 | - |
| 13 | 37.0 | 1.99, m/ 1.53, m |
| 14 | 19.7 | 1.62, m |
| 15 | 46.7 | 2.14, m/ 2.04, m |
| 16 | 152.2 | - |
| 17 | 104.4 | 5.20, s/ 4.86, s |
| 18 | 27.9 | 1.24, s |
| 19 | 177.1 | - |
| 22 | 92.8 | 5.57, d, <i>J</i> 7.6Hz |
| 24 | 76.7 | 3.92, m |
| 25 | 63.4 | 3.53, m |
| 26 | 85.9 | 4.05, m |
| 27 | 75.9 | 4.01, m |
| 28 | 101.8 | 4.99, d, <i>J</i> 7.9Hz |
| 30 | 70.1 | 3.32, m |
| 31 | 71.1 | 3.21, m |
| 32 | 69.8 | 3.39, m |
| 33 | 70.6 | 3.18, m |
| 34 | 102.5 | 4.76, d, <i>J</i> 7.9Hz |
| 36 | 76.5 | 3.72, m |
| 37 | 74.6 | 3.25, m |
| 38 | 76.2 | 3.66, m |
| 39 | 73.8 | 3.34, m |
| 40 | 103.5 | 4.64, m |
| 42 | 76.9 | 3.69, m |
| 43 | 76.4 | 3.65, m |
| 44 | 76.6 | 3.42, m |
| 45 | 76.5 | 3.45, m |
| 46 | 95.6 | 4.64, m |
| 48 | 76.2 | 3.59, m |
| 49 | 74.4 | 3.30, m |
| 50 | 76.6 | 3.68, m |
| 51 | 80.5 | 3.48, m |
| 59 | 60.9 | 3.78, m |
| 61 | 61.0 | 3.86, m |
| 66 | 62.0 | 3.91, m |
| 74 | 61.8 | 3.84, m |
| 76 | 61.0 | 3.67, m |
| 78 | 15.9 | 0.92, s |

[00201] Key GHMBC correlations between H45 to C46, H28 to C27 and H26 to C34 (and vice versa) confirmed the connectivity of the 5 sugar molecules. Based on all the observed 2D correlations and the chemical shift signals the structure of Reb D4 as that shown 5 in FIG. 17. The structure of Reb D4 was deduced as 13-[(2-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 9: Taste test of rebaudiosides

[00202] A sensory evaluation of rebaudiosides was performed using sucrose as a control. The sucrose sample was purchased from Sigma-Aldrich and was used to prepared control samples at three different concentrations of 1.0%, 3.0%, and 6.0% sucrose in bottled water (w/v) at room temperature. The rebaudioside was prepared at 300ppm for sensory evaluation by adding a corresponding mass into 1000 mL of bottled water. The mixture was 10 stirred at room temperature and the steviol glycoside sample was then evaluated against several control sucrose samples at 1.0%, 3.0%, and 6.0% by a panel of 13 volunteer human subjects. 15 The results of the sensory evaluation are shown in Table 6.

Table 6. Sensory evaluation of Reb W1, Reb W2 and Reb D4 compared to sucrose

| Rebaudioside | Fold increase in sweetness relative to sucrose |
|--------------|--|
| Reb WB1 | 133 |
| Reb WB2 | 79 |
| Reb D4 | 109 |

20

STATEMENT OF INDUSTRIAL APPLICABILITY / TECHNICAL FIELD

[00203] This disclosure has applicability in the food, feed, beverage, and pharmacological industries. This disclosure relates generally to a method for the biosynthetic 25 production of steviol glycosides *via* a modified microbial strain.

Literature Cited and Incorporated by Reference:

1. Brandle, J. E. et al., (1998). *Stevia Rebaudiana: Its Agricultural, Biological, and Chemical Properties*, CANADIAN J. PLANT SCIENCE. 78 (4): 527–36.

5

2. Ceunen, S., and J.M. C. Geuns, *Steviol Glycosides: Chemical Diversity, Metabolism, and Function*, J. NAT. PROD., 2013, 76 (6), pp 1201–28 (2013).

3. Du J et al., (2011), *Engineering microbial factories for synthesis of value-added products*, J

10 IND MICROBIOL BIOTECHNOL. 38: 873-90.

4. GRAS Notices, USA Food and Drug Administration, United States Health & Human Services. (2016) (relevant to steviol glycosides & polyglycosides).

15 5. Häusler A, and Münch T., (1997), *Microbial production of natural flavors*, ASM NEWS 63:551-59.

6. Prakash I., et al.; *Isolation and Characterization of a Novel Rebaudioside M Isomer from a Bioconversion Reaction of Rebaudioside A and NMR Comparison Studies of Rebaudioside M Isolated from Stevia rebaudiana Bertoni and Stevia rebaudiana Morita*, BIOMOLECULES, 2014 Jun; 4(2): 374–89. (Published online 2014 Mar 31. 2014).

7. Prakash I., et al., *Development of Next Generation Stevia Sweetener: Rebaudioside M*, FOODS, 2014, 3:162-175.

25

8. Shockley JM. Et a., (2003), *Arabidopsis contains a large superfamily of acyl-activating enzymes: phylogenetic and biochemical analysis reveals a new class of acyl-coenzyme A synthetases*. PLANT PHYSIOL 132 1065–76.

30

Sequences of Interest:**UGT76G1 sequence:**

Amino Acid Sequence: (SEQ ID NO: 1)

MENKTETTVRRRRRIILFPVPFQGHINPILQLANVLYSKGFSITIFHTNFNPKTSNYPHF
5 TFRFILDNDPQDERISNLPTHGPLAGMRIPIINEHGAELRRELELLMLASEEDEEVSCLI
TDALWYFAQSVADSLNLLRLVLMTSSLNFNAHVSLPQFDELGYLDPDDKTRLEEQA
SGFPMLKVKDIIKSAYSNWQILKEILGKMIKQTAKASSGVIWNSFKELEESELETVIREIPA
PSFLIPLPKHLTASSSSLLDHDRTVFQWLDQQPPSSVLYVSFGSTSEVDEKDFLEIARGL
VDSKQSFLWVVRPGFVKGSTWVEPLPDGFLGERGRIVKWPQQEVLAHGAIGAFWT
10 HSGWNSTLESVCEGVPMIFSDFGLDQPLNARYMSDVLKVGVLNGWERGEIANAIR
RVMVDEEGEYIRQNARVLKQKADVSLMKGGSSYESLESLVSYISSL

DNA sequence: (SEQ ID NO: 2)

ATGGAGAATAAGACAGAAACAACCGTAAGACGGAGGCGGAGGATTATCTTGTCC
15 CTGTACCATTCAAGGCCATATTAATCCGATCCTCCAATTAGCAAACGTCCTCTAC
TCCAAGGGATTTCAATAACAATCTCCATACTAACTTAAACAAGCCTAAAACGAG
TAATTATCCTCACTTACATTCAAGGTTCATTCTAGACAACGACCCCTCAGGATGAGC
GTATCTCAAATTACCTACGCATGGCCCTTGGCAGGTATGCGAATACCAATAATC
AATGAGCATGGAGCCGATGAACCTCGTCGAGTTAGAGCTTCTATGCTCGCAA
20 GTGAGGAAGACGAGGAAGTTCGTGCCTAATAACTGATGCGCTTGGTACTCGCC
CAATCAGTCGAGACTCACTGAATCTACGCCGTTGGCCTTATGACAAGTTCTTAC
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25 GGAAAAATGATAAAGCAAACCAAAGCGTCCTCTGGAGTAATCTGGAACCTCTCA
AGGAGTTAGAGGAATCTGAACCTGAAACGGTCATCAGAGAAATCCCCGCTCCCTC
GTTCTTAATTCCACTACCCAAGCACCTACTGCAAGTAGCAGTTCCCTCCTAGATC
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30 GAGGGCTCGTGGATAGCAAACAGAGCTTCCTGTGGTAGTGAGACCGGGATTGCGT
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CCTTTGGACCCACTCTGGTGGATTCTACTCTGAAAGTGTCTGTGAAGGCGTTC
CAATGATATTTCTGATTTGGGCTTGACCAGCCTCTAACGCTCGCTATATGTCTG
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CAACGCCATACGCCGGTAATGGTGGACGAGGAAGGTGAGTACATACGTACAGAAC
5 GCTCGGGTTTAAAACAAAAAGCGGACGTCAGCCTATGAAGGGAGGTAGCTCCT
ATGAATCCCTAGAATCCTGGTAAGCTATATATCTTCGTTATAA

CP1 sequence:

Amino Acid: (SEQ ID NO:3)

10 MNWQILKEILGKMIKQTKASSGVIWNSFKELEESELETVIREIPAPSFLIPLPKHLTASSS
SLLDHDRTVFQWLDQQPPSSVLYVSFGSTSEVDEKDFLEIARGLVDSKQSFLWVVRPG
FVKGSTWVEPLPDGFLGERGRIVKWPQQEVLAHGAIGAFWTHSGWNSTLESVCEGV
PMIFSDFGLDQPLNARYMSDVLKVGVYLENGWERGEIANAIRVMVDEEHEYIRQNA
RVLKQKADVSLMKGGSSYESLESLVSYISSLENKTETTVRRRRRIILFPVPFQGHINPIL
15 QLANVLYSKGFSITIFHTNFNKPCTSYPHFTFRFILDNDPQDERISNLPTHGPLAGMRI
PIINEHGADELRRELELLMLASEEDEEVSCLITDALWYFAQSVADSLNLRLVLMTSSL
FNFHAHVSLPQFDELGYLDPDDKTRLEEQASGFPMVKDIKSAYS

DNA sequence: (SEQ ID NO:4)

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CGTCGTGGCGTTATCTGAACTCCTCAAAGAACTGGAAGAATCAGAACTGGA
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GGATCAGCAACCGCCGTATCGGTGCTGTATGTTCTTCATTGGTAGCACCTCTGAAG
25 TCGATGAAAAAGACTTCTGGAAATCGCTCGCGGCTGGTGGATAGTAAACAGTC
CTTCCTGTGGTGGTTCGTCCGGTTTGAAAGGCAGCACGTGGTTGAACCGC
TGCCGGATGGCTTCCTGGGTGAACGCAGCGTATTGTCAAATGGTGCCGCAGCA
AGAAGTGCTGGCACATGGTGCTATCGCGCGTTGGACCCACTCTGGTTGGAACA
GTACGCTGGAATCCGTTGCGAAGGTGTCCCGATGATTTCAGCGATTTGGCCTG
30 GACCAGCCGCTGAATGCCGCTATATGTCTGATGTTCTGAAAGTCGGTGTACCT
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ACGTGAGCCTGATGAAAGGCGGTAGCTCTTATGAATCACTGGAATCGCTGGTTAG
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CTGTGGTATTCGCCCAGAGCGTTGCAGATTCTCTGAACCTGCGCCGTCTGGTCCT
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B-glu1 sequence:

15 Amino Acid: (SEQ ID NO:5)

MTQLDVESLIQELTLNEKVQLLSGSDFWHTTPVRLGIPKMRLSDGPNGVRGTFKFFNG
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ESFGEDPVVNGLSSAAMINGLQGKYIAATMKHYVCNDLEMDRNCIDAQVSHRALRE
VYLLPFQIAVRDANPRAIMTAYNKANGEHVSQSKFLLDEVLRKEWGDGLLMSDWF
20 GVYDAKSSITNGLDLEMPGPPQCRVHSATDHAINSGEIHINDVDERVRSLLSLINYCHQ
SGVTEEDPETSDNNTPETIEKLRKISRESIVLLKDDDRNRSILPLKKSDKIAVIGNNAKQ
AAAYCGGGSASVLSYHTTPFDSIJKSRLEDSNTPAYTIGADAYKNLPLGPQM TDSDGK
PGFDAKFFVGSPTSKDRKLIDHFQLTNSQVFLVDYYNEQIPENKEFYVDVEGQFIPED
GTYNFGLTVFGTGRLFVDDKLVSDSSQNQTPGDSFFGLAAQEVIHSIHLVKGKAYKIK
25 VLYGSSVTRTYEIAASVAFEGGAFTFGAAKQRNEDEEIARAVEIAKANDVVLCIGLN
QDFESEGFDRPDIKIPGATNKMVS AVLKANPNTVIVNQTGTPVEMPWASDAPVILQA
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5 GTGACCTTCCCCTGCGTTTGGAGGATAACCCGGCGTACCTGAACCTCCAGAGCAA
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15

UGT85C2 sequence:

Amino Acid: (SEQ ID NO: 7)

MDAMATTEKKPHVIFIPFPAQSHIKAMLKLAQLLHHKGLQITFVNTDFIHNQFLESSGP
HCLDGAPGFRFETIPDGVSHPPEASIPIRESLRSIETNFLDRFIDLVTKLPPPTCIISDGF
20 LSVFTIDAEEKLGIPLVMMYWTLAACGFMGFYHIHSLIEKGFAPLKDasyltngylDTV
IDWVPGMEGIRLKDFPLDWSTDLNDKVLMTTEAPQRSHKVSHHIFHTFDELEPSIIKT
LSLRYNHIYTIGPLQLLDDQIPEEKKQTGITSLHGYSLVKEEPECFQWLQSKEPNSVVYV
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WCSQEVLKHPVGFLTHCGWGSTIESLSAGVPMICWPYSWDQLTNCRYICKEWEV
25 GLEMGTVKRDEVKRLVQELMGEGGHKMRNKAQDWKEKARIAIAAPNGSSLNIDKM
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DNA (SEQ ID NO: 8)

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CGGTTTCTGACCCACTGCGGCTGGGTAGCACGATTGAATCTCTGAGTGCTGGT
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TGTAAAGAATGGGAAGTCGGCCTGGAAATGGGTACGAAAGTGAAGCGTGACGAA
20 GTTAAACGCCTGGTCCAAGAACTGATGGCGAAGGCAGTCATAAAATGCGTAACA
AAGCGAAGGATTGGAAAGAAAAGGCCGCATTGCGATTGCGCCGAACGGCAGCA
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A

25 **HV1 sequence:**

Amino Acid sequence: (SEQ ID NO: 9)

MDGNSSSPLHVVICPWLALGHLLPCLDIAERLASRGHRVSFVSPRNIARLPPLRPAV
APLVDFVALPLPHVDGLPEGAESTNDVPYDKFELHRKAFDGLAAPFSEFLRAACAEGA
GSRPDWLIVDTFHHAAAAAVENKVPCVMLLGAATVIAGFARGVSEHAAA AVGKE
30 RPAAEAPSFETERRKLMTTQNAGMTVAERYFLTLMRSSDLVAIRSCEWEPEPESVAALT
TLAGKPVVPLGLLPPSPEGGRGVSKEDAAVRWLDAQPAKSVVYVALGSEVPLRAEQV
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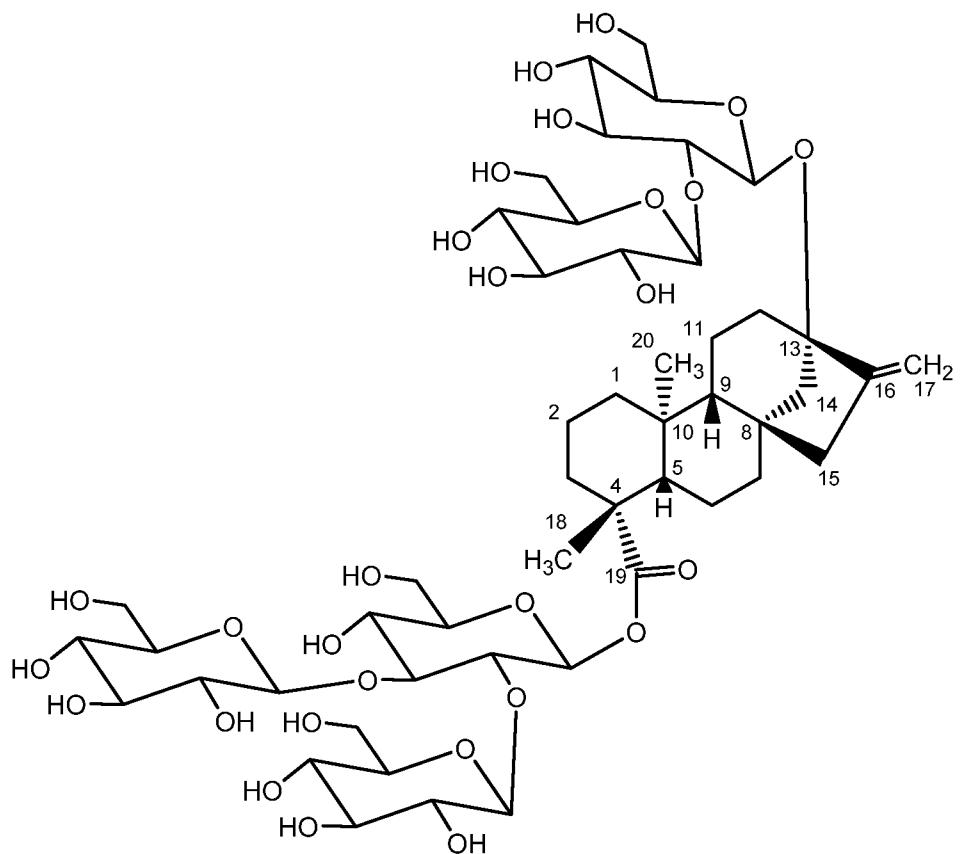
GAVAAFLTHCGWNSTIEGLLFGHPLIMLPISSDQGPNARLMEGRKVGMQVPRDESCGS
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DNA sequence: (SEQ ID NO: 10)

5 ATGGATGGTAACTCCTCCTCGCCGCTGCATGTGGTCATTGTCCGTGGCTGGC
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CCGGCTGTTGCACCGCTGGTTGATTCTCGCACTGCCGTGCCGATGTTGACGG
TCTGCCGGAGGGTGCAGAACGACCAATGATGTGCCGTATGACAAATTGAACTG
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GGGTGCAGCAACGGTATCGCTGGTTCGCGCGTGGTAGCGAACATGCGGCG
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15 GTCGCAAGCTGATGACCACGCAGAACATGCCCTCCGCATGACCGTGGCAGAACGCTA
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GCCGCAGTGCTGCCGCCGGTTCGAAGAACGTACCCCGCCGTGGTCTGGTTG
CACGGGTTGGGTGCCGCAGATTGGCGTTCTGGCTCATGGTGCAGGTGGCTGCGTT
TGACCCACTGTGGCTGGAAACTCTACGATCGAAGGCCTGCTGGTCACTCCGCTG
25 ATTATGCTGCCGATCAGCTCTGATCAGGGTCCGAATGCGCGCCTGATGGAAGGCC
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AACGTTGTATTGACGGTTTATCCAGCAACTGCGCAGTTACAAGGCGTGA

CLAIMS

1. A steviol glycoside of interest produced by a transformed cellular system growing within a medium.
2. The steviol glycoside of interest of claim 1, wherein said transformed cellular system is selected from the group consisting of: yeast, non-steviol glycoside producing plants, algae and bacteria.
3. The steviol glycoside of interest of claim 1, wherein said steviol glycoside is Reb D4 of structure:

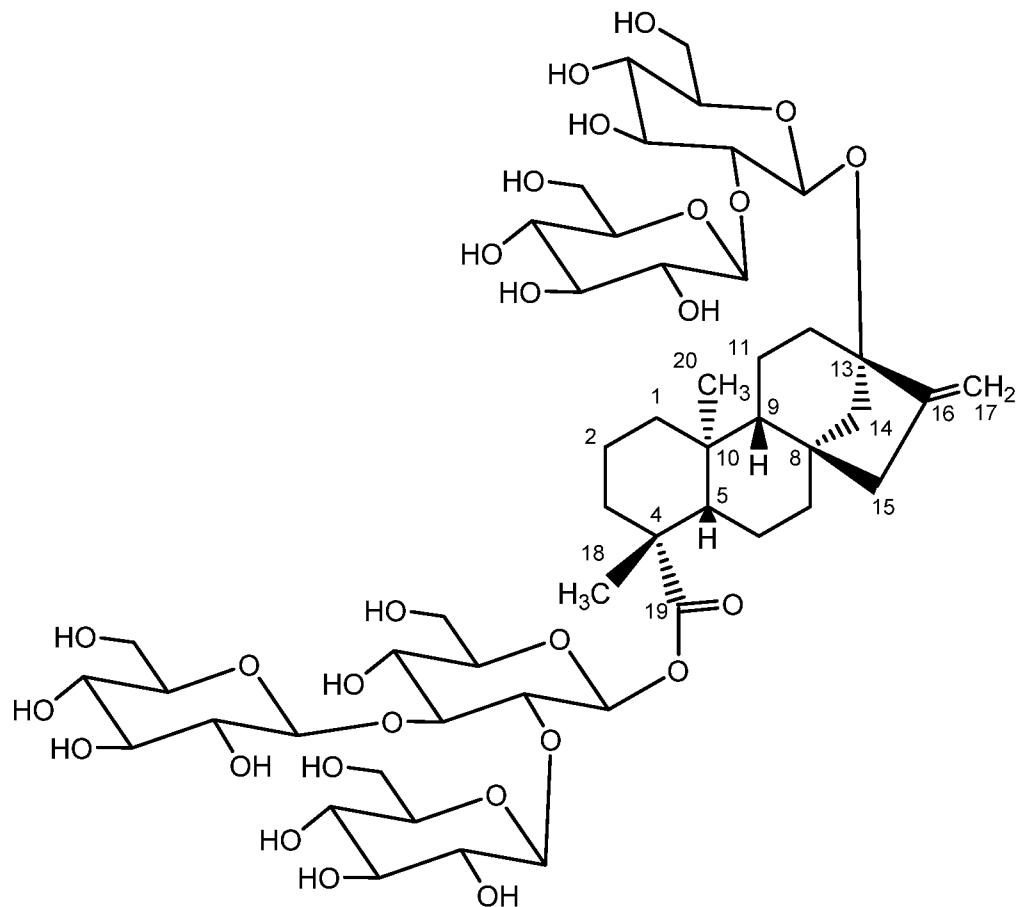


4. The steviol glycoside of interest of claim 1, wherein said steviol glycoside is Reb WB1.
5. The steviol glycoside of interest of claim 1, wherein said steviol glycoside is Reb WB2.
6. The steviol glycoside of interest of claim 1, wherein the source material is steviol.

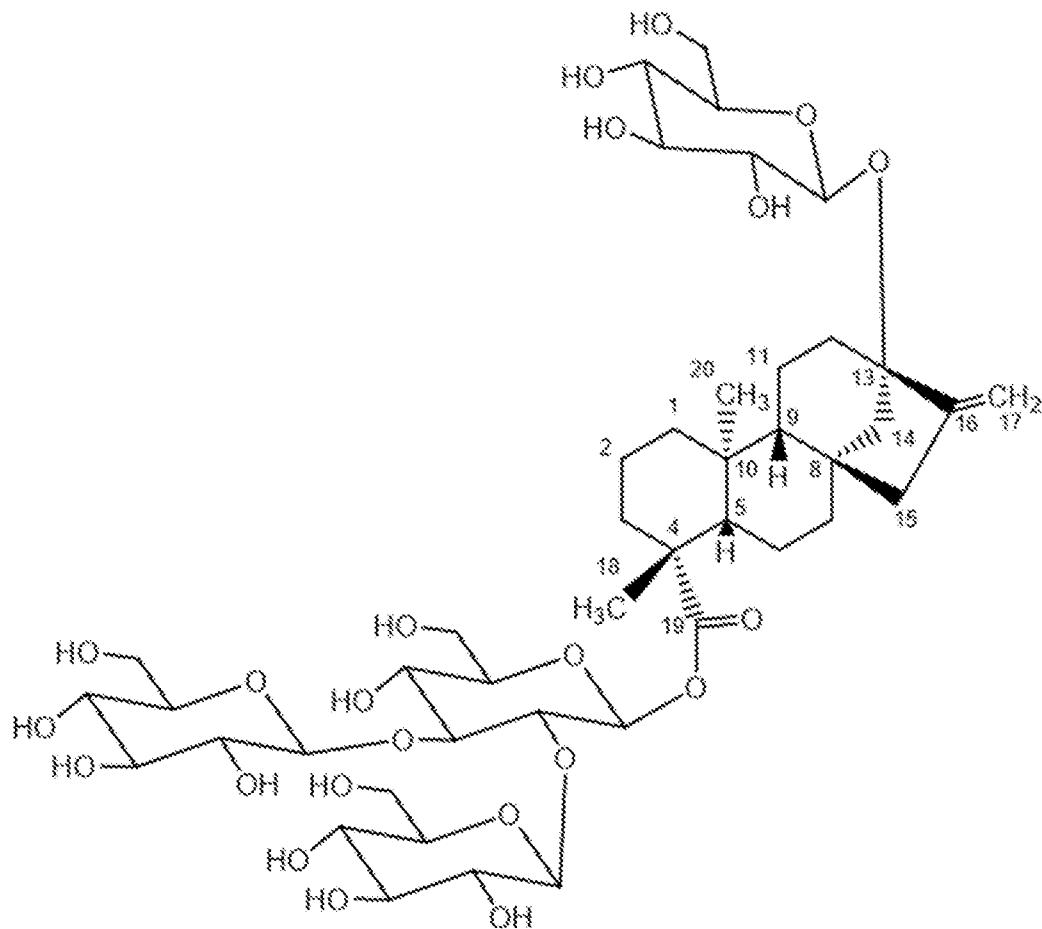
7. The steviol glycoside of interest of claim 2, wherein said cellular system is *E. Coli*.
8. The steviol glycoside of interest of claim 2, wherein said steviol glycoside content is at least 70% pure.
9. A consumable product comprising a sweetening amount of the steviol glycoside of claim 3.
10. A consumable product comprising a sweetening amount of the steviol glycoside of claim 4.
11. A consumable product comprising a sweetening amount of the steviol glycoside of claim 5.
12. The consumable product of claim 9, being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.
13. A CP1 recombinant polypeptide comprising a DNA sequence having at least 80% identity to SEQ ID NO:3.
14. The recombinant polypeptide of claim 13, wherein the amino acid sequence has at least 80% identity to SEQ ID NO:4.
15. The steviol glycoside of interest of claim 2, wherein said cellular system is a bacteria and is selected from the group consisting of *Escherichia*; *Salmonella*; *Bacillus*; *Acinetobacter*; *Streptomyces*; *Corynebacterium*; *Methylosinus*; *Methylomonas*; *Rhodococcus*; *Pseudomonas*; *Rhodobacter*; *Synechocystis*; *Saccharomyces*; *Zygosaccharomyces*; *Kluyveromyces*; *Candida*; *Hansenula*; *Debaryomyces*; *Mucor*; *Pichia*; *Torulopsis*; *Aspergillus*; *Arthrobotrys*; *Brevibacterium*; *Microbacterium*; *Arthrobacter*; *Citrobacter*; *Escherichia*; *Klebsiella*; *Pantoea*; *Salmonella*; *Corynebacterium*; *Clostridium*; and *Clostridium acetobutylicum*.
16. A biosynthetic method of making a steviol glycoside of interest comprising:
 - a) expressing an CP1 enzyme in a transformed cellular system;
 - b) growing the cellular system in a medium; and
 - c) producing the steviol glycoside of interest.
17. The method of claim 16, further comprising incubating a recombinant sucrose synthase with the substrate.

18. The method of claim 17, further comprising incubating a recombinant UDP glycosyltransferase UGT85C2 with the sucrose synthase, the substrate, and the CP1 recombinant polypeptide.
19. The method of claim 18, further comprising adding a beta glucosidase enzyme to the reaction mixture.
20. The method of claim 17, wherein the sucrose synthase is selected from the group consisting of an *Arabidopsis* sucrose synthase 1, an *Arabidopsis* sucrose synthase 3 and a *Vigna radiata* sucrose synthase.
21. The method of claim 17, wherein the sucrose synthase is an *Arabidopsis thaliana* sucrose synthase 1.
22. The method of claim 16, wherein the steviol glycosides produced are a mixture of Reb D4 and Reb M.
23. The steviol glycoside of interest of claim 2, wherein the method of production further comprises: i) purifying a crude product; and, ii) removing solvents under vacuum to provide a concentrated product.
24. The steviol glycoside of interest of claim 23, wherein said crude product is purified by column chromatography.
25. The steviol glycoside of interest of claim 23, wherein said crude product is purified by acid-base extraction.
26. The steviol glycoside of interest of claim 23, wherein said crude product is purified by vacuum distillation.
27. The steviol glycoside of interest of claim 23, further comprising purifying said steviol glycoside using a semi-preparative HPLC.
28. The method of claim 16, wherein said steviol glycoside is Reb WB1.
29. The method of claim 16, wherein said steviol glycoside is Reb WB2.
30. The method of claim 16, wherein said steviol glycoside is Reb D4.
31. The method of claim 16, wherein said steviol glycoside is Reb M.
32. The method of claim 16, further comprising the use of HV1 (SEQ ID NO:9).
33. The method of claim 32, further comprising the use of UGT76G1 (SEQ ID NO:1).

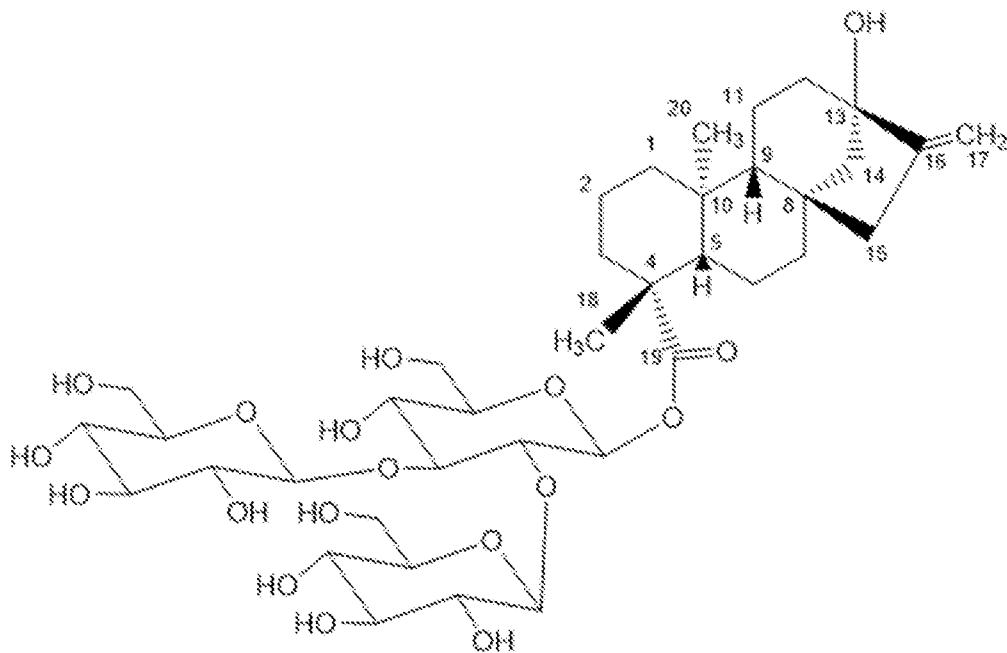
34. A steviol glycoside Reb D4 having the structure:



35. A composition comprising the steviol glycoside of claim 34, wherein said steviol glycoside content in the composition is at least 70% pure.
36. A consumable product comprising a sweetening amount of the steviol glycoside of claim 34.
37. The consumable product of claim 36, being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.
38. A composition comprising a mixture of Reb D4 and Reb M.
39. A steviol glycoside Reb WB1 having the structure:



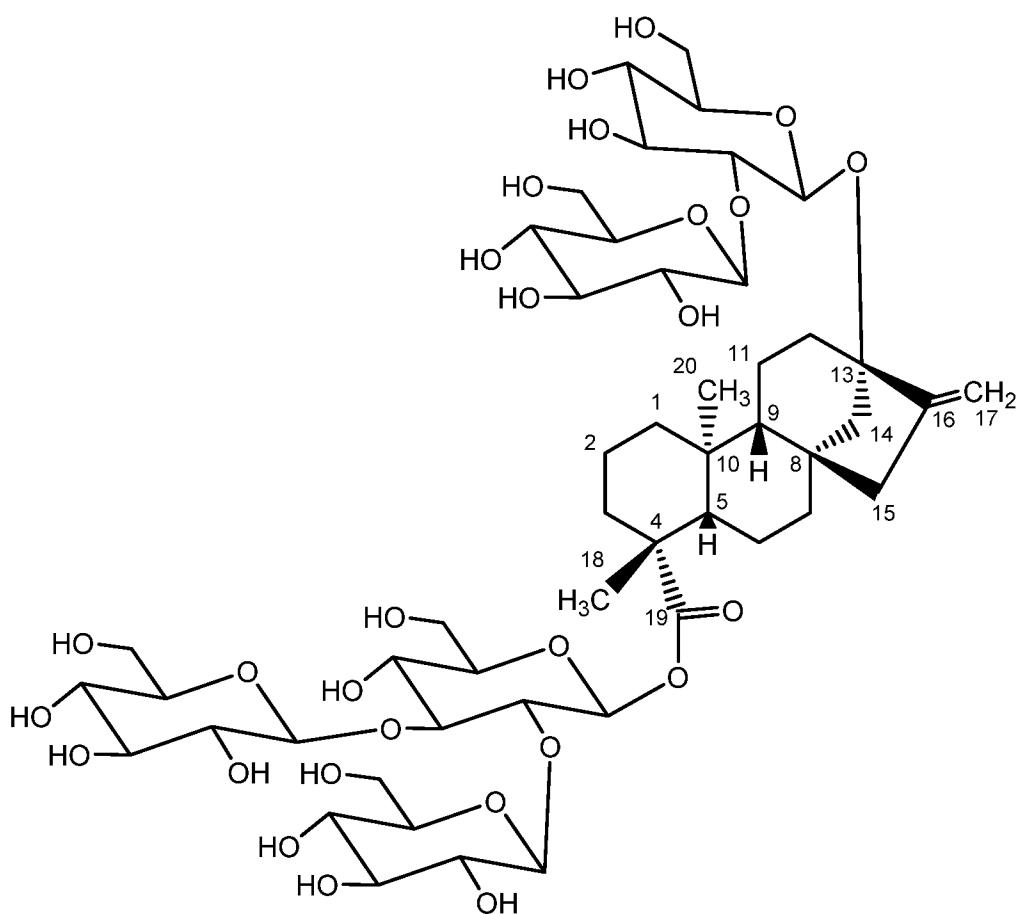
40. A composition comprising the steviol glycoside of claim 39, wherein said steviol glycoside content in the composition is at least 70% pure.
41. A consumable product comprising a sweetening amount of the steviol glycoside of claim 39.
42. The consumable product of claim 41, being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.
43. A steviol glycoside Reb WB2 having the structure:

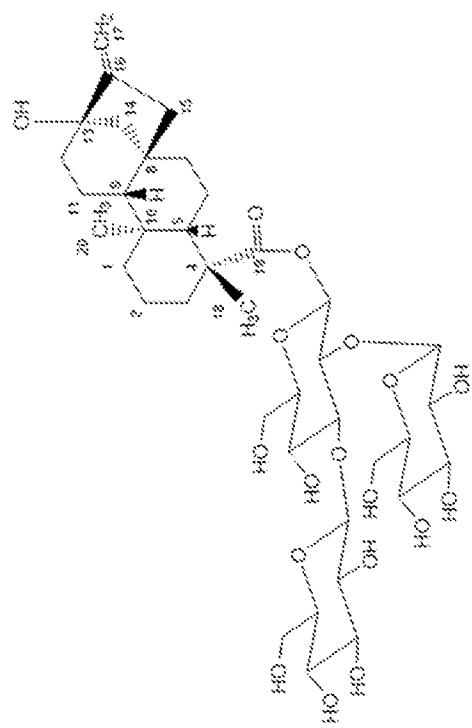


44. A composition comprising the steviol glycoside of claim 43, wherein said steviol glycoside content in the composition is at least 70% pure.
45. A consumable product comprising a sweetening amount of the steviol glycoside of claim 43.
46. The consumable product of claim 45, being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.
47. A method of producing rebaudioside M, comprising cultivating a recombinant cell under suitable growth conditions where said recombinant cell exhibits the ability to produce steviol glycosides, the method comprising:
 - contacting said recombinant cell with a reaction composition containing stevioside, sucrose synthase and sucrose; wherein said recombinant cell expresses a first UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said stevioside substrate to produce rebaudioside E;
 - wherein said recombinant cell expresses a second UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said Rebaudioside E to produce rebaudioside D4; and,

wherein said recombinant cell expresses a third UDP-glycosyltransferase (UGT) or a catalytically active portion thereof capable of using said Rebaudioside D4 to produce rebaudioside M.

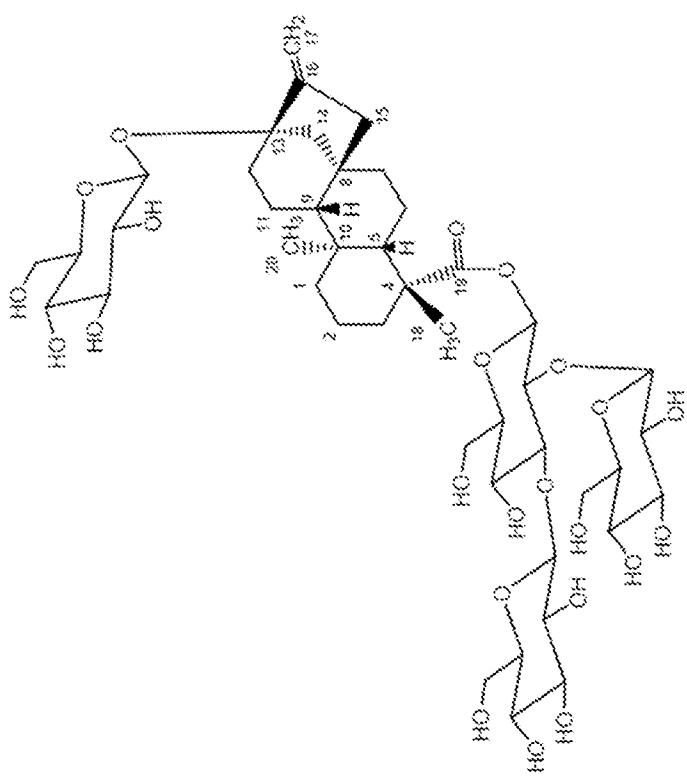
48. The method of claim 47, further comprising a sucrose synthase gene or a catalytically active portion thereof being expressed in said recombinant cell.
49. The method of claim 47, further comprising a sucrose synthase being added to the reaction composition.
50. Reb M produced by the method of claim 47.
51. A recombinant cell that expresses the biosynthetic pathway of claim 47.
52. The recombinant cell of claim 51, wherein the cell is a yeast cell.
53. The recombinant cell of claim 51, wherein the cell is a bacterial cell.
54. The recombinant cell of claim 51, wherein the cell is a plant cell.

**FIG. 1**



rebaudioside WB2

FIG. 2



rebaudioside WB1

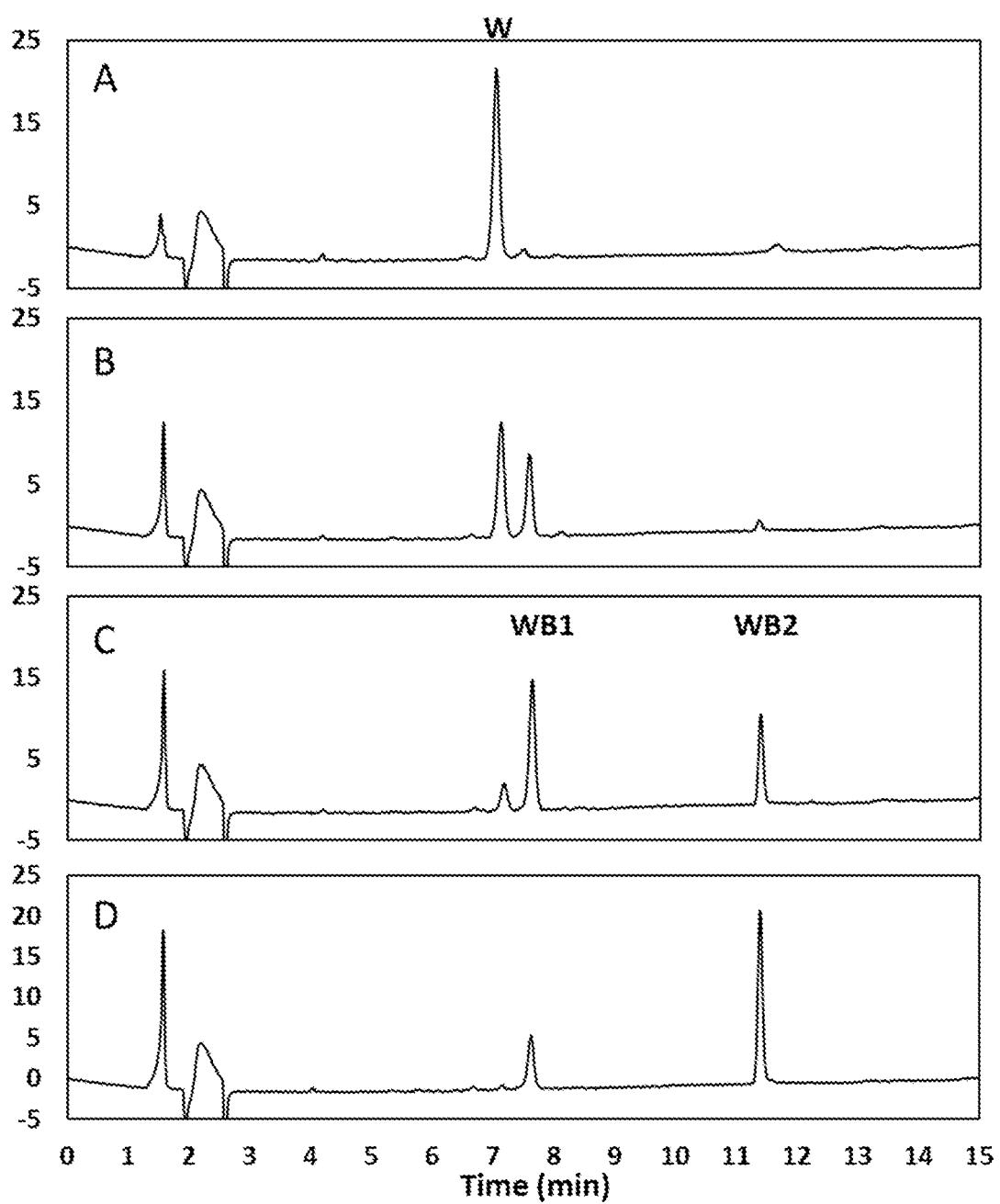


FIG. 3

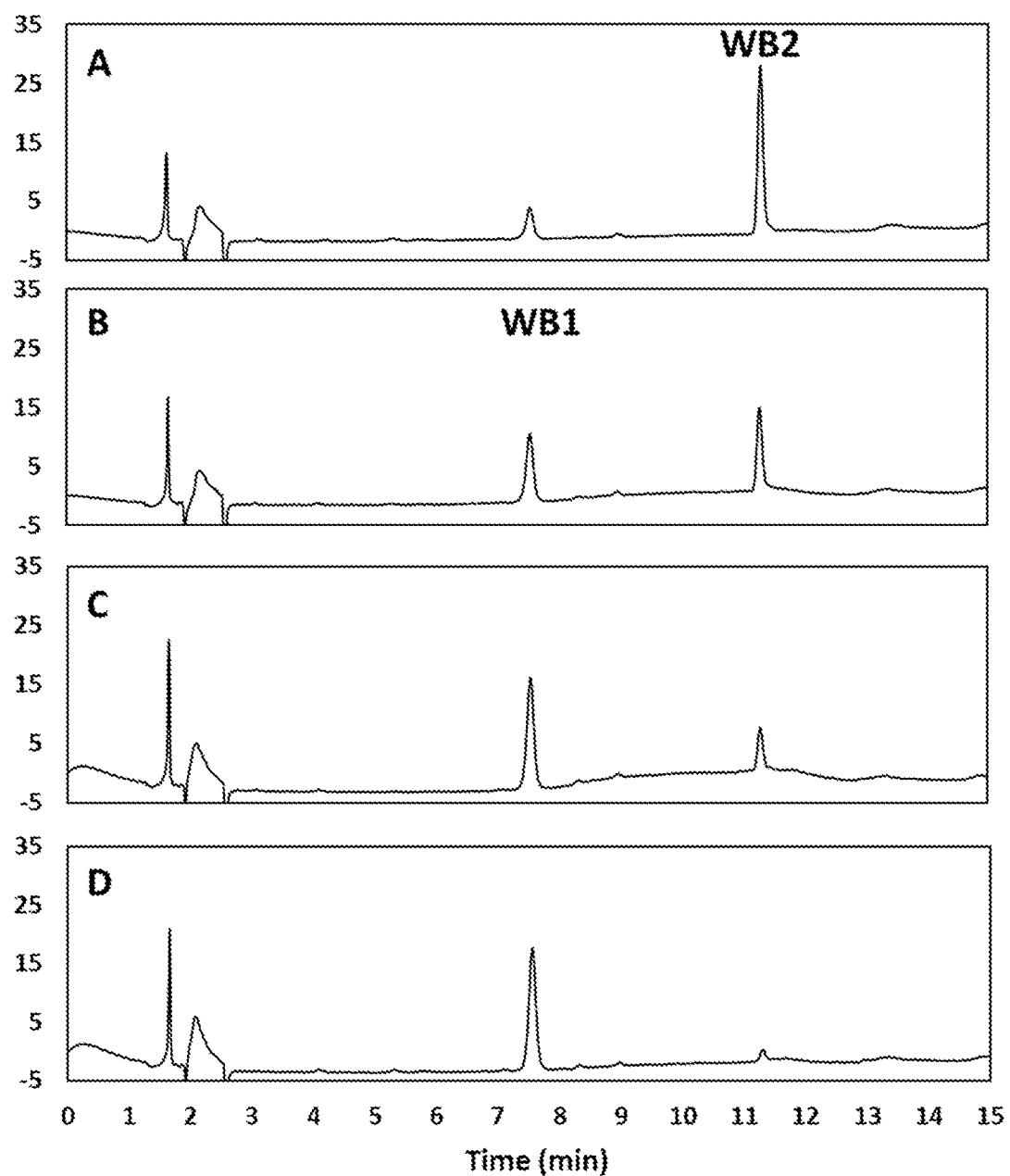


FIG. 4

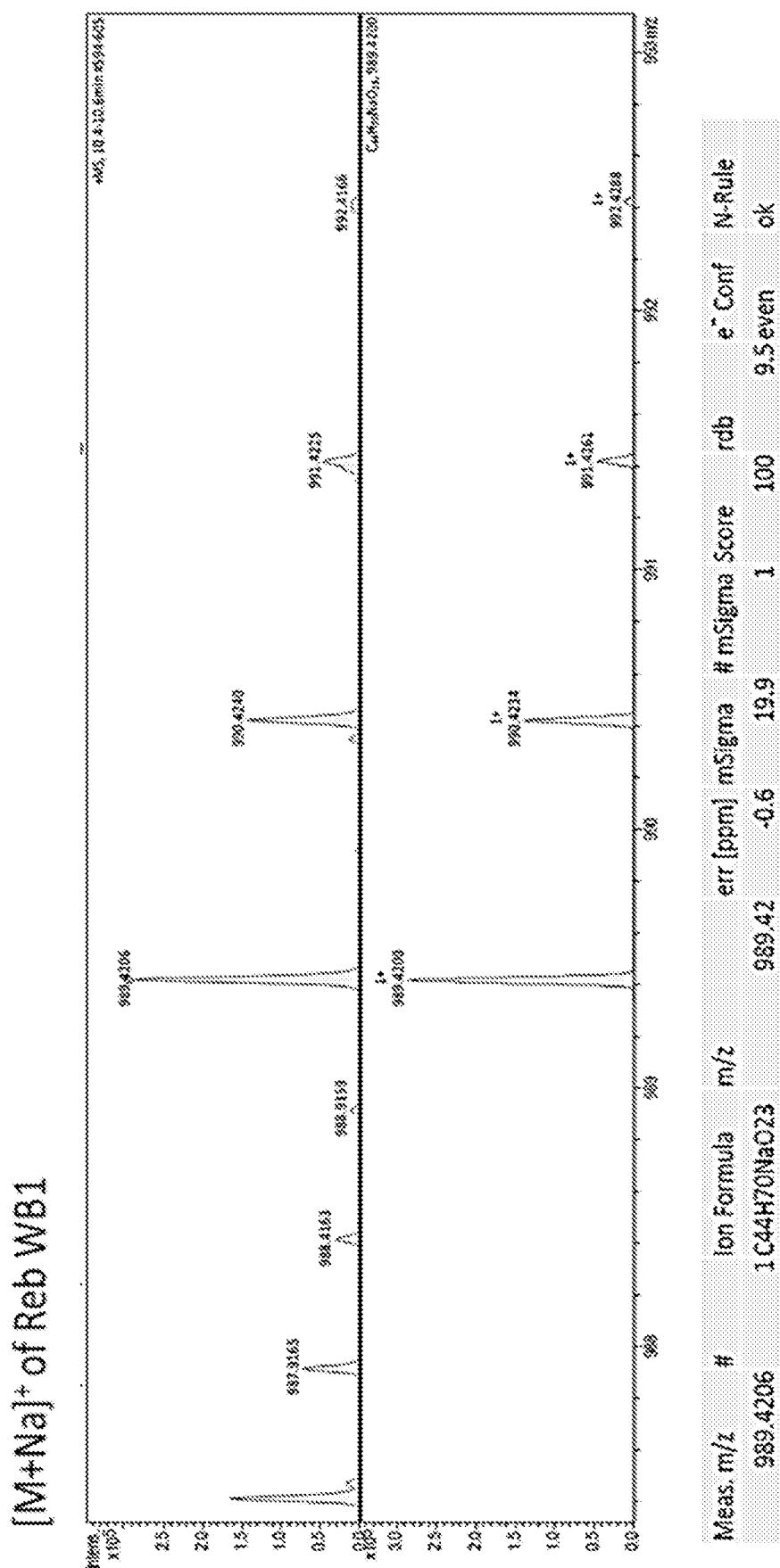


FIG. 5

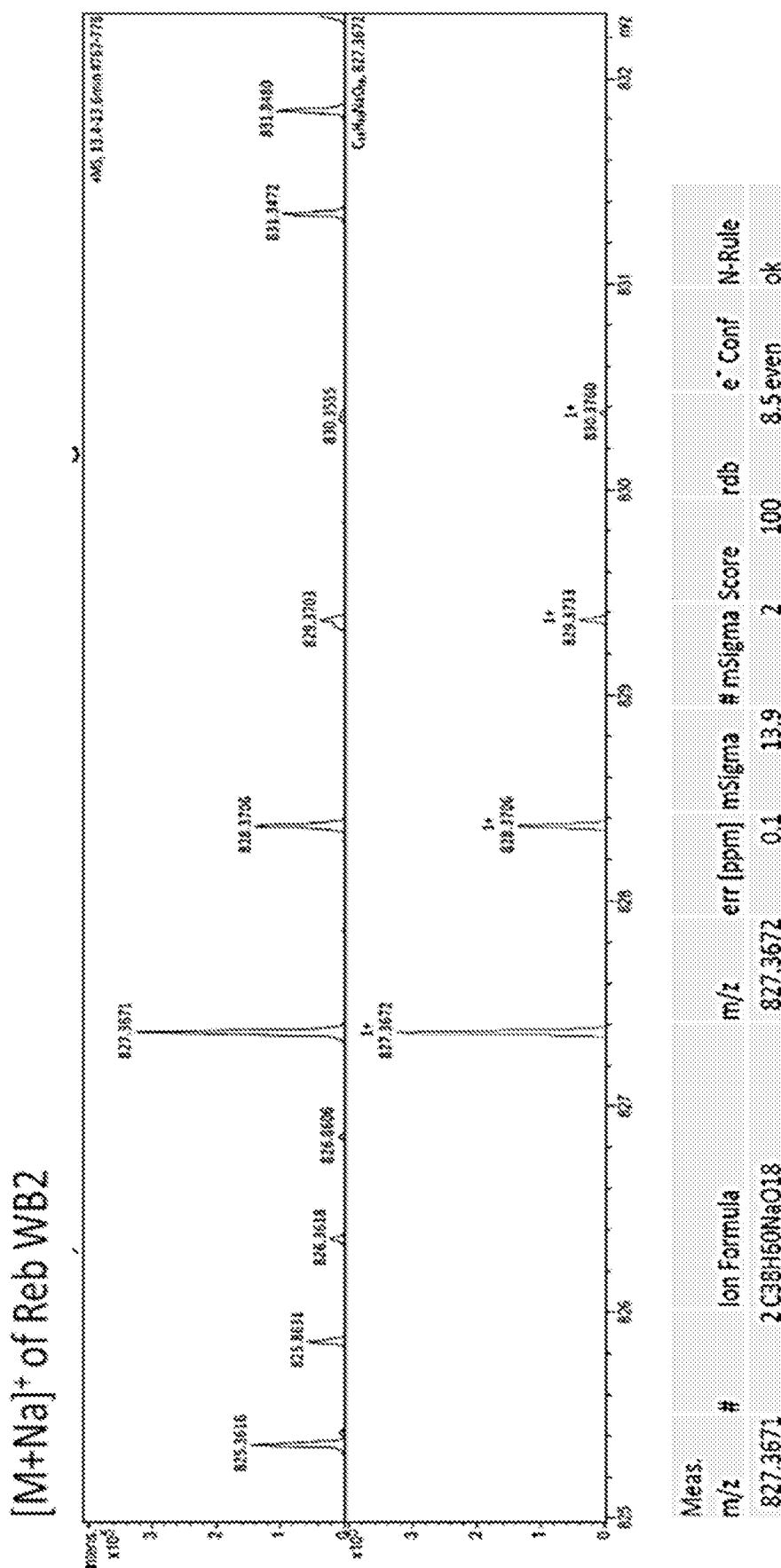


FIG. 5 (continued)

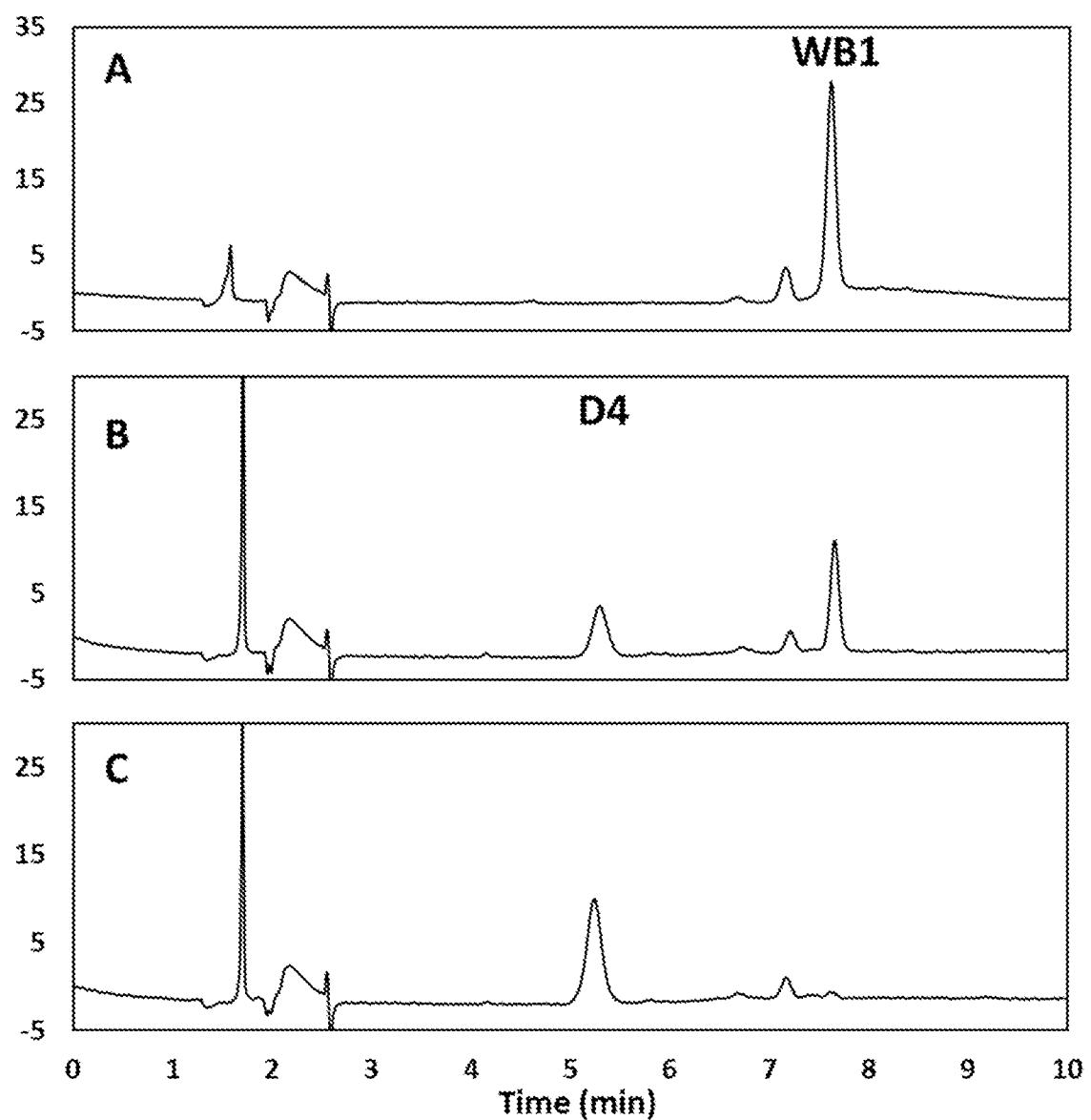
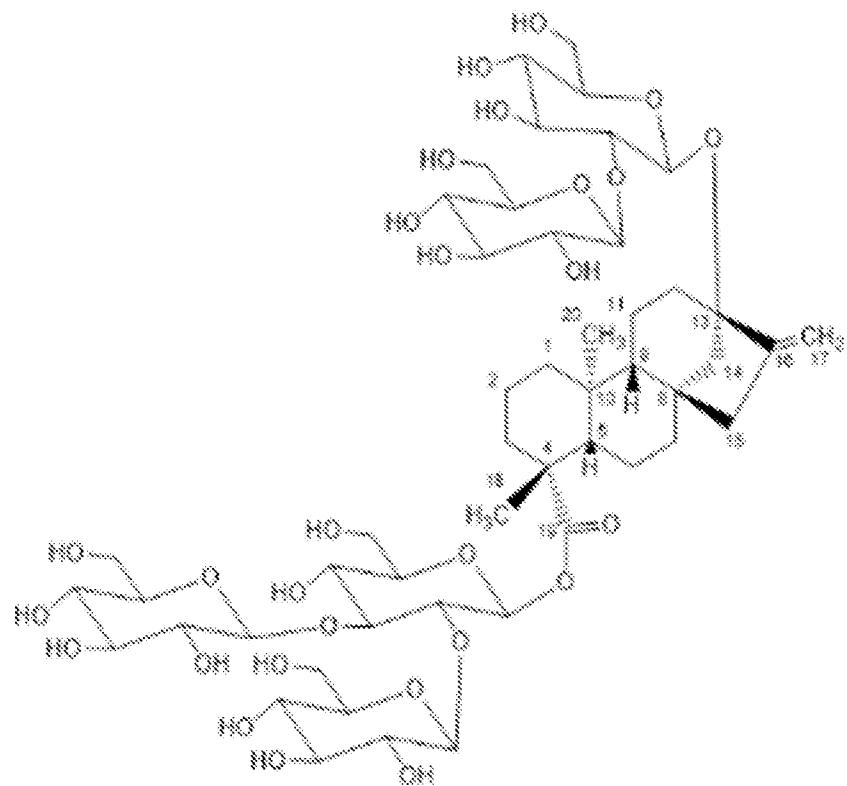


FIG. 6



Rebaudioside D4

FIG. 7A

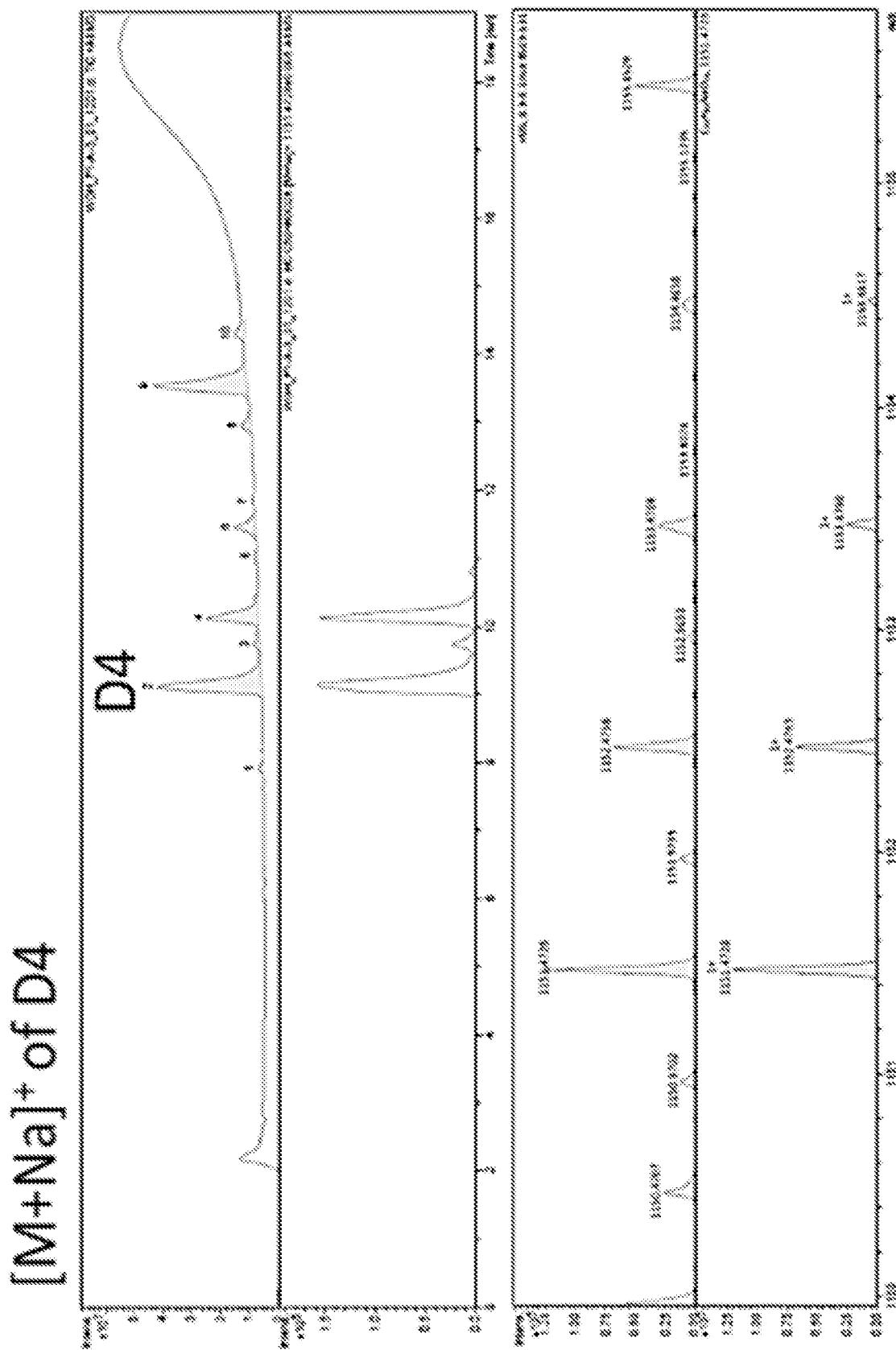


FIG. 7B

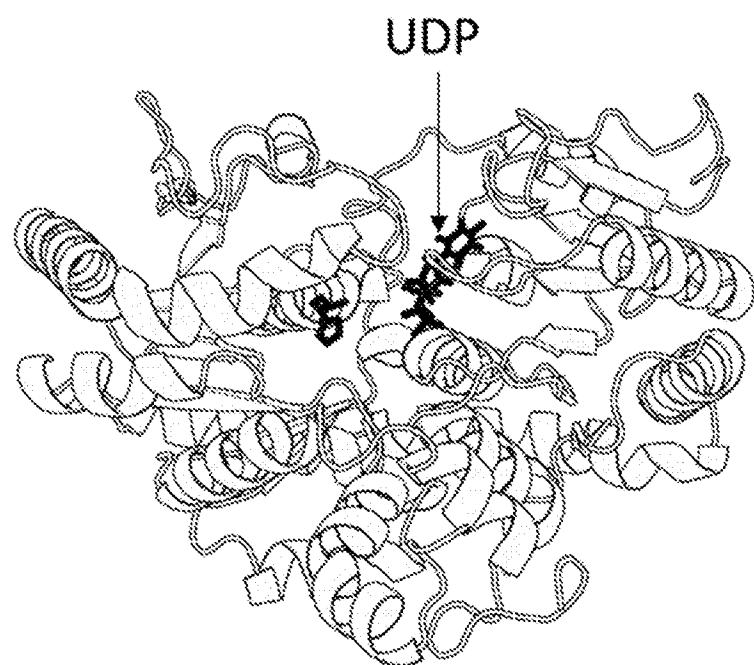


FIG. 8

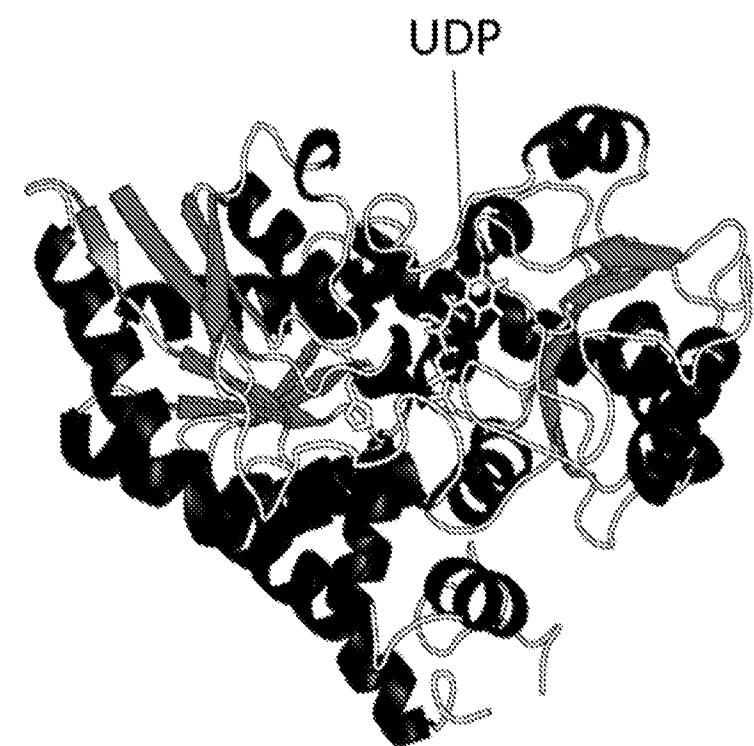


FIG. 9

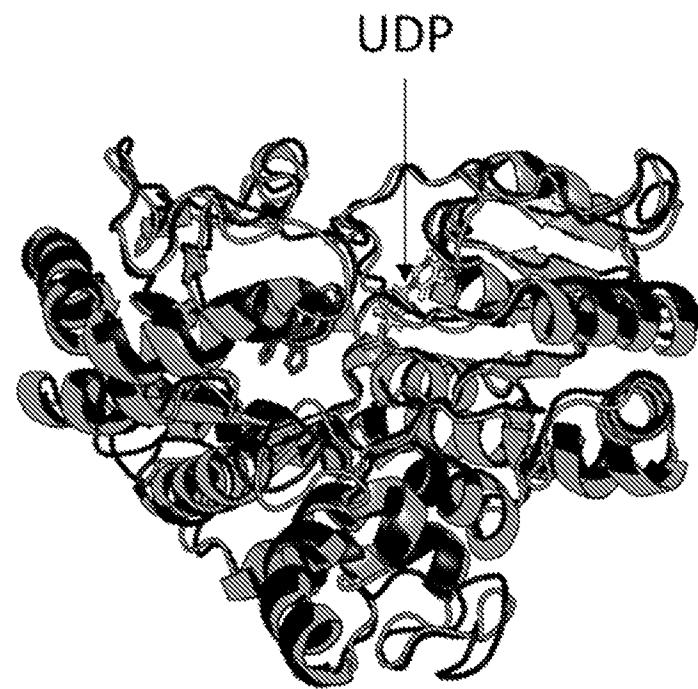


FIG. 10

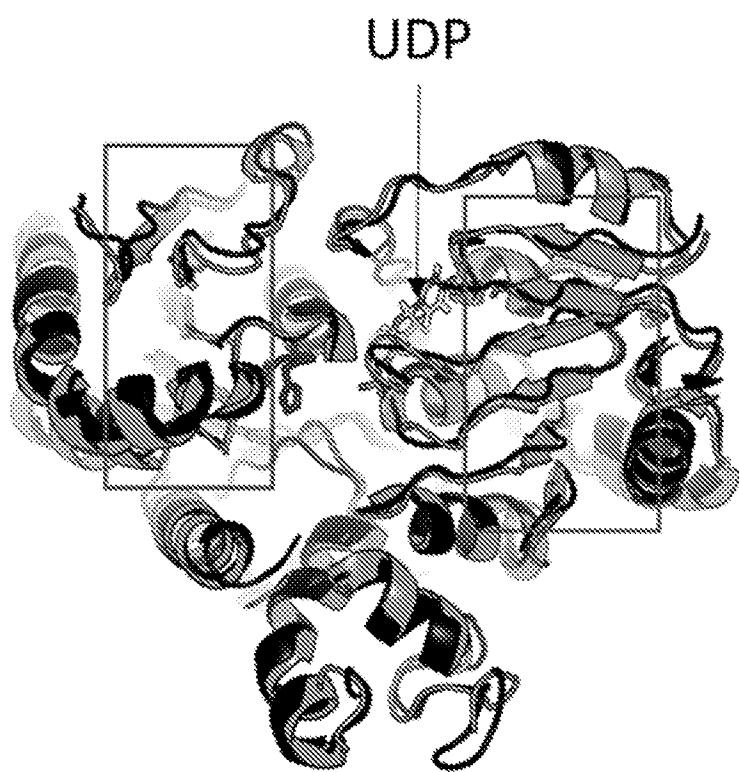


FIG. 11

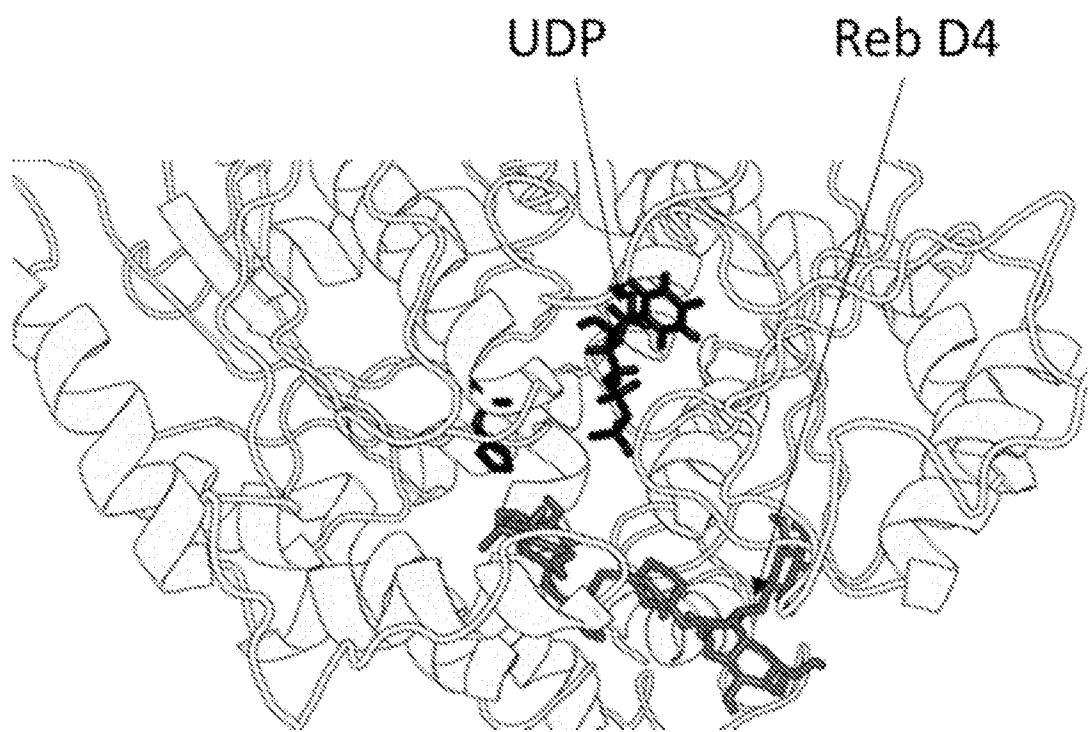


FIG. 12

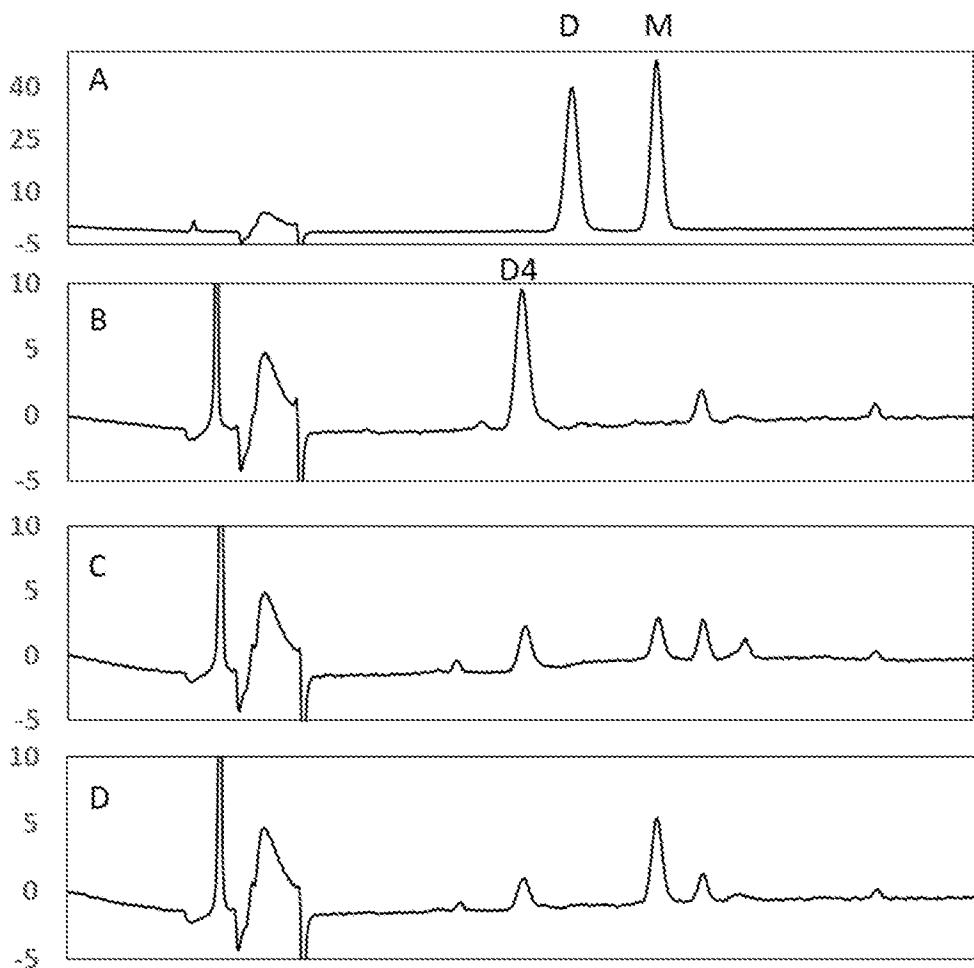


FIG. 13

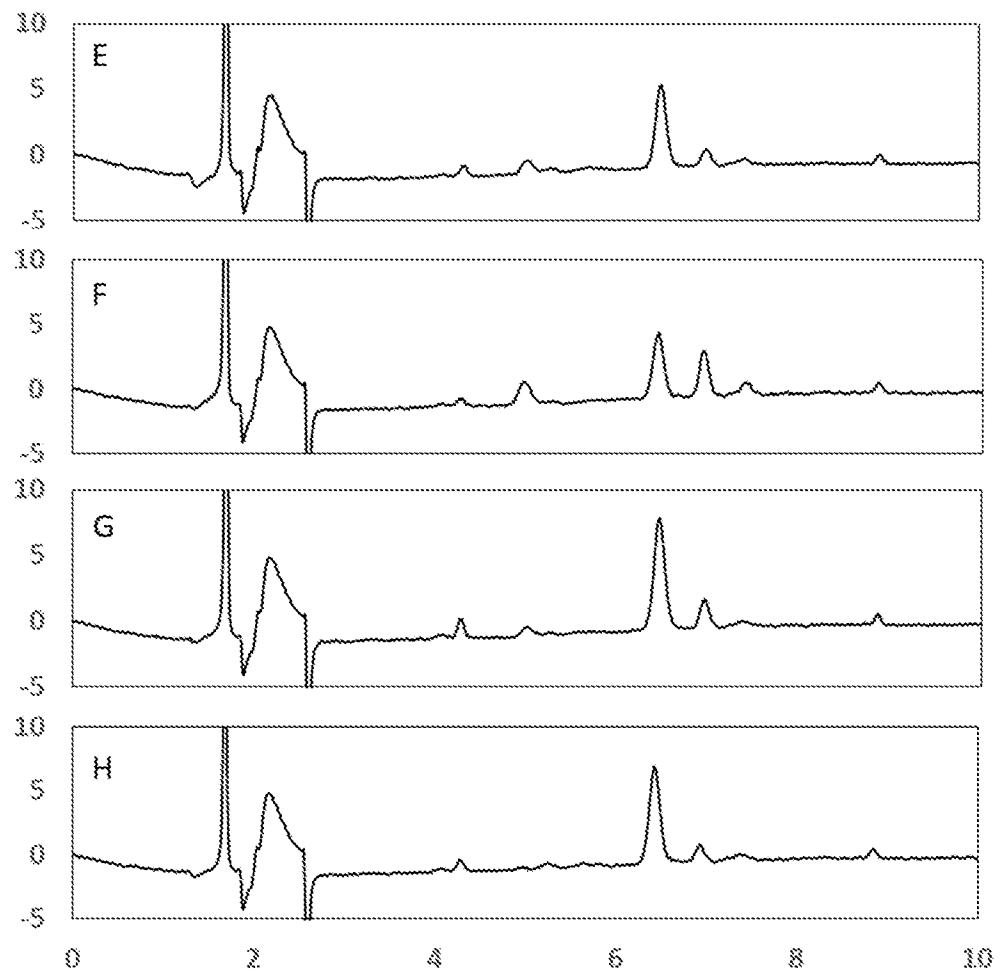


FIG. 13 (continued)

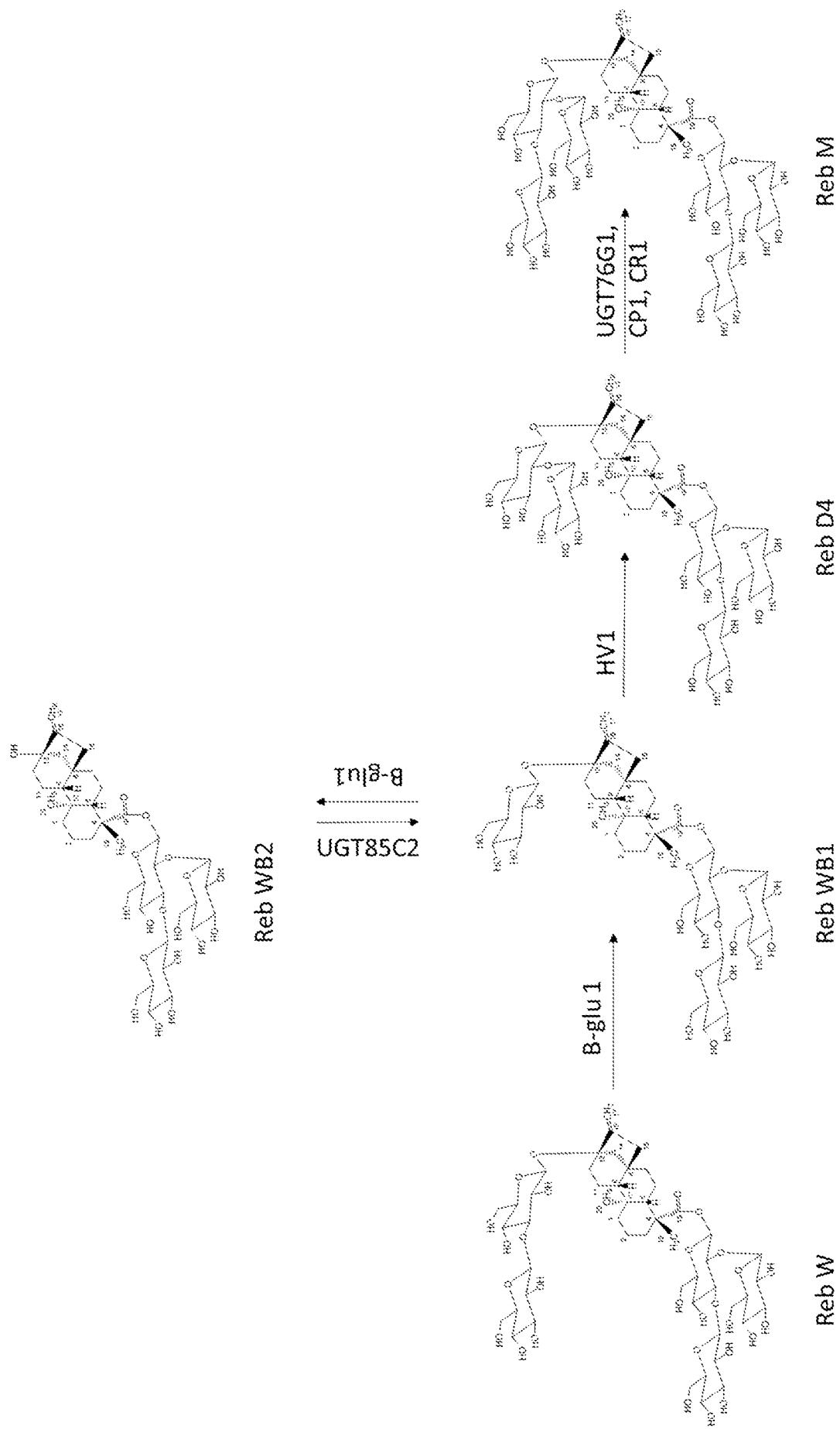


FIG. 14

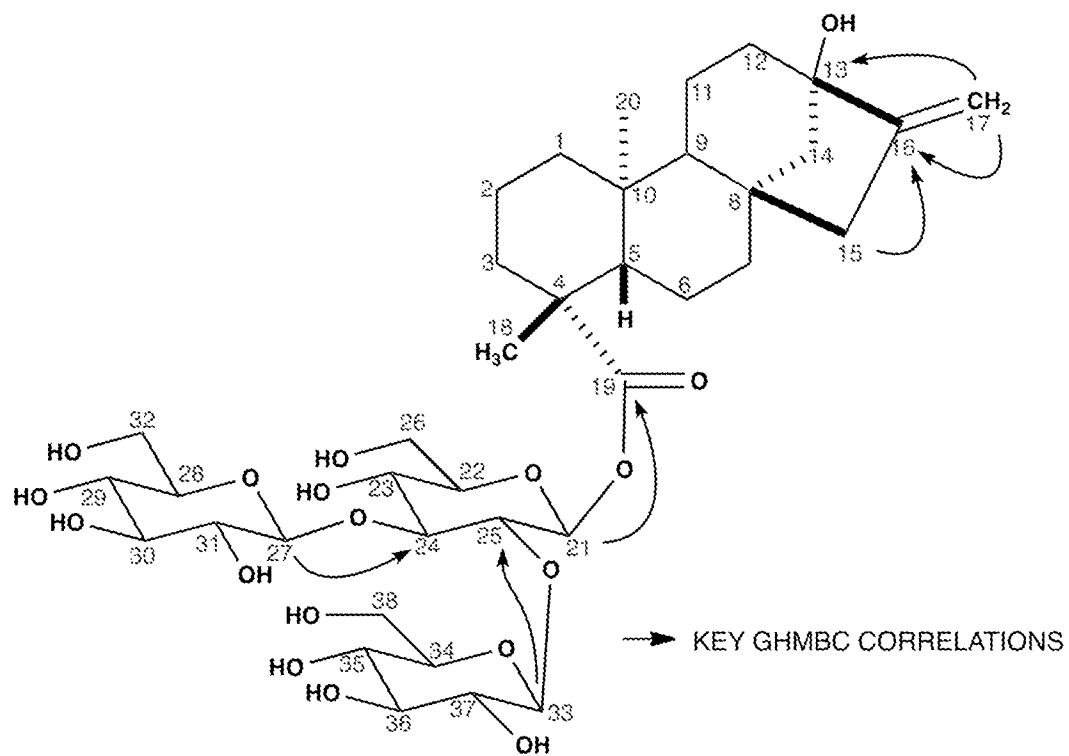


FIG. 15

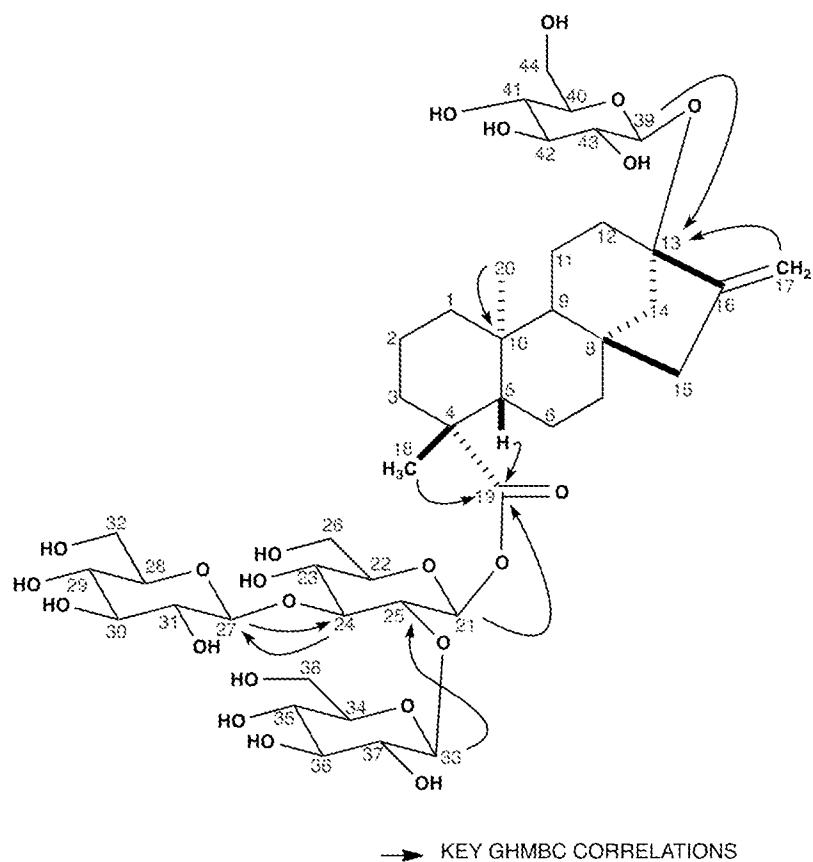


FIG. 16

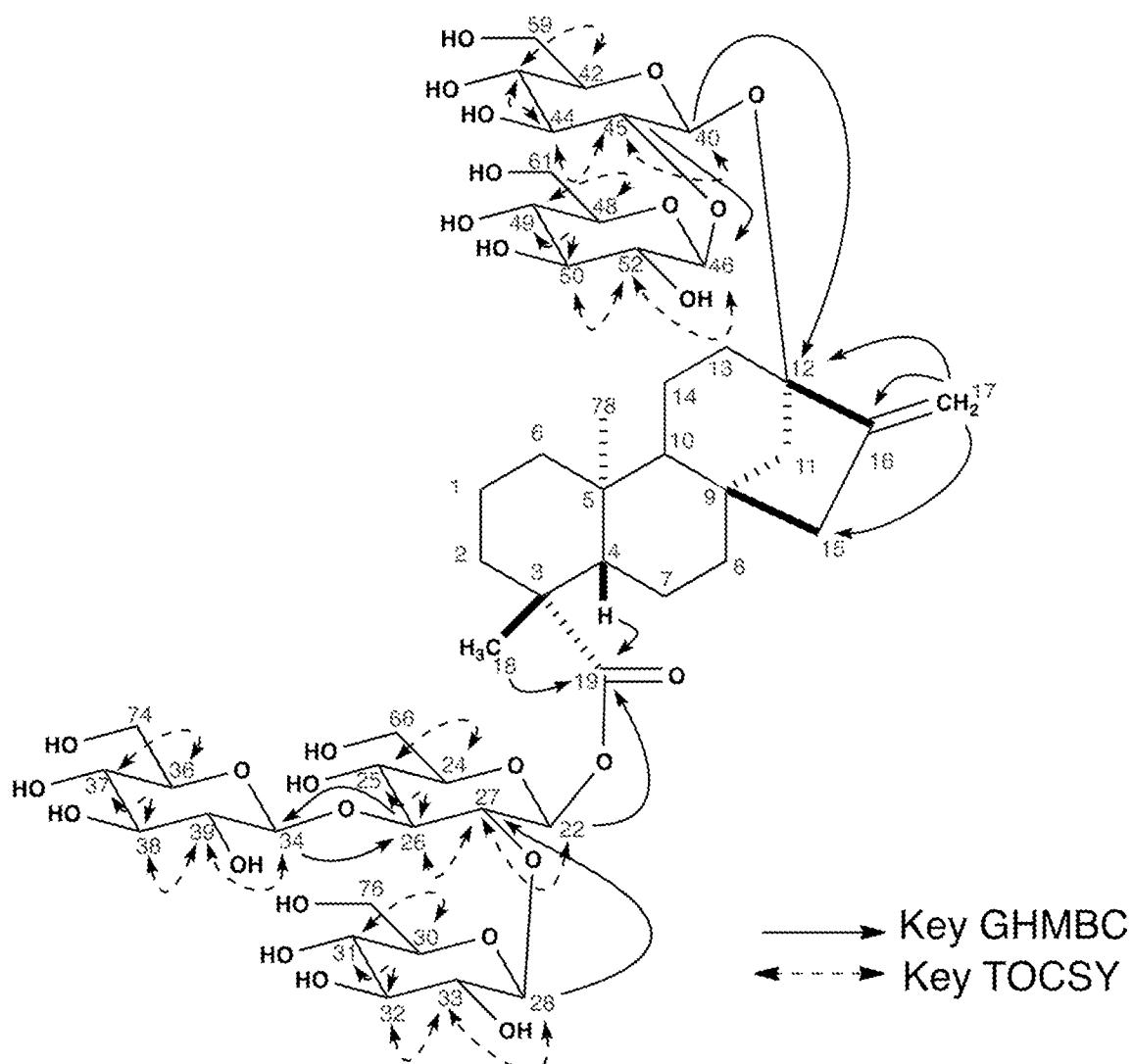


FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/056457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A23L 2/60; A23L 27/30; C12N 1/21 (2017.01)

CPC - A23V 2002/00; A23V 2250/258; C12N 9/1048 (2017.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 426/648; 426/655; 426/658 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-------------------------|
| X | WO 2016/073740 A1 (MANUS BIOSYNTHESIS, INC.) 12 May 2016 (12.05.2016) entire document | 1, 2, 6, 7, 13-16, 31 |
| -- | | ----- |
| Y | US 2011/0091634 A1 (ABELYAN et al) 21 April 2011 (21.04.2011) entire document | 8, 17-21, 23-27, 32, 33 |
| Y | US 2016/0298159 A1 (PEPSICO INC.) 13 October 2016 (13.10.2016) entire document | 8, 23-27 |
| Y | WO 2013/026151 A1 (JUST-BIO INC. et al) 28 February 2013 (28.02.2013) entire document | 17-21 |
| Y | US 2016/0095338 A1 (CONAGEN INC.) 07 April 2016 (07.04.2016) entire document | 19 |
| A | US 2016/0186225 A1 (EVOLVA SA) 30 June 2016 (30.06.2016). entire document | 32, 33 |
| A | TALHA et al. "Analysis of stevioside in Stevia rebaudiana," Journal of Medicinal Plants Research, 23 March 2012 (23.03.2012), Vol. 6, Pgs. 2216-2219. entire document | 1-54 |

| | | | |
|--------------------------|--|--------------------------|--------------------------|
| <input type="checkbox"/> | Further documents are listed in the continuation of Box C. | <input type="checkbox"/> | See patent family annex. |
|--------------------------|--|--------------------------|--------------------------|

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier application or patent but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 "&" document member of the same patent family

| | |
|---|--|
| Date of the actual completion of the international search | Date of mailing of the international search report |
| 19 December 2017 | 18 JAN 2018 |
| Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300 | Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/056457

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. forming part of the international application as filed:
 in the form of an Annex C/ST.25 text file.
 on paper or in the form of an image file.
 - b. furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. furnished subsequent to the international filing date for the purposes of international search only:
 in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOS: 1-10 were searched.