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(54) **HIGH-PURITY STEVIOL GLYCOSIDES**

(71) Applicant: **PURECIRCLE USA INC.**, WESTCHESTER, IL (US)

(72) Inventors: **Avetik MARKOSYAN**, Negeri Sembilan (MY); **Saravanan A/L RAMANDACH**, Negeri Sembilan (MY); **Mohamad Afzaal BIN HASIM**, Negeri Sembilan (MY); **Khairul NIZAM BIN NAWI**, Negeri Sembilan (MY); **Siew Yin CHOW**, Negeri Sembilan (MY)

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C12N 9/10	(2006.01)

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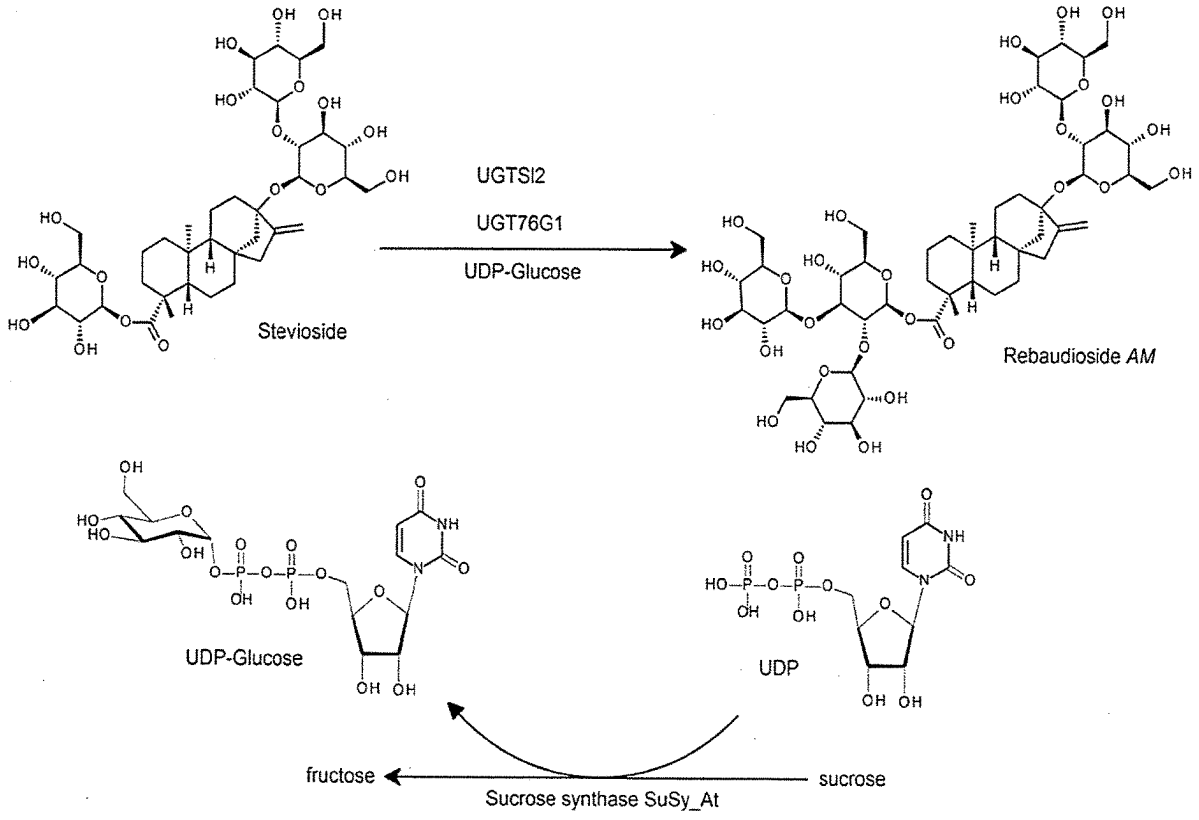
(57) **ABSTRACT**

Methods of preparing highly purified steviol glycosides, particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, and rebaudioside AM are described. The methods include utilizing enzyme preparations and recombinant microorganisms for converting various staring compositions to target steviol glycosides. The highly purified rebaudiosides are useful as non-caloric sweetener, flavor enhancer, sweetness enhancer, and foaming suppressor in edible and chewable compositions such as any beverages, confectioneries, bakery products, cookies, and chewing gums.

Specification includes a Sequence Listing.



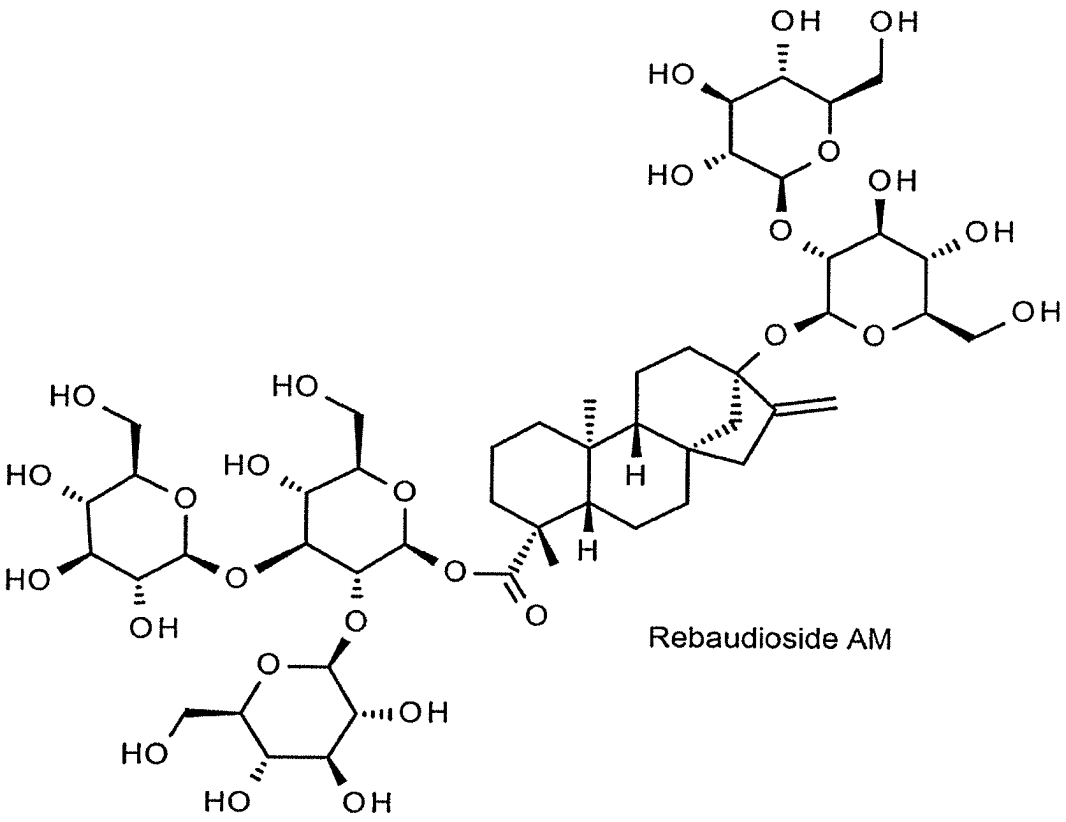


FIG. 1

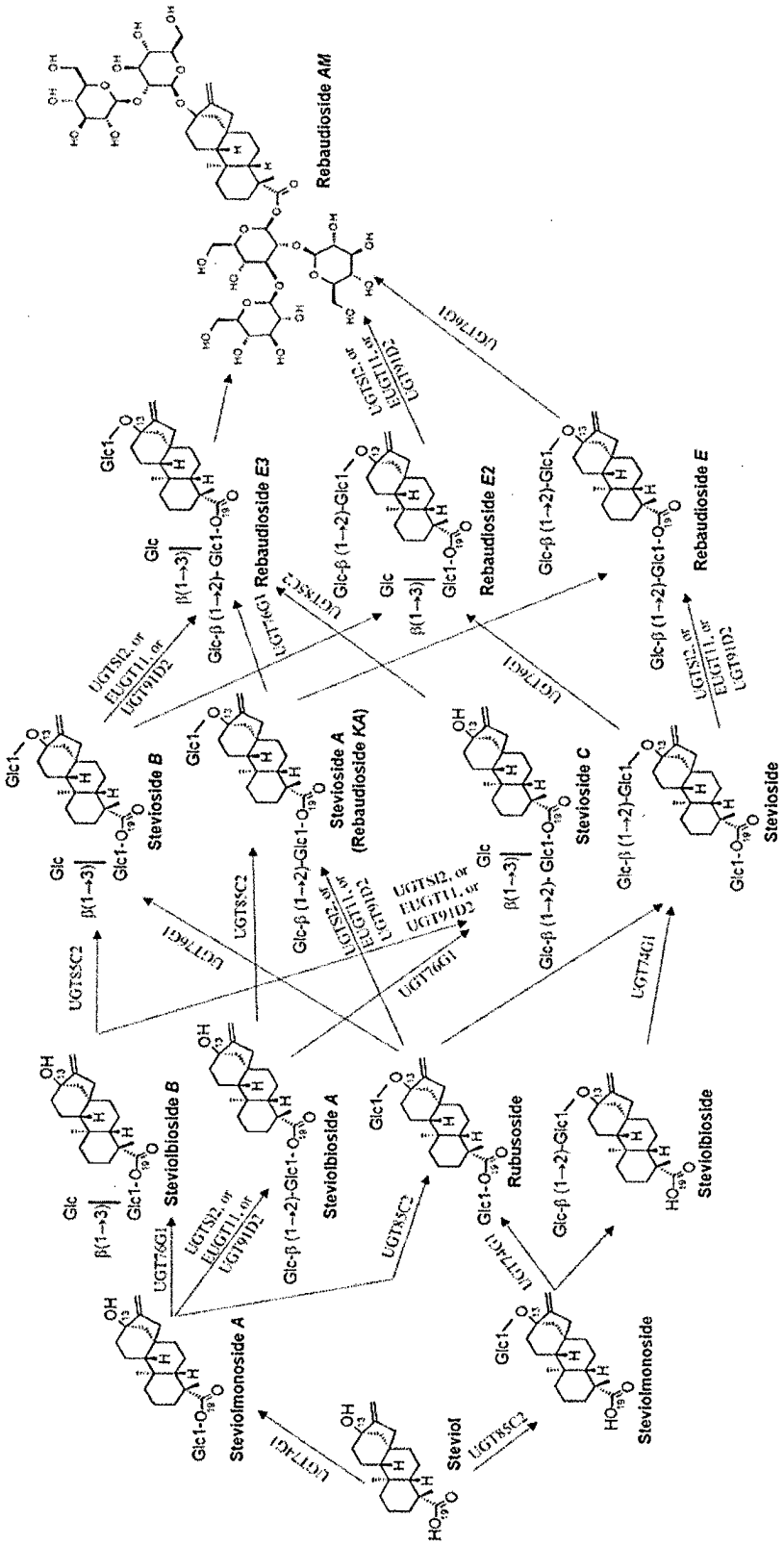

Rebaudioside AM
Rebaudioside E3
Rebaudioside E2
Rebaudioside E
Stevioside B
Stevioside A
(Rebaudioside KA)
Stevioside C
Stevioside
Steviolbioside B
Steviolbioside A
Rubusoside
Steviolbioside
Steviolmonoside A
Steviol
Steviolmonoside
UGT76G1
UGT85C2
UGTSI2, or EUGT11, or UGT91D2

FIG. 2

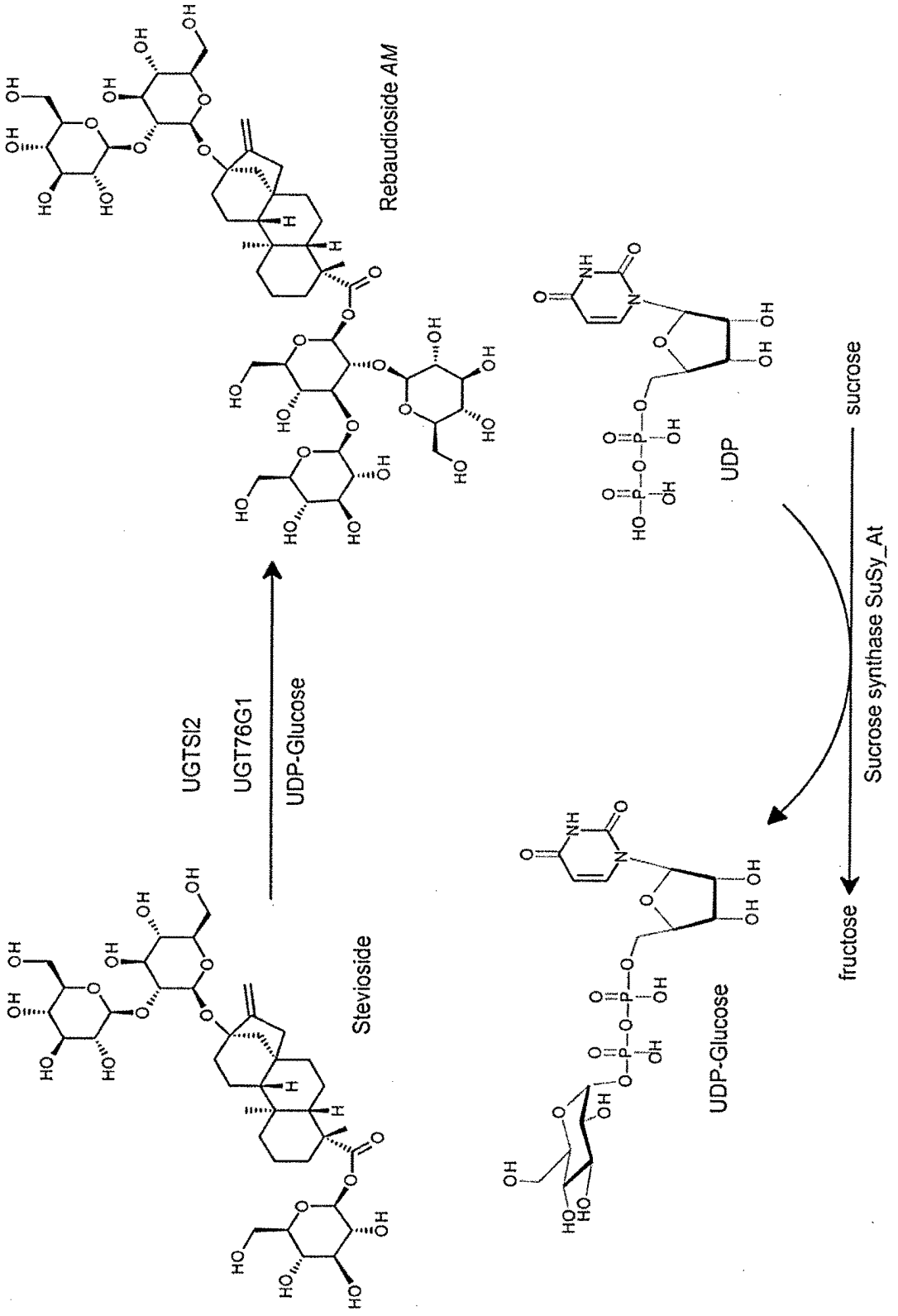

Rebaudioside AM
UGTSl2
UGT76G1
UDP-Glucose
Stevioside
UDP
sucrose
Sucrose synthase SuSy_At
fructose
UDP-Glucose

FIG. 3

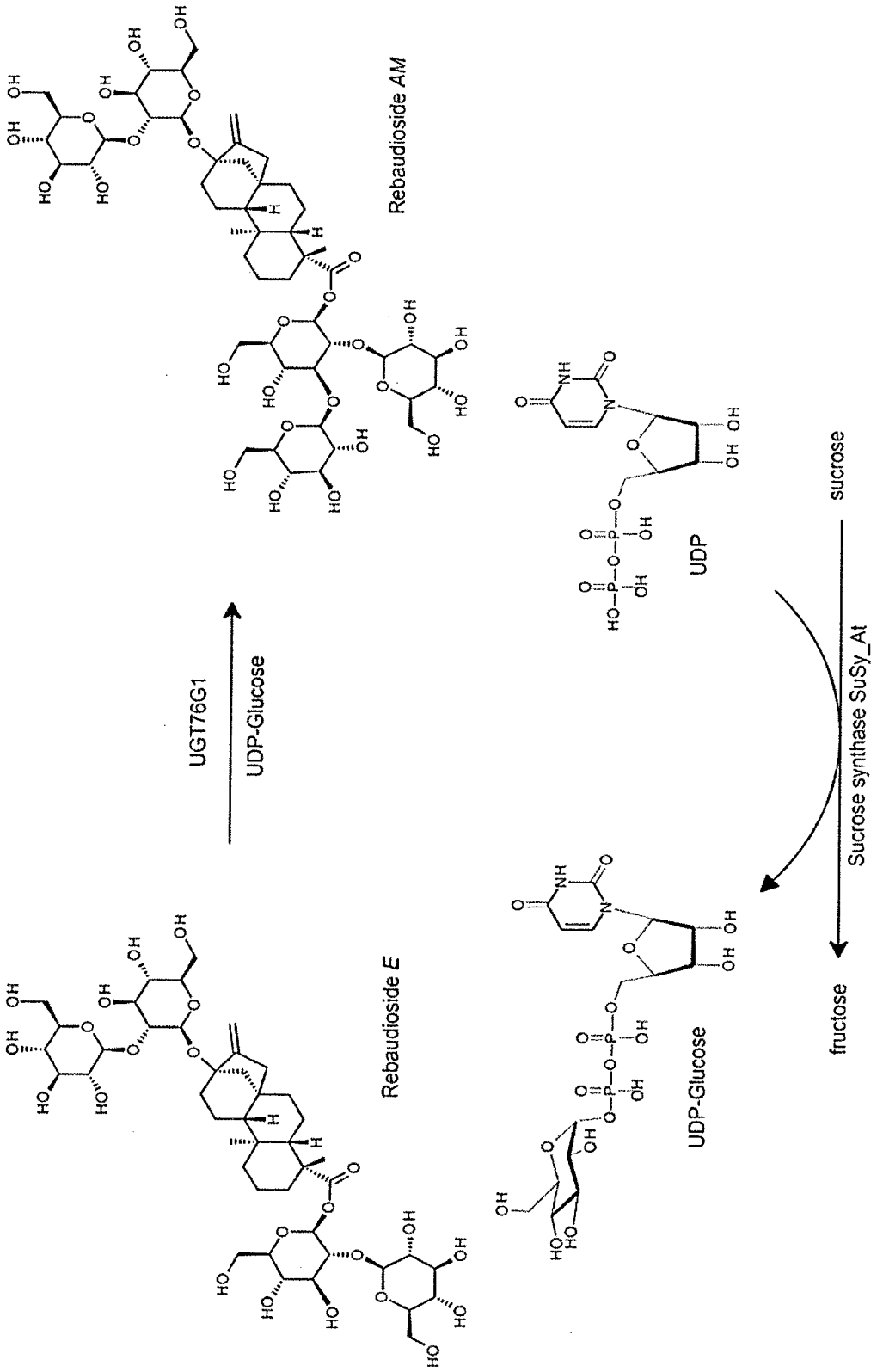

Rebaudioside AM
UGT76G1
UDP-Glucose
Rebaudioside E
UDP
sucrose
Sucrose synthase SuSy_At
UDP-Glucose
fructose

FIG. 4

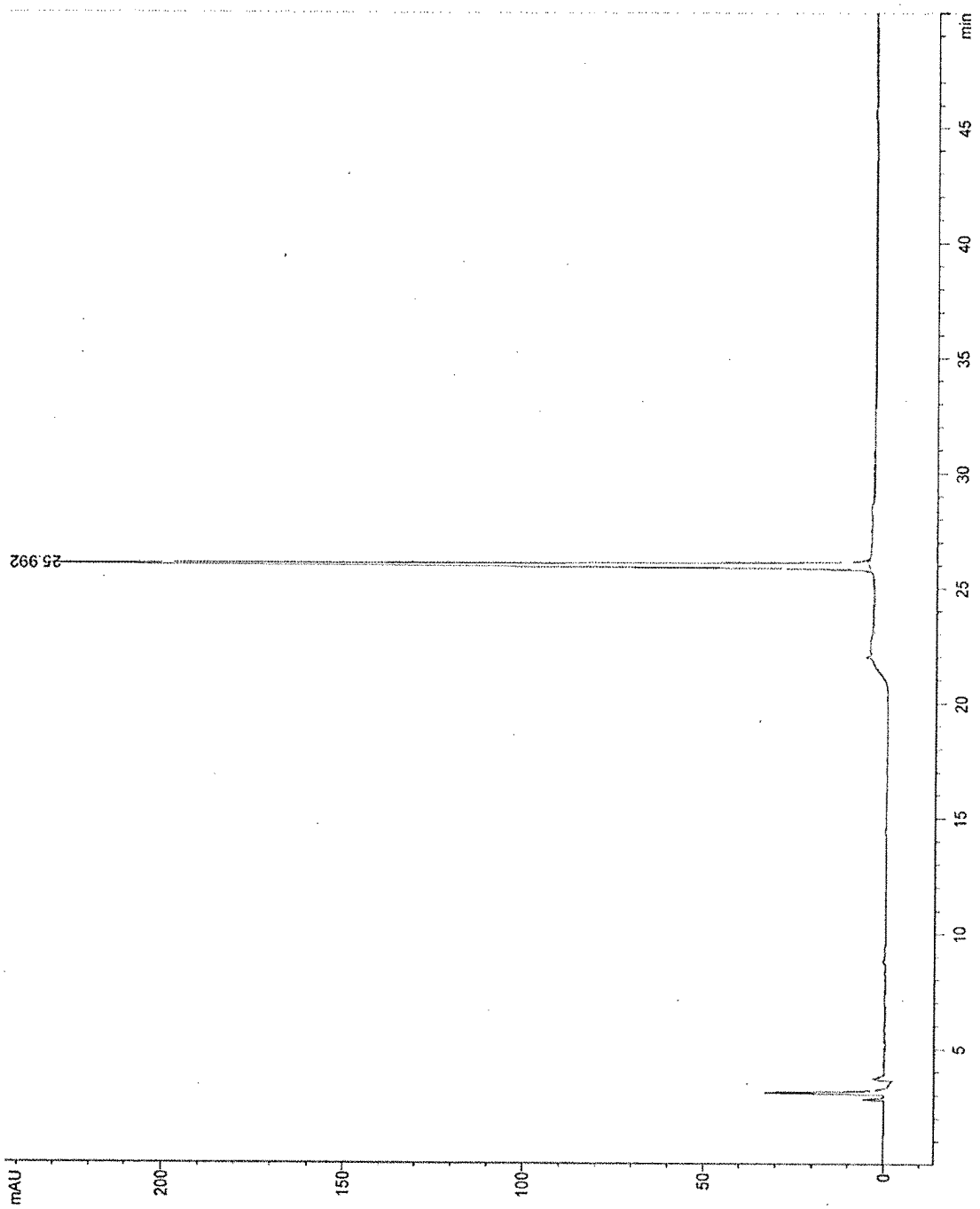


FIG. 5

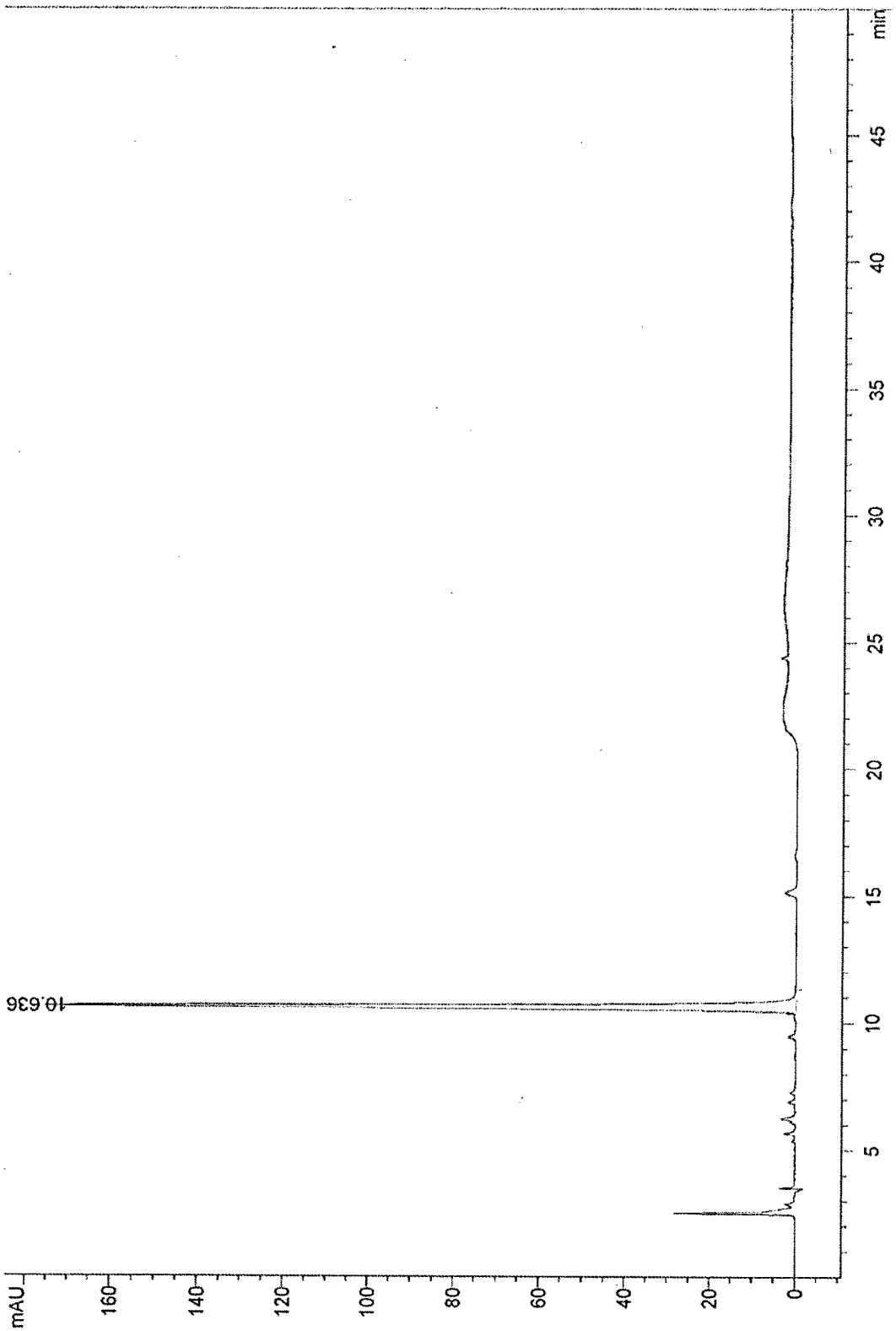


FIG. 6

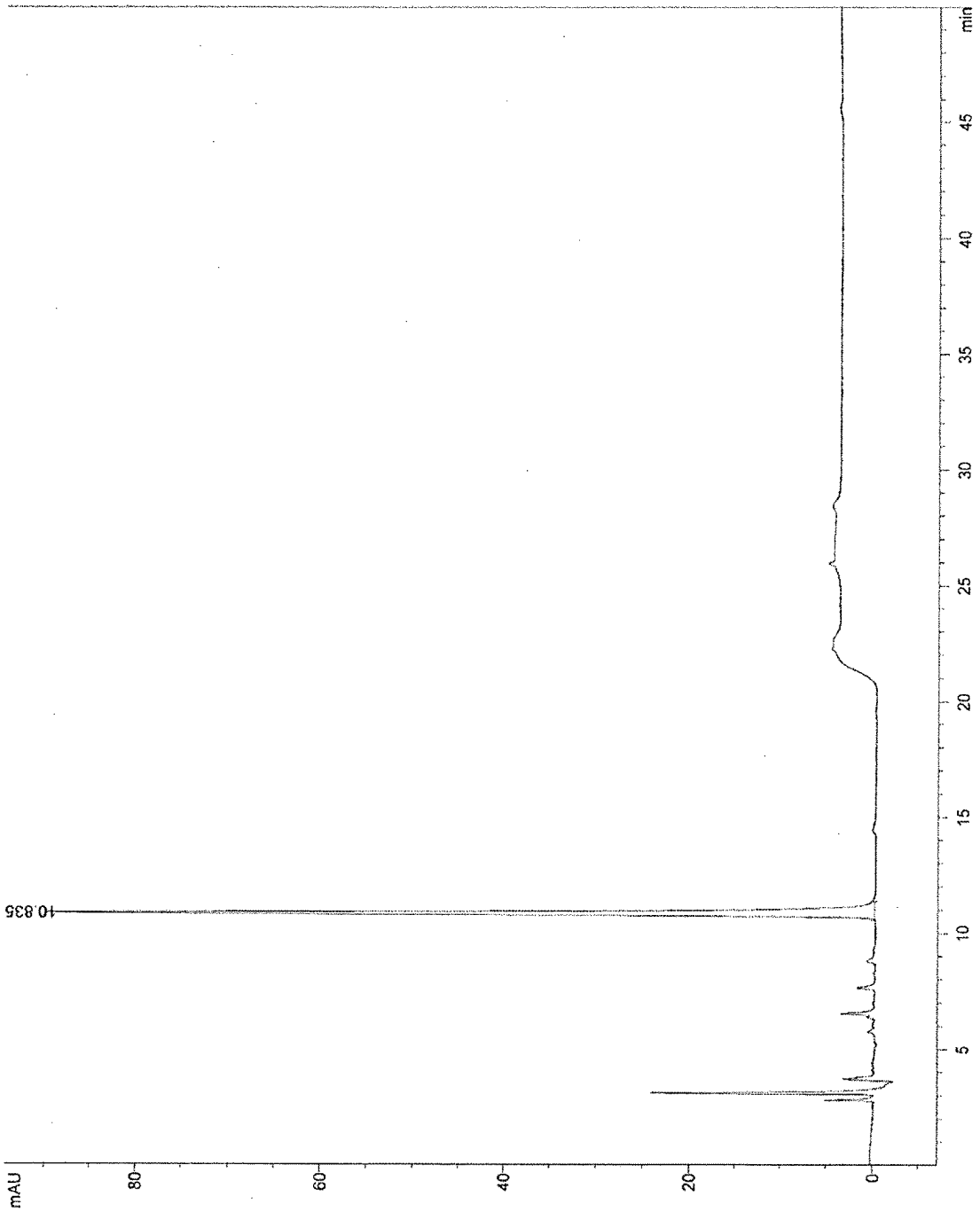


FIG. 7

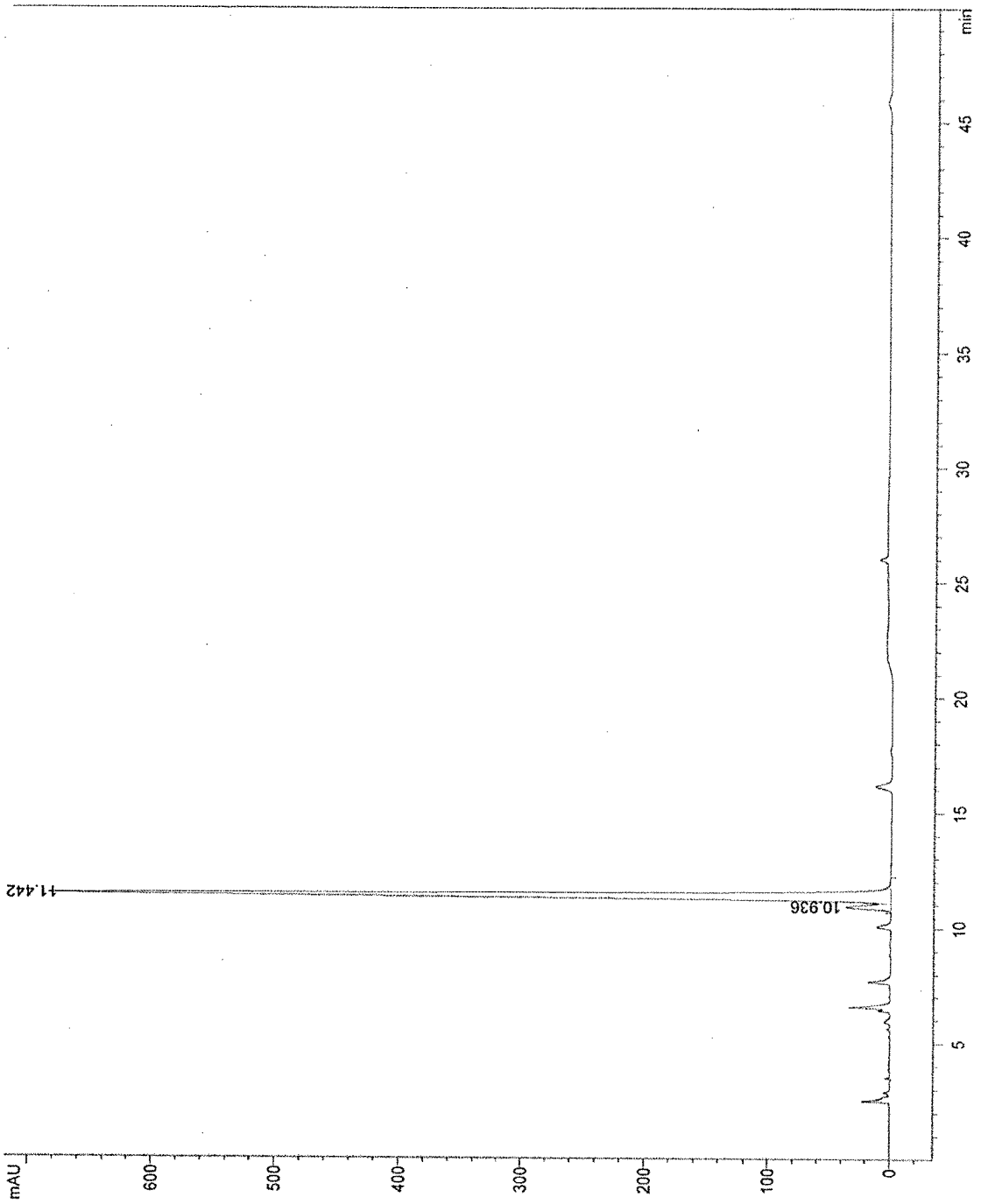

mAU
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
45
min
11.442
10.936

FIG. 8

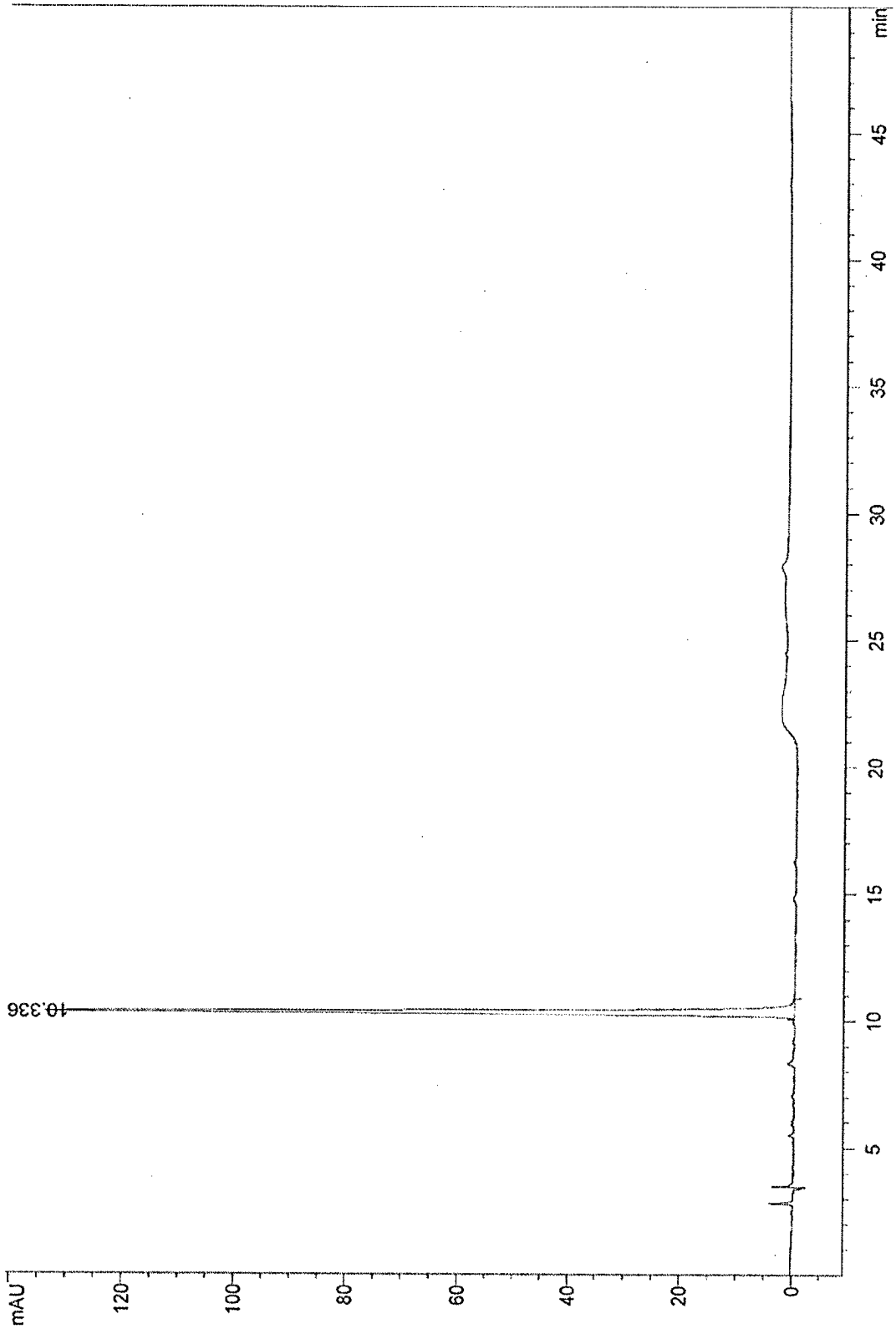
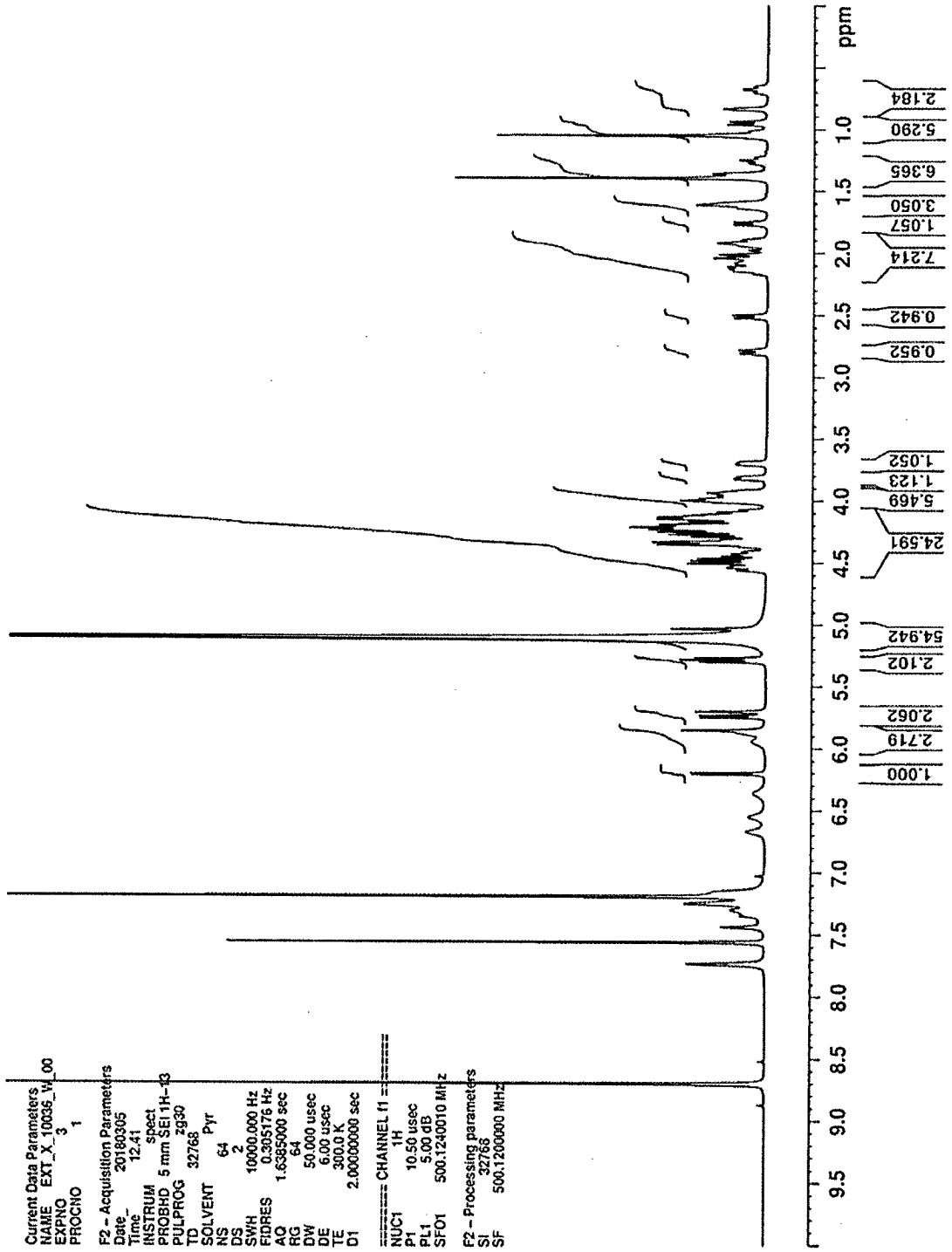


FIG. 9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PROCNO 1
F2 - Acquisition Parameters
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Time 12.41
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PULPROG zg30
TD 32768
SOLVENT Pyr
NS 64
DS 2
SWH 10000.000 Hz
FIDRES 0.305176 Hz
AQ 1.6385000 sec
RG 64
DW 50.000 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 10.50 usec
PL1 5.00 dB
SFO1 500.1240010 MHz
F2 - Processing parameters
SI 32768
SF 500.1200000 MHz
ppm
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
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1.5
1.0
1.000
2.719
2.062
2.102
54.942
24.591
5.469
1.123
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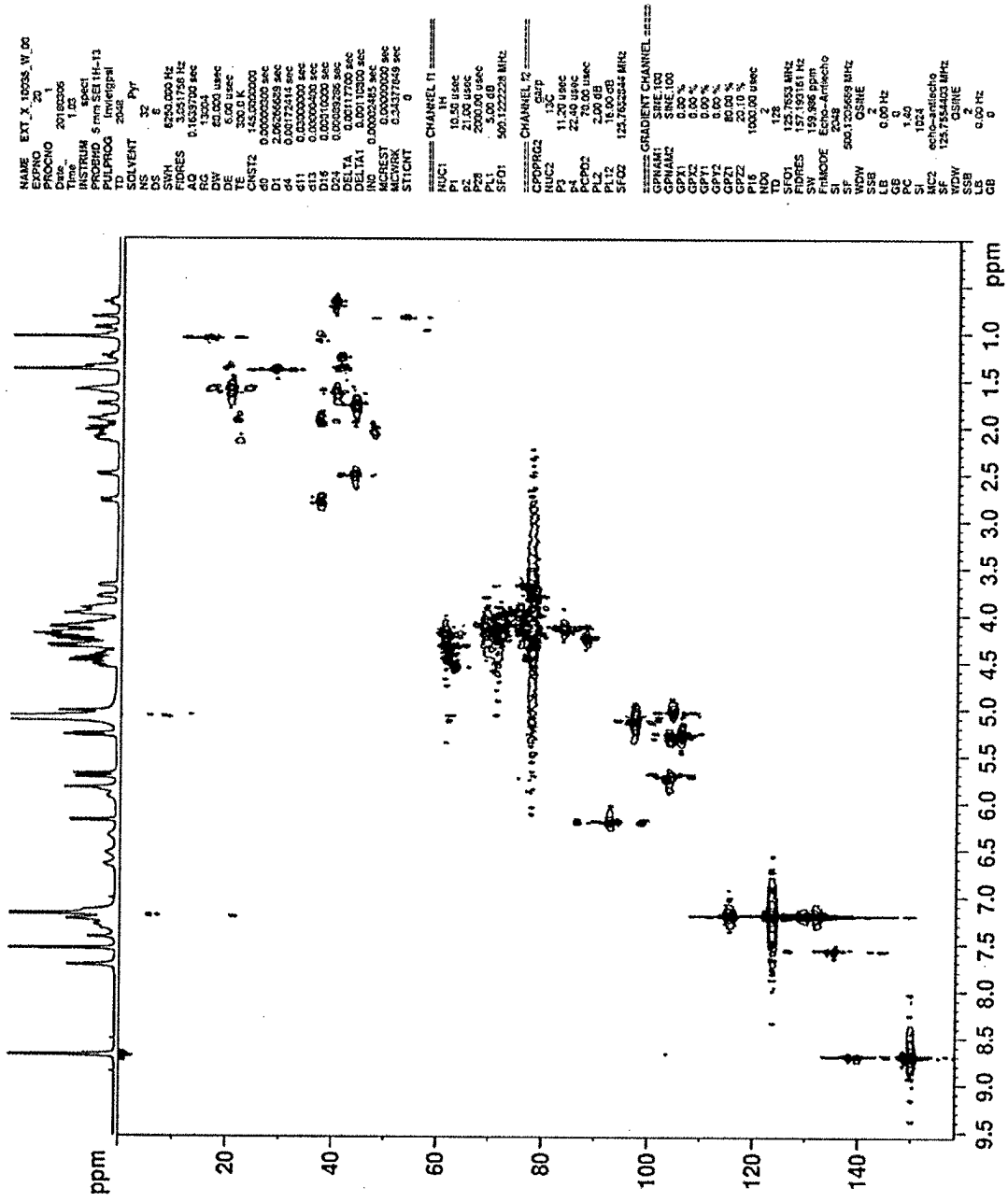
0.952
0.942
7.214
1.057
3.050
6.365
5.290
2.184

FIG. 10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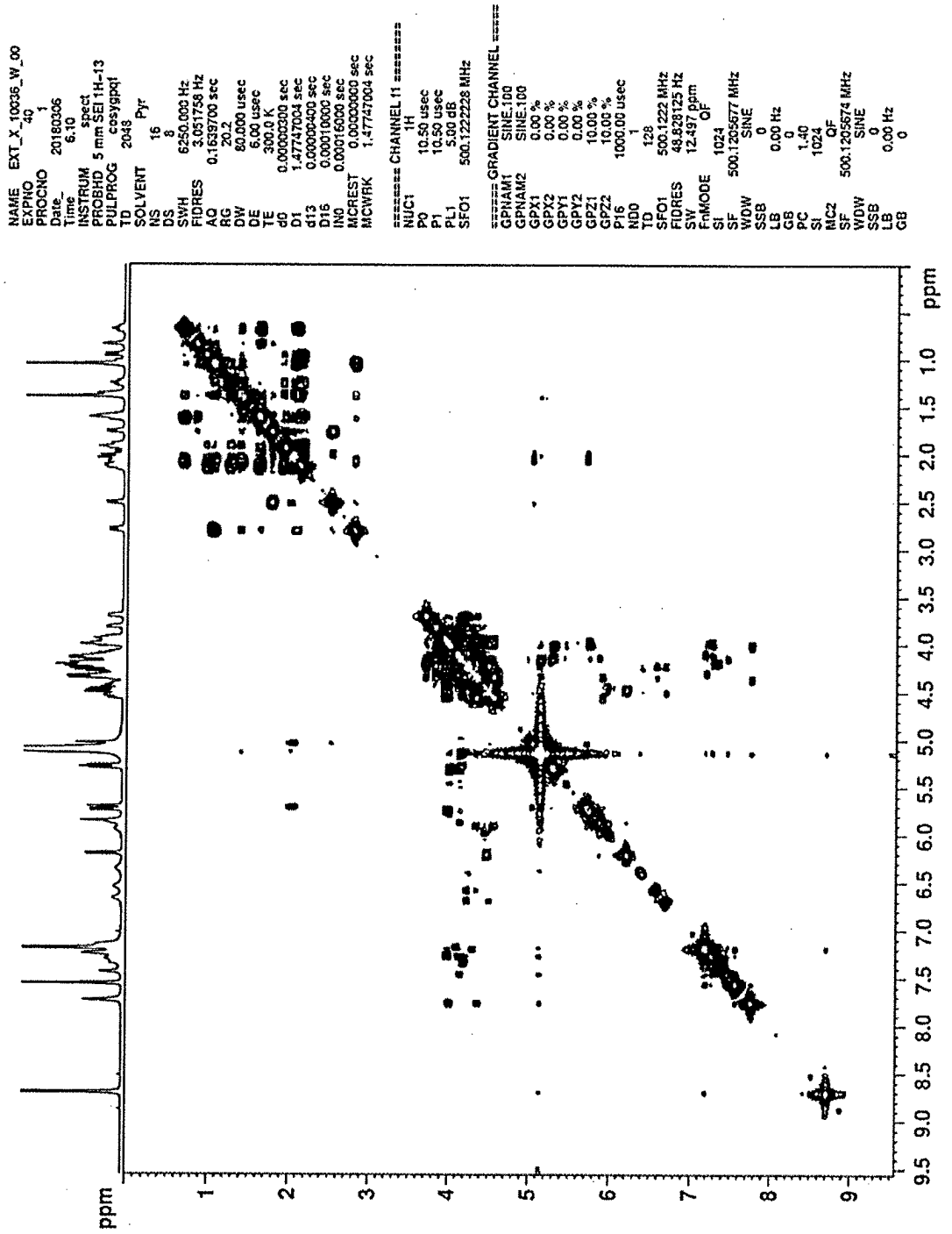
PULPROG invietgpsi
TD 2048
SOLVENT Pyr
NS 32
DS 8
SWH 8250.000 Hz
FIDRES 3.251758 Hz
AQ 0.16537700 sec
RG 1334.1
DW 62.000 usec
DE 6.00 usec
TE 330.0 K
CNST2 145.0000000
D0 0.000003300 sec
D1 2.00000000 sec
D4 0.00172414 sec
d11 0.03000000 sec
d13 0.00004400 sec
D16 0.00010000 sec
D24 0.00689655 sec
DELTA 0.00172500 sec
DELTA1 0.00115000 sec
IN0 0.00002485 sec
MCREST 0.00000000 sec
MCWRK 0.34377649 sec
ST1CNT 0
CHANNEL f1
NUC1 1H
P1 10.50 usec
P2 21.00 usec
PCPD2 200.00 usec
PL1 3.00 dB
SFO1 500.1222228 MHz
CHANNEL f2
CPDPRG2 garp
NUC2 13C
P3 11.20 usec
P4 22.40 usec
PCPD2 70.00 usec
PL2 2.00 dB
PL12 16.00 dB
SFO2 125.7652044 MHz
GRADIENT CHANNEL
GPNAM1 SINE.100
GPNAM2 SINE.100
GPX1 0.00 %
GPX2 0.00 %
GPY1 0.00 %
GPY2 0.00 %
GPZ1 80.00 %
GPZ2 -20.10 %
P16 1000.00 usec
ND0 2
TD 128
SFO1 125.7553 MHz
FIDRES 157.193161 Hz
SW 159.896 ppm
FnMODE Echo-Antiecho
SI 2048
SF 500.1225659 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.40
SI 1024
MC2 echo-antiecho
SF 125.7554403 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0
ppm
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm
20 40 60 80 100 120 140

FIG. 11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TD 2048
SOLVENT Pyr
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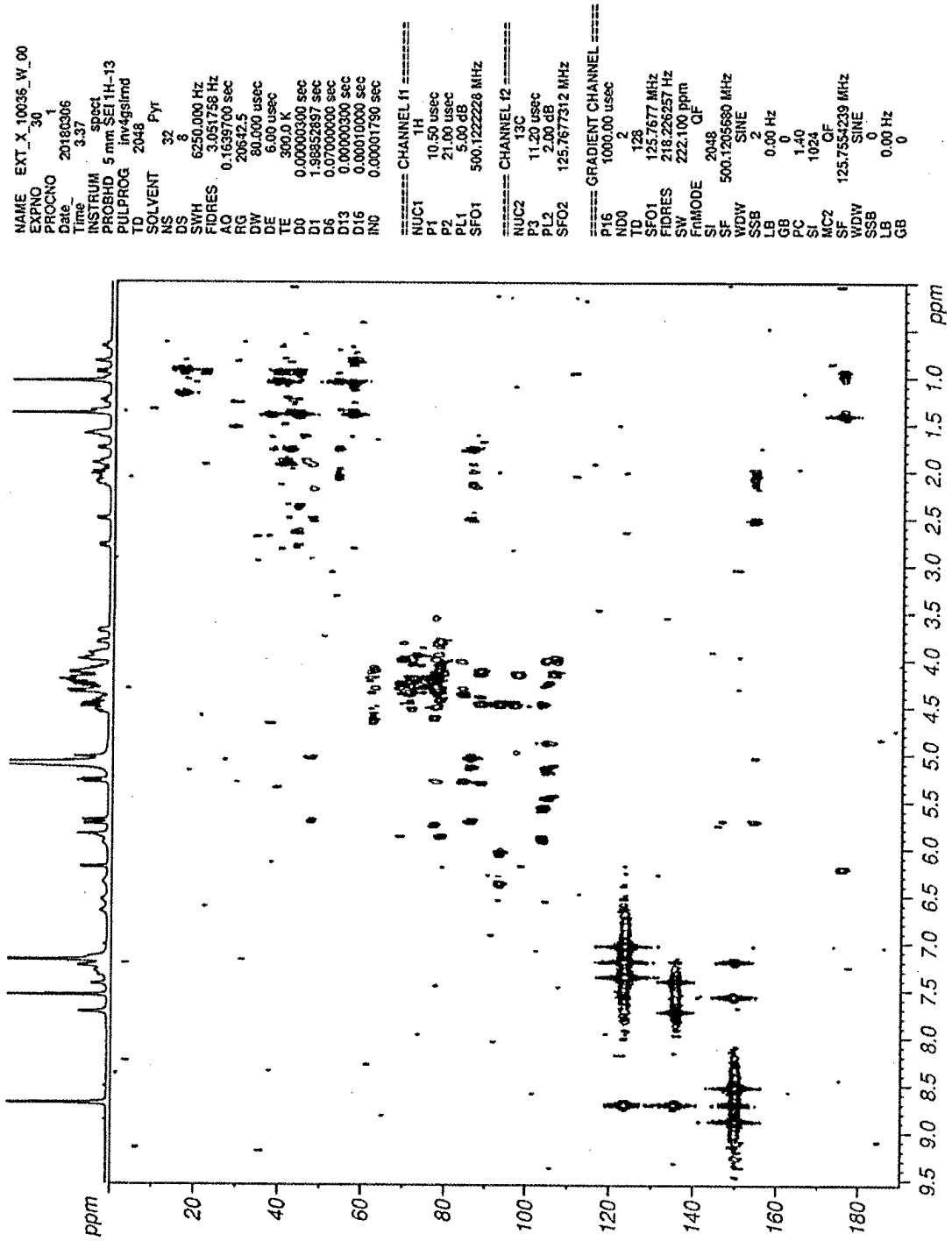
DS 8
SWH 6250.003 Hz
FIDRES 3.051758 Hz
AQ 0.1639700 sec
RG 20.2
DW 80.000 usec
DE 6.00 usec
TE 300.0 K
d0 0.00000300 sec
D1 1.47747004 sec
d13 0.00000400 sec
D16 0.00010000 sec
IN0 0.00016000 sec
MCREST 0.00000000 sec
MCWRK 1.47747004 sec
======= CHANNEL f1 ========
NUC1 1H
P0 10.50 usec
P1 10.50 usec
PL1 5.00 dB
SFO1 500.1222228 MHz
====== GRADIENT CHANNEL =====
GPNAM1 SINE.100
GPNAM2 SINE.100
GPX1 0.00 %
GPX2 0.00 %
GPY1 0.00 %
GPY2 0.00 %
GPZ1 10.00 %
GPZ2 10.00 %
P16 1000.00 usec
ND0 1
TD 128
SFO1 500.1222 MHz
FIDRES 48.828125 Hz
SW 12.497 ppm
FnMODE QF
SI 1024
SF 500.1205677 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
SI 1024
MC2 QF
SF 500.1205674 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
ppm
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm
1 2 3 4 5 6 7 8 9

FIG. 12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PROCNO 1
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Time 3.37
INSTRUM spect
PROBHD 5 mm SEI 1H-13
PULPROG inv4gsimd
TD 2048
SOLVENT Pyr
NS 32
DS 8
SWH 6250.000 Hz
FIDRES 3.051758 Hz
AQ 0.16397000 sec
RG 20642.5
DW 80.000 usec
DE 6.00 usec
TE 300.0 K
D0 0.00000300 sec
D1 1.98852897 sec
D6 0.07000000 sec
D13 0.00000300 sec
D16 0.00010000 sec
IN0 0.00001790 sec
======== CHANNEL f1 ========
NUC1 1H
P1 10.50 usec
P2 21.00 usec
PL1 5.00 dB
SFO1 500.1222228 MHz
======== CHANNEL f2 ========
NUC2 13C
P3 11.20 usec
PL2 2.00 dB
SFO2 125.7677312 MHz
====== GRADIENT CHANNEL =====
P16 1000.00 usec
ND0 2
TD 128
SFO1 125.7677 MHz
FIDRES 218.226257 Hz
SW 222.100 ppm
FnMODE QF
SI 2048
SF 500.1205680 MHz
WDW SINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.40
SI 1024
MC2 QF
SF 125.7554239 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
ppm
20
40
60
80
100
120
140
160
180
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm

FIG. 13

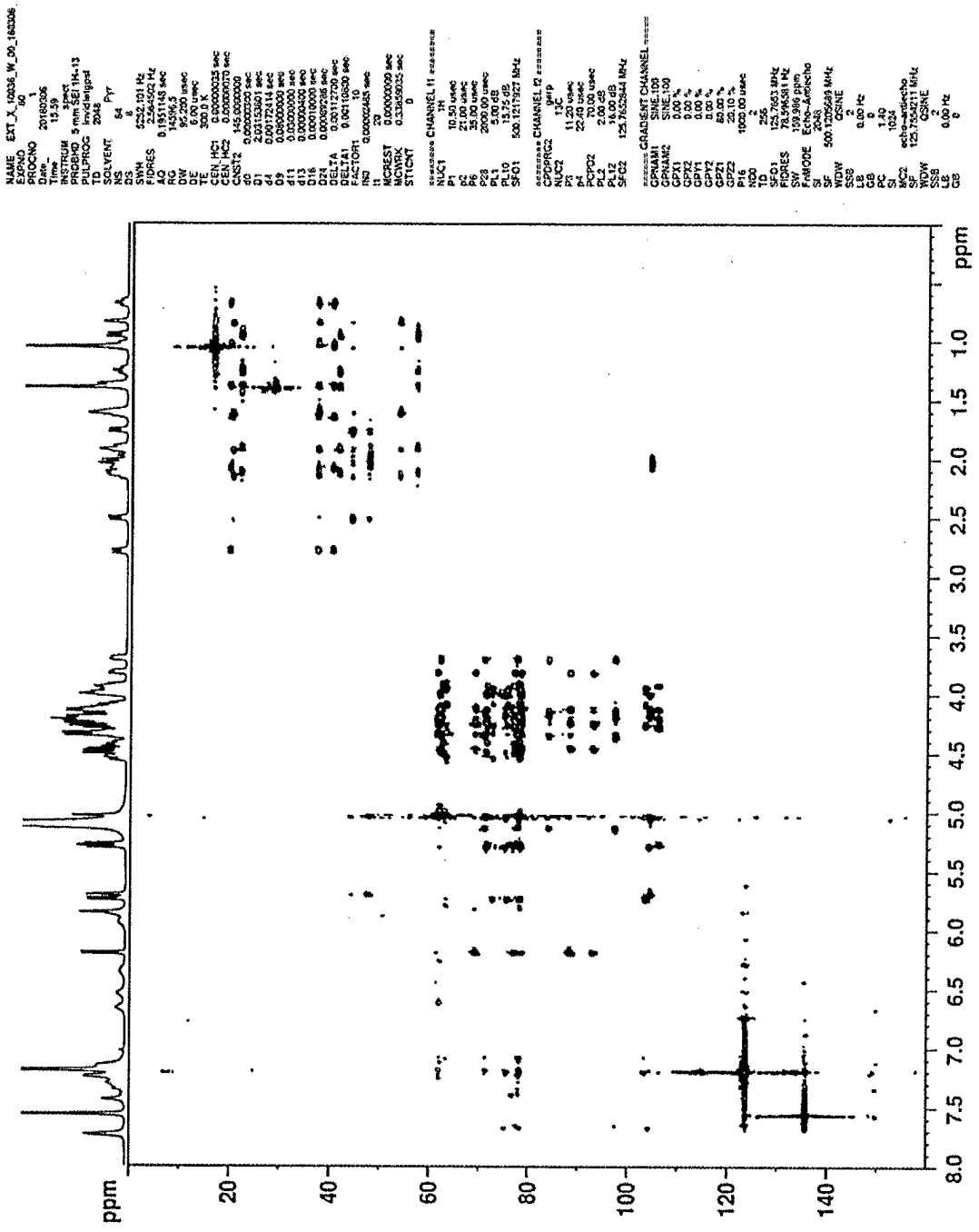
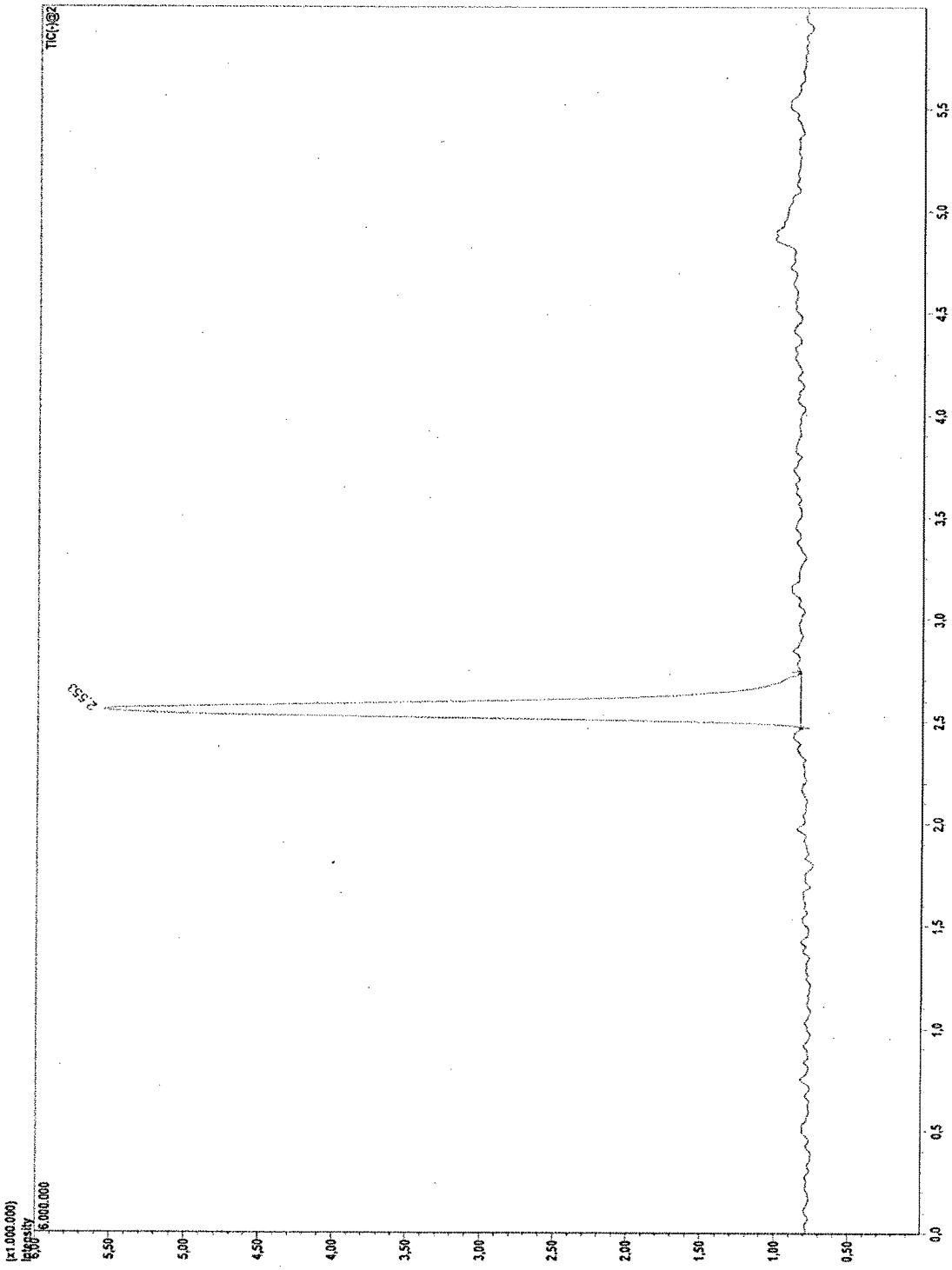


FIG. 14


TIC(+)@2
2.553
(x1,000,000)
Intensity
6,000,000
6,50
6,00
5,50
5,00
4,50
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3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,0
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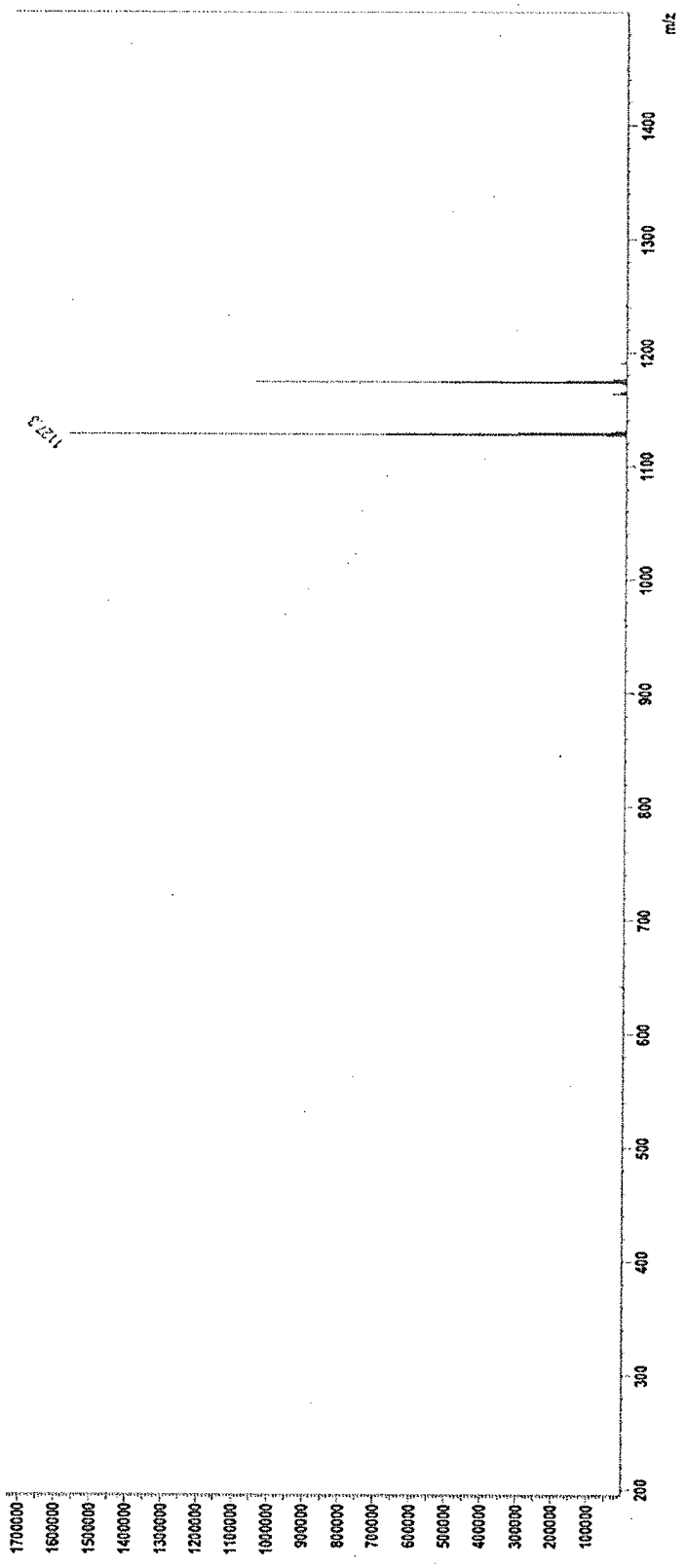
4,0
4,5
5,0
5,5

FIG. 15a


m/z
1123.3
200
300
400
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400000
500000
600000
700000
800000
900000
1000000
1100000
1200000
1300000
1400000
1500000
1600000
1700000

FIG. 15b

HIGH-PURITY STEVIOL GLYCOSIDES

TECHNICAL FIELD

[0001] The present invention relates to a process for preparing compositions comprising steviol glycosides, including highly purified steviol glycoside compositions.

BACKGROUND OF THE INVENTION

[0002] High intensity sweeteners possess a sweetness level that is many times greater than the sweetness level of sucrose. They are essentially non-caloric and are commonly used in diet and reduced-calorie products, including foods and beverages. High intensity sweeteners do not elicit a glycemic response, making them suitable for use in products targeted to diabetics and others interested in controlling for their intake of carbohydrates.

[0003] Steviol glycosides area class of compounds found in the leaves of *Stevia rebaudiana* Bertoni, a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America. They are characterized structurally by a single base, steviol, differing by the presence of carbohydrate residues at positions C13 and C19. They accumulate in *Stevia* leaves, composing approximately 10%-20% of the total dry weight. On a dry weight basis, the four major glycosides found in the leaves of *Stevia* typically include stevioside (9.1%), rebaudioside A (3.8%), rebaudioside C (0.6-1.0%) and dulcoside A (0.3%). Other known steviol glycosides include rebaudioside B, C, D, E, F and M, steviolbioside and rubusoside.

[0004] Although methods are known for preparing steviol glycosides from *Stevia rebaudiana*, many of these methods are unsuitable for use commercially.

[0005] Accordingly, there remains a need for simple, efficient, and economical methods for preparing compositions comprising steviol glycosides, including highly purified steviol glycoside compositions.

SUMMARY OF THE INVENTION

[0006] The present invention provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell and/or enzyme preparation, thereby producing a composition comprising a target steviol glycoside.

[0007] The starting composition can be any organic compound comprising at least one carbon atom. In one embodiment, the starting composition is selected from the group consisting of steviol glycosides, polyols or sugar alcohols, various carbohydrates.

[0008] The target steviol glycoside can be any steviol glycoside. In one embodiment, the target steviol glycoside is steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or a synthetic steviol glycoside.

[0009] In one embodiment, the target steviol glycoside is rebaudioside AM.

[0010] In some preferred embodiments enzyme preparation comprising one or more enzymes, or a microbial cell comprising one or more enzymes, capable of converting the starting composition to target steviol glycosides are used. The enzyme can be located on the surface and/or inside the cell. The enzyme preparation can be provided in the form of a whole cell suspension, a crude lysate or as purified enzyme(s). The enzyme preparation can be in freeform or immobilized to a solid support made from inorganic or organic materials.

[0011] In some embodiments, a microbial cell comprises the necessary enzymes and genes encoding thereof for converting the starting composition to target steviol glycosides. Accordingly, the present invention also provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell comprising at least one enzyme capable of converting the starting composition to target steviol glycosides, thereby producing a medium comprising at least one target steviol glycoside.

[0012] The enzymes necessary for converting the starting composition to target steviol glycosides include the steviol biosynthesis enzymes, UDP-glucosyltransferases (UGTs) and/or UDP-recycling enzyme.

[0013] In one embodiment, the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

[0014] In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzymes.

[0015] In one embodiment the steviol biosynthesis enzymes are selected from the group including geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated H M G-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.

[0016] The UDP-glucosyltransferase can be any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol and/or a steviol glycoside substrate to provide the target steviol glycoside.

[0017] As used hereinafter, the term "SuSy_AT", unless specified otherwise, refers to sucrose synthase having amino-acid sequence "SEQ ID 1" as described in Example 1.

[0018] As used hereinafter, the term "UGTS12", unless specified otherwise, refers to UDP-glucosyltransferase having amino-acid sequence "SEQ ID 2" as described in Example 1.

[0019] As used hereinafter, the term "UGT76G1", unless specified otherwise, refers to UDP-glucosyltransferase having amino-acid sequence "SEQ ID 3" as described in Example 1.

[0020] In one embodiment, steviol biosynthesis enzymes and UDP-glucosyltransferases are produced in a microbial cell. The microbial cell may be, for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc. In another embodiment, the UDP-glucosyltransferases are synthesized.

[0021] In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2,

UGT76G1, UGT91D2, UGTS12, EUGT11 and UGTs having substantial (>85%, >86%, >87%, >88%, >89%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, >99%) amino-acid sequence identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.

[0022] In one embodiment, steviol biosynthesis enzymes, UGTs and UDP-glucose recycling system are present in one microorganism (microbial cell). The microorganism may be for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.

[0023] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capableof adding at least one glucose unit to steviol orany starting steviol glycoside bearing an —OH functional group at C13 to give a target steviol glycoside having an —O-glucose beta glucopyranoside glycosidic linkage at C13. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2, or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0024] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing a —COOH functional group at C19 to give a target steviol glycoside having a —COO-glucose beta-glucopyranoside glycosidic linkage at C19. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1, or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0025] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1→2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0026] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1→3 glucopyranoside glycosidic linkage(s) at the newly formed bond glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGT76G1, or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0027] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C13 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1→2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0028] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capableof adding at least one glucose unit to steviol to form steviolmonoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0029] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside A. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0030] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0031] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside A. In a particular embodiment, the UDP-glucosyltransferase is UGTSI2 or a UGT having >85% amino-acid sequence identity with UGTSI2. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0032] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0033] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0034] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form steviolbioside.

[0035] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0036] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransfer-

ase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0037] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside A. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0038] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0039] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0040] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside A (rebaudioside KA). In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0041] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside.

[0042] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside to form stevioside. In a particular embodiment, the UDP-glucosyl transferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0043] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0044] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E2.

[0045] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0046] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E.

[0047] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside C to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0048] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E2. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0049] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0050] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E3 to form rebaudioside AM.

[0051] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E2 to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0052] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0053] Optionally, the method of the present invention further comprises using more than one UGT on a starting composition, to give a target steviol glycoside(s) having more than one glucose unit than the starting composition. In a particular embodiment, the UDP-glucosyltransferases are UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any UGT having >85% amino-acid sequence identity with UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any combination thereof, capable of adding more than one glucose unit to a starting composition to give a steviol glycoside(s) having more than one glucose unit than the starting composition.

[0054] In one embodiment, the UDP-glucosyltransferases are any UDP-glucosyltransferases capable of adding overall two glucose unit to stevioside to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferases are

selected from UGTS12, EUGT11, UGT91D2, UGT76G1 or any UGT having >85% amino-acid sequence identity with UGTS12, EUGT11, UGT91D2, UGT76G1 or any combination thereof. In another particular embodiment, the UDP-glucosyltransferases are UGTS12 and UGT76G1.

[0055] Optionally, the method of the present invention further comprises recycling UDP to provide UDP-glucose. In one embodiment, the method comprises recycling UDP by providing a recycling catalyst and a recycling substrate, such that the biotransformation of steviol and/or the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose.

[0056] In one embodiment, the recycling catalyst is sucrose synthase SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At.

[0057] In one embodiment, the recycling substrate is sucrose.

[0058] Optionally, the method of the present invention further comprises the use of transglycosidases that use oligo- or poly-saccharides as the sugar donor to modify recipient target steviol glycoside molecules. Non-limiting examples include cyclodextrin glycosyltransferase (CGTase), fructofuranosidase, amylase, saccharase, glucosucrase, beta-h-fructosidase, beta-fructosidase, sucrase, fructosylinvertase, alkaline invertase, acid invertase, fructofuranosidase. In some embodiments, glucose and sugar(s) other than glucose, including but not limited to fructose, xylose, rhamnose, arabinose, deoxyglucose, galactose are transferred to the recipient target steviol glycosides. In one embodiment, the recipient steviol glycoside is rebaudioside AM.

[0059] Optionally, the method of the present invention further comprises separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition. The target steviol glycoside can be separated by at least one suitable method, such as, for example, crystallization, separation by membranes, centrifugation, extraction, chromatographic separation or a combination of such methods.

[0060] In one embodiment, the target steviol glycoside can be produced within the microorganism. In another embodiment, the target steviol glycoside can be secreted out in the medium. In one another embodiment, the released steviol glycoside can be continuously removed from the medium. In yet another embodiment, the target steviol glycoside is separated after the completion of the conversion reaction.

[0061] In one embodiment, separation produces a composition comprising greater than about 80% by weight of the target steviol glycoside on an anhydrous basis, i.e., a highly purified steviol glycoside composition. In another embodiment, separation produces a composition comprising greater than about 90% by weight of the target steviol glycoside. In particular embodiments, the composition comprises greater than about 95% by weight of the target steviol glycoside. In other embodiments, the composition comprises greater than about 99% by weight of the target steviol glycoside.

[0062] The target steviol glycoside can be in any polymorphic or amorphous form, including hydrates, solvates, anhydrous or combinations thereof.

[0063] Purified target steviol glycosides can be used in consumable products as a sweetener, flavor modifier, flavor with modifying properties and/or foaming suppressor. Suitable consumable products include, but are not limited to, food, beverages, pharmaceutical compositions, tobacco products, nutraceutical compositions, oral hygiene compositions, and cosmetic compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0064] FIG. **1** shows the chemical structure of rebaudioside AM.

[0065] FIG. **2** shows the pathways of producing rebaudioside AM and various steviol glycosides from steviol.

[0066] FIG. **3** shows the biocatalytic production of rebaudioside AM from stevioside using the enzymes UGTS12 and UGT76G1 and concomitant recycling of UDP to UDP-glucose via sucrose synthase SuSy_At.

[0067] FIG. **4** shows the biocatalytic production of rebausioside AM from rebaudioside E using the enzyme UGT76G1 and concomitant recycling of UDP to UDP-glucose via sucrose synthase SuSy_At.

[0068] FIG. **5** shows the HPLC chromatogram of stevioside. The peak with retention time of 25.992 minutes corresponds to stevioside.

[0069] FIG. **6** shows the HPLC chromatogram of the product of the biocatalytic production of rebaudioside AM from stevioside. The peak with retention time of 10.636 minutes corresponds to rebaudioside AM.

[0070] FIG. **7** shows the HPLC chromatogram of rebaudioside E. The peak with retention time of 10.835 minutes corresponds to rebaudioside E.

[0071] FIG. **8** shows the HPLC chromatogram of the product of the biocatalytic production of rebaudioside AM from rebaudioside E. The peaks with retention time of 10.936 and 11.442 minutes correspond to rebaudioside E and rebaudioside AM respectively.

[0072] FIG. **9** shows the HPLC chromatogram of rebaudioside AM after purification by methanol crystallization. The peak with retention time of 10.336 minutes corresponds to rebaudioside AM.

[0073] FIG. **10** shows the ^{1}HNMR spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0074] FIG. **11** shows the HSQC spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0075] FIG. **12** shows the H,H COSY spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0076] FIG. **13** shows the HMBC spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0077] FIG. **14** shows the HSQC-TOCSY spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0078] FIG. **15***a* and FIG. **15***b* show the LC chromatogram and mass spectrum of rebaudioside AM respectively.

DETAILED DESCRIPTION

[0079] The present invention provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell and/or enzyme preparation, thereby producing a composition comprising a target steviol glycoside.

[0080] One object of the invention is to provide an efficient biocatalytic method for preparing target steviol glycosides, particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or a synthetic steviol glycoside from various starting compositions.

[0081] As used herein, the abbreviation term "reb" refers to "rebaudioside". Both terms have the same meaning and may be used interchangeably.

[0082] As used herein, "biocatalysis" or "biocatalytic" refers to the use of natural or genetically engineered biocatalysts, such as enzymes, or cells including microorganisms, comprising one or more enzyme, capableof single or multiple step chemical transformations on organic compounds. Biocatalysis processes include fermentation, biosynthesis, bioconversion and biotransformation processes. Both isolated enzyme, and whole-cell biocatalysis methods are known in the art. Biocatalyst protein enzymes can be naturally occurring or recombinant proteins.

[0083] As used herein, the term "steviol glycoside(s)" refers to a glycoside of steviol, including, but not limited to, naturally occurring steviol glycosides, e.g. steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

Starting Composition

[0084] As used herein, "starting composition" refers to any composition (generally an aqueous solution) containing one or more organic compound comprising at least one carbon atom.

[0085] In one embodiment, the starting composition is selected from the group consisting of steviol, steviol glycosides, polyols and various carbohydrates.

[0086] The starting composition steviol glycoside is selected from the group consisting of steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 or other glycoside of steviol occurring in *Stevia rebaudiana* plant, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

[0087] In one embodiment, the starting composition is steviol.

[0088] In another embodiment, the starting composition steviol glycoside is steviolmonoside.

[0089] In yet another embodiment, the starting composition steviol glycoside is steviolmonoside A.

[0090] In still another embodiment, the starting composition steviol glycoside is rubusoside.

[0091] In yet another embodiment, the starting composition steviol glycoside is steviolbioside.

[0092] In yet another embodiment, the starting composition steviol glycoside is steviolbioside A.

[0093] In yet another embodiment, the starting composition steviol glycoside is steviolbioside B.

[0094] In still another embodiment, the starting composition steviol glycoside is stevioside.

[0095] In yet another embodiment, the starting composition steviol glycoside is stevioside A, also known as rebaudioside KA.

[0096] In still another embodiment, the starting composition steviol glycoside is stevioside B.

[0097] In still another embodiment, the starting composition steviol glycoside is stevioside C.

[0098] In another embodiment, the starting composition steviol glycoside is rebaudioside E.

[0099] In another embodiment, the starting composition steviol glycoside is rebaudioside E2.

[0100] In another embodiment, the starting composition steviol glycoside is rebaudioside E3.

[0101] The term "polyol" refers to a molecule that contains more than one hydroxyl group. A polyol may be a diol, triol, or a tetraol which contain 2, 3, and 4 hydroxyl groups, respectively. A polyol also may contain more than four hydroxyl groups, such as a pentaol, hexaol, heptaol, or the like, which contain 5, 6, or 7 hydroxyl groups, respectively. Additionally, a polyol also may be a sugar alcohol, polyhydric alcohol, or polyalcohol which is a reduced form of carbohydrate, wherein the carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. Examples of polyols include, but are not limited to, erythritol, maltitol, mannitol, sorbitol, lactitol, xylitol, inositol, isomalt, propylene glycol, glycerol, threitol, galactitol, hydrogenated isomaltulose, reduced isomalto-oligosaccharides, reduced xylo-oligosaccharides, reduced gentio-oligosaccharides, reduced maltose syrup, reduced glucose syrup, hydrogenated starch hydrolyzates, polyglycitols and sugar alcohols or any other carbohydrates capable of being reduced.

[0102] The term "carbohydrate" refers to aldehyde or ketone compounds substituted with multiple hydroxyl groups, of the general formula $(CH_2O)_n$, wherein n is 3-30, as well as their oligomers and polymers. The carbohydrates of the present invention can, in addition, be substituted or deoxygenated at one or more positions. Carbohydrates, as used herein, encompass unmodified carbohydrates, carbohydrate derivatives, substituted carbohydrates, and modified carbohydrates. As used herein, the phrases "carbohydrate derivatives", "substituted carbohydrate", and "modified carbohydrates" are synonymous. Modified carbohydrate means any carbohydrate wherein at least one atom has been added, removed, or substituted, or combinations thereof. Thus, carbohydrate derivatives or substituted carbohydrates include substituted and unsubstituted monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The carbohydrate derivatives or substituted carbohydrates optionally can be deoxygenated at any corresponding C-position, and/or substituted with one or more moieties such as hydrogen, halogen, haloalkyl, carboxyl, acyl, acyloxy, amino, amido, carboxyl derivatives, alkylamino, dialkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfo, mercapto, imino, sulfonyl, sulfenyl, sulfinyl, sulfamoyl, carboalkoxy, carboxamido, phosphonyl, phosphinyl, phosphoryl, phosphino, thioester, thioether, oximino, hydrazino, carbamyl, phospho, phosphonato, or any other viable functional group provided the carbohydrate derivative or substituted carbohydrate functions to improve the sweet taste of the sweetener composition.

[0103] Examples of carbohydrates which may be used in accordance with this invention include, but are not limited to, tagatose, trehalose, galactose, rhamnose, various cyclodextrins, cyclic oligosaccharides, various types of maltodextrins, dextran, sucrose, glucose, ribulose, fructose, threose, arabinose, xylose, lyxose, allose, altrose, mannose, idose, lactose, maltose, invert sugar, isotrehalose, neotrehalose, isomaltulose, erythrose, deoxyribose, gulose, idose, talose, erythrulose, xylulose, psicose, turanose, cellobiose, amylopectin, glucosamine, mannosamine, fucose, glucuronic acid,

gluconic acid, glucono-lactone, abequose, galactosamine, beet oligosaccharides, isomalto-oligosaccharides (isomaltose, isomaltotriose, panose and the like), xylo-oligosaccharides (xylotriose, xylobiose and the like), xylo-terminated oligosaccharides, gentio-oligosaccharides (gentiobiose, gentiotriose, gentiotetraose and the like), sorbose, nigero-oligosaccharides, palatinose oligosaccharides, fructooligosaccharides (kestose, nystose and the like), maltotetraol, maltotriol, malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and the like), starch, inulin, inulo-oligosaccharides, lactulose, melibiose, raffinose, ribose, isomerized liquid sugars such as high fructose corn syrups, coupling sugars, and soybean oligosaccharides. Additionally, the carbohydrates as used herein may be in either the D- or L-configuration.

[0104] The starting composition may be synthetic or purified (partially or entirely), commercially available or prepared.

[0105] In one embodiment, the starting composition is glycerol.

[0106] In another embodiment, the starting composition is glucose.

[0107] In still another embodiment, the starting composition is sucrose.

[0108] In yet another embodiment, the starting composition is starch.

[0109] In another embodiment, the starting composition is maltodextrin.

[0110] In yet another embodiment, the starting composition is cellulose.

[0111] In still another embodiment, the starting composition is amylose.

[0112] The organic compound(s) of starting composition serve as a substrate(s) for the production of the target steviol glycoside(s), as described herein.

Target Steviol Glycoside

[0113] The target steviol glycoside of the present method can be any steviol glycoside that can be prepared by the process disclosed herein. In one embodiment, the target steviol glycoside is selected from the group consisting of steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or other glycoside of steviol occurring in *Stevia rebaudiana* plant, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

[0114] In one embodiment, the target steviol glycoside is steviolmonoside.

[0115] In another embodiment, the target steviol glycoside is steviolmonoside A.

[0116] In another embodiment, the target steviol glycoside is steviolbioside.

[0117] In another embodiment, the target steviol glycoside is steviolbioside A.

[0118] In another embodiment, the target steviol glycoside is steviolbioside B.

[0119] In another embodiment, the target steviol glycoside is rubusoside.

[0120] In another embodiment, the target steviol glycoside is stevioside.

[0121] In another embodiment, the target steviol glycoside is stevioside A (rebaudioside KA).

[0122] In another embodiment, the target steviol glycoside is stevioside B.

[0123] In another embodiment, the target steviol glycoside is stevioside C.

[0124] In another embodiment, the target steviol glycoside is rebaudioside E.

[0125] In another embodiment, the target steviol glycoside is rebaudioside E2.

[0126] In another embodiment, the target steviol glycoside is rebaudioside E3.

[0127] In another embodiment, the target steviol glycoside is rebaudioside AM.

[0128] The target steviol glycoside can be in any polymorphic or amorphous form, including hydrates, solvates, anhydrous or combinations thereof.

[0129] In one embodiment, the present invention is a biocatalytic process for the production of steviolmonoside.

[0130] In one embodiment, the present invention is a biocatalytic process for the production of steviolmonoside A.

[0131] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside.

[0132] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside A.

[0133] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside B.

[0134] In one embodiment, the present invention is a biocatalytic process for the production of rubusoside.

[0135] In one embodiment, the present invention is a biocatalytic process for the production of stevioside.

[0136] In one embodiment, the present invention is a biocatalytic process for the production of stevioside A (rebaudioside KA).

[0137] In one embodiment, the present invention is a biocatalytic process for the production of stevioside B.

[0138] In one embodiment, the present invention is a biocatalytic process for the production of stevioside C.

[0139] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E.

[0140] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E2.

[0141] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E3.

[0142] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside AM.

[0143] In a particular embodiment, the present invention provides for the biocatalytic process for the production of rebaudioside AM from a starting composition comprising stevioside and UDP-glucose.

[0144] In another particular embodiment, the present invention provides for the biocatalytic process for the production of rebaudioside AM from a starting composition comprising rebaudioside E and UDP-glucose.

[0145] Optionally, the method of the present invention further comprises separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition. The target steviol glycoside can be separated by any suitable method, such as, for example, crystallization, separation by membranes, centrifugation, extraction, chromatographic separation or a combination of such methods.

[0146] In particular embodiments, the process described herein results in a highly purified target steviol glycoside composition. The term "highly purified", as used herein,

refers to a composition having greater than about 80% by weight of the target steviol glycoside on an anhydrous (dried) basis. In one embodiment, the highly purified target steviol glycoside composition contains greater than about 90% by weight of the target steviol glycoside on an anhydrous (dried) basis, such as, for example, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% or greater than about 99% target steviol glycoside content on a dried basis.

[0147] In one embodiment, when the target steviol glycoside is reb AM, the process described herein provides a composition having greater than about 90% reb AM content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is reb AM, the process described herein provides a composition comprising greater than about 95% reb AM content by weight on a dried basis.

Microorganisms and Enzyme Preparations

[0148] In one embodiment of present invention, a microorganism (microbial cell) and/or enzyme preparation is contacted with a medium containing the starting composition to produce target steviol glycosides.

[0149] The enzyme can be provided in the form of a whole cell suspension, a crude lysate, a purified enzyme or a combination thereof. In one embodiment, the biocatalyst is a purified enzyme capable of converting the starting composition to the target steviol glycoside. In another embodiment, the biocatalyst is a crude lysate comprising at least one enzyme capableof converting the starting composition to the target steviol glycoside. In still another embodiment, the biocatalyst is a whole cell suspension comprising at least one enzyme capable of converting the starting composition to the target steviol glycoside.

[0150] In another embodiment, the biocatalyst is one or more microbial cells comprising enzyme(s) capable of converting the starting composition to the target steviol glycoside. The enzyme can be located on the surface of the cell, inside the cell or located both on the surface of the cell and inside the cell.

[0151] Suitable enzymes for converting the starting composition to target steviol glycosides include, but are not limited to, the steviol biosynthesis enzymes and UDP-glucosyltransferases (UGTs). Optionally it may include UDP recycling enzyme(s).

[0152] In one embodiment, the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

[0153] In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (M E P/DOX P) enzymes.

[0154] In one embodiment the steviol biosynthesis enzymes are selected from the group including geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated H M G-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.

[0155] The UDP-glucosyltransferase can be any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol and/or a steviol glycoside substrate to provide the target steviol glycoside.

[0156] In one embodiment, steviol biosynthesis enzymes and UDP-glucosyltransferases are produced in a microbial cell. The microbial cell may be, for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc. In another embodiment, the UDP-glucosyltransferases are synthesized.

[0157] In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2, UGTS12, EUGT11 and UGTs having substantial (>85%, >86%, >87%, >88%, >89%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, >99%) amino-acid sequence identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.

[0158] In one embodiment, steviol biosynthesis enzymes, UGTs and UDP-glucose recycling system are present in one microorganism (microbial cell). The microorganism may be for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.

[0159] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing an —OH functional group at C13 to give a target steviol glycoside having an —O-glucose beta glucopyranoside glycosidic linkage at C13. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2, or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0160] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing a —COOH functional group at C19 to give a target steviol glycoside having a —COO-glucose beta-glucopyranoside glycosidic linkage at C19. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1, or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0161] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1→2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0162] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta

1→3 glucopyranoside glycosidic linkage(s) at the newly formed bond glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGT76G1, or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0163] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C13 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1→2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0164] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0165] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside A. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0166] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0167] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside A. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0168] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0169] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0170] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form steviolbioside.

[0171] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0172] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is E UGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0173] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside A. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0174] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0175] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0176] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside A (rebaudioside KA). In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0177] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside.

[0178] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside to form stevioside. In a particular embodiment, the UDP-glucosyl transferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0179] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence

identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0180] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E2.

[0181] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0182] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E.

[0183] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside C to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0184] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E2. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0185] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0186] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E3 to form rebaudioside AM.

[0187] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E2 to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0188] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0189] Optionally, the method of the present invention further comprises using more than one UGT on a starting composition, to give a target steviol glycoside(s) having more than one glucose unit than the starting composition. In a particular embodiment, the UDP-glucosyltransferases are UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any UGT having >85% amino-acid sequence identity with UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any combination thereof, capable of adding more than one glucose unit to a starting composition to give a steviol glycoside(s) having more than one glucose unit than the starting composition.

[0190] In one embodiment, the UDP-glucosyltransferases are any UDP-glucosyltransferases capable of adding overall two glucose unit to stevioside to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferases are selected from UGTS12, EUGT11, UGT91D2, UGT76G1 or any UGT having >85% amino-acid sequence identity with UGTS12, EUGT11, UGT91D2, UGT76G1 or any combination thereof. In another particular embodiment, the UDP-glucosyltransferases are UGTS12 and UGT76G1.

[0191] Optionally, the method of the present invention further comprises recycling UDP to provide UDP-glucose. In one embodiment, the method comprises recycling UDP by providing a recycling catalyst and a recycling substrate, such that the biotransformation of steviol and/or the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose. The UDP recycling enzyme can be sucrose synthase SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At and the recycling substrate can be sucrose.

[0192] Optionally, the method of the present invention further comprises the use of transglycosidases that use oligo- or poly-saccharides as the sugar donor to modify recipient target steviol glycoside molecules. Non-limiting examples include cyclodextrin glycosyltransferase (CGTase), fructofuranosidase, amylase, saccharase, glucosucrase, beta-h-fructosidase, beta-fructosidase, sucrase, fructosylinvertase, alkaline invertase, acid invertase, fructofuranosidase. In some embodiments, glucose and sugar(s) other than glucose, including but not limited to fructose, xylose, rhamnose, arabinose, deoxyglucose, galactose are transferred to the recipient target steviol glycosides. In one embodiment, the recipient steviol glycoside is rebaudioside AM.

[0193] In another embodiment, the UDP-glucosyltransferase capable of adding at least one glucose unit to starting composition steviol glycoside has >85% amino-acid sequence identity with UGTs selected from the following listing of Geninfo identifier numbers, preferably from the group presented in Table 1, and Table 2.

397567	30680413	115480946	147798902	218193594	225443294
454245	32816174	116310259	147811764	218193942	225444853
1359905	32816178	116310985	147827151	219885307	225449296
1685003	34393978	116788066	147836230	222615927	225449700
1685005	37993665	116788606	147839909	222619587	225454338

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2191136	37993671	116789315	147846163	222623142	225454340
2501497	37993675	119394507	147855977	222625633	225454342
2911049	39104603	119640480	148905778	222625635	225454473
4218003	41469414	122209731	148905999	222636620	225454475
4314356	41469452	125526997	148906835	222636621	225458362
13492674	42566366	125534279	148907340	222636628	225461551
13492676	42570280	125534461	148908935	222636629	225461556
15217773	42572855	125540090	148909182	224053242	225461558
15217796	44890129	125541516	148909920	224053386	225469538
15223396	46806235	125545408	148910082	224055535	225469540
15223589	50284482	125547340	148910154	224056138	226316457
15227766	51090402	125547520	148910612	224056160	226492603
15230017	51090594	125554547	148910769	224067918	226494221
15231757	52839682	125557592	156138791	224072747	226495389
15234056	56550539	125557593	156138797	224080189	226495945
15234195	62734263	125557608	156138799	224091845	226502400
15234196	62857204	125559566	156138803	224094703	226507980
15238503	62857206	125563266	165972256	224100653	226531147
15239523	62857210	125571055	168016721	224100657	226532094
15239525	62857212	125579728	171674071	224101569	238477377
15239543	75265643	125588307	171906258	224103105	240254512
15239937	75285934	125589492	183013901	224103633	242032615
15240305	75288884	125599469	183013903	224103637	242032621
15240534	77550661	125601477	186478321	224109218	242038423
15982889	77556148	126635837	187373030	224114583	242043290
18086351	82791223	126635845	187373042	224116284	242044836
18418378	83778990	126635847	190692175	224120552	242051252
18418380	89953335	126635863	194701936	224121288	242056217
18418382	110741436	126635867	195620060	224121296	242056219
19743740	110743955	126635883	209954691	224121300	242056663
19911201	115438196	126635887	209954719	224130358	242059339
20149064	115438785	133874210	209954725	224140703	242059341
20260654	115441237	133874212	209954733	224143404	242060922
21435782	115454819	145358033	210063105	224143406	242067411
21553613	115456047	147772508	210063107	224144306	242067413
21593514	115457492	147776893	212275846	224285244	242076258
22759895	115459312	147776894	216296854	225431707	242076396
23955910	115464719	147776895	217074506	225435532	242084750
26452040	115471069	147786916	218185693	225436321	242091005
28393204	115471071	147798900	218187075	225440041	242095206
30679796	115474009	147798901	218189427	225441116	242345159
242345161	297724601	326492035	356523945	357140904	359486938
255536859	297725463	326493430	356523957	357165849	359487055
255538228	297728331	326500410	356523959	357165852	359488135
255541676	297738632	326506816	356523961	357168415	359488708
255547075	297745347	326507826	356523963	357437837	359493630
255552620	297745348	326508394	356524387	357442755	359493632
255552622	297795735	326509445	356524403	357442757	359493634
255555343	297796253	326511261	356527181	357445729	359493636
255555361	297796257	326511866	356533209	357445731	359493815
255555363	297796261	326512412	356533852	357445733	359495856
255555365	297797587	326517673	356534718	357446799	359495858
255555369	297798502	326518800	356535480	357446805	359495869
255555373	297799226	326521124	356542996	357452779	359495871
255555377	297805988	326525567	356543136	357452781	359497638
255556812	297807499	326525957	356543932	357452783	359807261
255556818	297809125	326526607	356549841	357452787	374256637
255563008	297809127	326527141	356549843	357452789	377655465
255564074	297811403	326530093	356554358	357452791	378405177
255564531	297820040	326534036	356554360	357452797	378829085
255572878	297821483	326534312	356558606	357452799	387135070
255577901	297825217	332071132	356560333	357470367	387135072
255583249	297832276	339715876	356560599	357472193	387135078
255583253	297832280	342306012	356560749	357472195	387135092
255583255	297832518	342306016	356566018	357474295	387135094
255585664	297832520	343457675	356566169	357474493	387135098
255585666	297840825	343457677	356566173	357474497	387135100
255634688	297840827	350534960	356567761	357474499	387135134
255644801	297847402	356498085	356574704	357490035	387135136
255645821	297849372	356499771	356576401	357493567	387135174
255647456	300078590	356499777	356577660	357497139	387135176
255648275	300669727	356499779	357114993	357497581	387135184
260279126	302142947	356501328	357115447	357497671	387135186
260279128	302142948	356502523	357115451	357500579	387135188
261343326	302142950	356503180	357115453	357504663	387135190
283132367	302142951	356503184	357116080	357504691	387135192

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283362112	302765302	356503295	357116928	357504699	387135194
289188052	302796334	356504436	357117461	357504707	387135282
295841350	302811470	356504523	357117463	357505859	387135284
296088529	302821107	356504765	357117829	357510851	387135294
296090415	302821679	356511113	357117839	357516975	387135298
296090524	319759260	356515120	357125059	359477003	387135300
296090526	319759266	356517088	357126015	359477998	387135302
297599503	320148814	356520732	357134488	359478043	387135304
297601531	326489963	356522586	357135657	359478286	387135312
297611791	326490273	356522588	357138503	359484299	387135314
297722841	326491131	356522590	357139683	359486936	387135316
387135318	449440433	460376293	460413408	462423864	475546199
387135320	449445896	460378310	460416351	470101924	475556485
387135322	449446454	460380744	462394387	470102280	475559699
387135324	449447657	460381726	462394433	470102858	475578293
387135326	449449002	460382093	462394557	470104211	475591753
387135328	449449004	460382095	462395646	470104264	475593742
388493506	449449006	460382754	462395678	470104266	475612072
388495496	449451379	460384935	462396388	470106317	475622476
388498446	449451589	460384937	462396389	470106357	475622507
388499220	449451591	460385076	462396419	470115448	475623787
388502176	449451593	460385872	462396542	470130404	482550481
388517521	449453712	460386018	462397507	470131550	482550499
388519407	449453714	460389217	462399998	470136482	482550740
388521413	449453716	460394872	462400798	470136484	482550999
388827901	449453732	460396139	462401217	470136488	482552352
388827903	449457075	460397862	462402118	470136492	482554970
388827907	449467555	460397864	462402237	470137933	482555336
388827909	449468742	460398541	462402284	470137937	482555478
388827913	449495638	460403139	462402416	470140422	482556454
393887637	449495736	460403141	462404228	470140426	482557289
393887646	449499880	460403143	462406358	470140908	482558462
393887649	449502786	460403145	462408262	470141232	482558508
393990627	449503471	460405998	462409325	470142008	482558547
397746860	449503473	460407578	462409359	470142010	482561055
397789318	449515857	460407590	462409777	470142012	482561555
413924864	449518643	460409128	462411467	470143607	482562795
414590349	449519559	460409134	462414311	470143939	482562850
414590661	449522783	460409136	462414416	470145404	482565074
414591157	449524530	460409459	462414476	473923244	482566269
414879558	449524591	460409461	462415526	474114354	482566296
414879559	449528823	460409463	462415603	474143634	482566307
414879560	449528825	460409465	462415731	474202268	482568689
414888074	449534021	460409467	462416307	474299266	482570049
431812559	460365546	460410124	462416920	474363119	482570572
449432064	460366882	460410126	462416922	474366157	482575121
449432066	460369823	460410128	462416923	474429346	
449433069	460369829	460410130	462416924	475432777	
449436944	460369831	460410132	462417401	475473002	
449438665	460369833	460410134	462419769	475489790	
449438667	460370755	460410213	462420317	475511330	
449440431	460374714	460411200	462423366	475516200	

TABLE 1

GI number	Accession	Origin
190692175	ACE87855.1	*Stevia rebaudiana*
41469452	AAS07253.1	*Oryza saliva*
62857204	BAD95881.1	*Ipomoea nil*
62857206	BAD95882.1	*Ipomoea purperea*
56550539	BAD77944.1	*Bellis perennis*
115454819	NP_001051010.1	*Oryza sativa Japonica* Group
115459312	NP_001053256.1	*Oryza sativa Japonica* Group
115471069	NP_001059133.1	*Oryza saliva Japonica* Group
115471071	NP_001059134.1	*Oryza saliva Japonica* Group
116310985	CAH67920.1	*Oryza sativa Indica* Group
116788066	ABK24743.1	*Picea sitchensis*
122209731	Q2V6J9.1	*Fragaria* × *ananassa*
125534461	EAY81009.1	*Oryza sativa Indica* Group
125559566	EAZ05102.1	*Oryza sativa Indica* Group
125588307	EAZ28971.1	*Oryza sativa Japonica* Group
148907340	ABR16806.1	*Picea sitchensis*
148910082	ABR18123.1	*Picea sitchensis*

TABLE 1-continued

GI number	Accession	Origin
148910612	ABR18376.1	*Picea sitchensis*
15234195	NP_194486.1	*Arabidopsis thaliana*
15239523	NP_200210.1	*Arabidopsis thaliana*
15239937	NP_196793.1	*Arabidopsis thaliana*
1685005	AAB36653.1	*Nicotiana tabacum*
183013903	ACC38471.1	*Medicago truncatula*
186478321	NP_172511.3	*Arabidopsis thaliana*
187373030	ACD03249.1	*Avena strigosa*
194701936	ACF85052.1	*Zea mays*
19743740	AAL92461.1	*Solanum lycopersicum*
212275846	NP_001131009.1	*Zea mays*
222619587	EEE55719.1	*Oryza sativa Japonica* Group
224055535	XP_002298527.1	*Populus trichocarpa*
224101569	XP_002334266.1	*Populus trichocarpa*
224120552	XP_002318358.1	*Populus trichocarpa*
224121288	XP_002330790.1	*Populus trichocarpa*
225444853	XP_002281094	*Vitis vinifera*

TABLE 1-continued

GI number	Accession	Origin
225454342	XP_002275850.1	*Vitis vinifera*
225454475	XP_002280923.1	*Vitis vinifera*
225461556	XP_002285222	*Vitis vinifera*
225469540	XP_002270294.1	*Vitis vinifera*
226495389	NP_001148083.1	*Zea mays*
226502400	NP_001147674.1	*Zea mays*
238477377	ACR43489.1	*Triticum aestivum*
240254512	NP_565540.4	*Arabidopsis thaliana*
2501497	Q43716.1	*Petunia* × *hybrida*
255555369	XP_002518721.1	*Ricinus communis*
26452040	BAC43110.1	*Arabidopsis thaliana*
296088529	CBI37520.3	*Vitis vinifera*
297611791	NP_001067852.2	*Oryza sativa Japonica* Group
297795735	XP_002865752.1	*Arabidopsis lyrata* subsp. *lyrata*
297798502	XP_002867135.1	*Arabidopsis lyrata* subsp. *lyrata*
297820040	XP_002877903.1	*Arabidopsis lyrata* subsp. *lyrata*
297832276	XP_002884020.1	*Arabidopsis lyrata* subsp. *lyrata*
302821107	XP_002992218.1	*Selaginella moellendorffii*
30680413	NP_179446.2	*Arabidopsis thaliana*
319759266	ADV71369.1	*Pueraria montana* var. *lobata*
326507826	BAJ86656.1	*Hordeum vulgare* subsp. *Vulgare*
343457675	AEM37036.1	*Brassica rapa* subsp. *oleifera*
350534960	NP_001234680.1	*Solanum lycopersicum*
356501328	XP_003519477.1	*Glycine max*
356522586	XP_003529927.1	*Glycine max*
356535480	XP_003536273.1	*Glycine max*
357445733	XP_003593144.1	*Medicago truncatula*
357452783	XP_003596668.1	*Medicago truncatula*
357474493	XP_003607531.1	*Medicago truncatula*
357500579	XP_003620578.1	*Medicago truncatula*
357504691	XP_003622634.1	*Medicago truncatula*
359477998	XP_003632051.1	*Vitis vinifera*
359487055	XP_002271587	*Vitis vinifera*
359495869	XP_003635104.1	*Vitis vinifera*
387135134	AFJ52948.1	*Linum usitatissimum*
387135176	AFJ52969.1	*Linum usitatissimum*
387135192	AFJ52977.1	*Linum usitatissimum*
387135282	AFJ53022.1	*Linum usitatissimum*
387135302	AFJ53032.1	*Linum usitatissimum*
387135312	AFJ53037.1	*Linum usitatissimum*
388519407	AFK47765.1	*Medicago truncatula*
393887646	AFN26668.1	*Barbarea vulgaris* subsp. *arcuata*
414888074	DAA64088.1	*Zea mays*
42572855	NP_974524.1	*Arabidopsis thaliana*
449440433	XP_004137989.1	*Cucumis sativus*
449446454	XP_004140986.1	*Cucumis sativus*
449449004	XP_004142255.1	*Cucumis sativus*
449451593	XP_004143546.1	*Cucumis sativus*
449515857	XP_004164964.1	*Cucumis sativus*
460382095	XP_004236775.1	*Solanum lycopersicum*
460409128	XP_004249992.1	*Solanum lycopersicum*
460409461	XP_004250157.1	*Solanum lycopersicum*
460409465	XP_004250159.1	*Solanum lycopersicum*
462396388	EMJ02187.1	*Prunus persica*
462402118	EMJ07675.1	*Prunus persica*
462409359	EMJ14693.1	*Prunus persica*
462416923	EMJ21660.1	*Prunus persica*
46806235	BAD17459.1	*Oryza saliva Japonica* Group
470104266	XP_004288529.1	*Fragaria vesca* subsp. *vesca*
470142008	XP_004306714.1	*Fragaria vesca* subsp. *vesca*
475432777	EMT01232.1	*Aegilops tauschii*
51090402	BAD35324.1	*Oryza sativa Japonica* Group

TABLE 2

GI number	Accession	Origin	Internal reference
460409128	XP.004249992.1	*Solanum lycopersicum*	UGTSI
460386018	XP.004238697.1	*Solanum lycopersicum*	—
460409134	XP.004249995.1	*Solanum lycopersicum*	—
460410132	XP.004250485.1	*Solanum lycopersicum*	UGTSI2

TABLE 2-continued

GI number	Accession	Origin	Internal reference
460410130	XP.004250484.1	*Solanum lycopersicum*	—
460410128	XP.004250483.1	*Solanum lycopersicum*	—
460378310	XP.004234916.1	*Solanum lycopersicum*	—
209954733	BAG80557.1	*Lycium barbarum*	UGTLB
209954725	BAG80553.1	*Lycium barbarum*	—

[0194] One embodiment of the present invention is a microbial cell comprising an enzyme, i.e. an enzyme capable of converting the starting composition to the target steviol glycoside. Accordingly, some embodiments of the present method include contacting a microorganism with a medium containing the starting composition to provide a medium comprising at least one target steviol glycoside.

[0195] The microorganism can be any microorganism possessing the necessary enzyme(s) for converting the starting composition to target steviol glycoside(s). These enzymes are encoded within the microorganism's genome.

[0196] Suitable microoganisms include, but are not limited to, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc.

[0197] In one embodiment, the microorganism is free when contacted with the starting composition.

[0198] In another embodiment, the microorganism is immobilized when contacted with the starting composition. For example, the microorganism may be immobilized to a solid support made from inorganic or organic materials. Non-limiting examples of solid supports suitable to immobilize the microorganism include derivatized cellulose or glass, ceramics, metal oxides or membranes. The microorganism may be immobilized to the solid support, for example, by covalent attachment, adsorption, cross-linking, entrapment or encapsulation.

[0199] In still another embodiment, the enzyme capable of converting the starting composition to the target steviol glycoside is secreted out of the microorganism and into the reaction medium.

[0200] The target steviol glycoside is optionally purified. Purification of the target steviol glycoside from the reaction medium can be achieved by at least one suitable method to provide a highly purified target steviol glycoside composition. Suitable methods include crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods.

Uses

[0201] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be used "as-is" or in combination with other sweeteners, flavors, food ingredients and combinations thereof.

[0202] Non-limiting examples of flavors include, but are not limited to, lime, lemon, orange, fruit, banana, grape, pear, pineapple, mango, berry, bitter almond, cola, cinnamon, sugar, cotton candy, vanilla and combinations thereof.

[0203] Non-limiting examples of other food ingredients include, but are not limited to, acidulants, organic and amino

acids, coloring agents, bulking agents, modified starches, gums, texturizers, preservatives, caffeine, antioxidants, emulsifiers, stabilizers, thickeners, gelling agents and combinations thereof.

[0204] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be prepared in various polymorphic forms, including but not limited to hydrates, solvates, anhydrous, amorphous forms and combinations thereof.

[0205] Highly purified target glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be incorporated as a high intensity natural sweetener in foodstuffs, beverages, pharmaceutical compositions, cosmetics, chewing gums, table top products, cereals, dairy products, toothpastes and other oral cavity compositions, etc.

[0206] Highly purified target glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be employed as a sweetening compound as the sole sweetener, or it may be used together with at least one naturally occurring high intensity sweeteners such as rebaudioside A, rebaudioside A2, rebaudioside A3, rebaudioside B, rebaudioside C, rebaudioside C2, rebaudioside D, rebaudioside D2, rebaudioside F, rebaudioside F2, rebaudioside F3, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside 12, rebaudioside 13, rebaudioside j, rebaudioside K, rebaudioside K2, rebaudioside L, rebaudioside M, rebaudioside M2, rebaudioside N, rebaudioside 0, rebaudioside 02, rebaudioside Q, rebaudioside Q2, rebaudioside Q3, rebaudioside R, rebaudioside S, rebaudioside T, rebaudioside T1, rebaudioside U, rebaudioside U2, rebaudioside V, rebaudioside W, rebaudioside W2, rebaudioside W3, rebaudioside Y, rebaudioside Z1, rebaudioside Z2, dulcoside A, dulcoside C, stevioside D, stevioside E, stevioside E2, stevioside F, mogrosides, brazzein, neohesperidin dihydrochalcone, glycyrrhizic acid and its salts, thaumatin, perillartine, pernandulcin, mukuroziosides, baiyunoside, phlomisoside-I, dimethyl-hexahydrofluorene-dicarboxylic acid, abrusosides, periandrin, carnosiflosides, cyclocarioside, pterocaryosides, polypodoside A, brazilin, hernandulcin, phillodulcin, glycyphyllin, phlorizin, trilobatin, dihydroflavonol, dihydroquercetin-3-acetate, neoastilibin, trans-cinnamaldehyde, monatin and its salts, selligueain A, hematoxylin, monellin, osladin, pterocaryoside A, pterocaryoside B, mabinlin, pentadin, miraculin, curculin, neoculin, chlorogenic acid, cynarin, Luo Han Guo sweetener, mogroside V, siamenoside and combinations thereof.

[0207] In a particular embodiment, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in a sweetener composition comprising a compound selected from the group consisting of rebaudioside A, rebaudioside A2, rebaudioside A3, rebaudioside B, rebaudioside C, rebaudioside C2, rebaudioside D, rebaudioside D2, rebaudioside F, rebaudioside F2, rebaudioside F3, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside I2, rebaudioside I3, rebaudioside J, rebaudioside K, rebaudioside K2, rebaudioside L, rebaudioside M, rebaudioside M2, rebaudioside N, rebaudioside O, rebaudioside O2, rebaudioside Q, rebaudioside Q2, rebaudioside Q3, rebaudioside R, rebaudioside S, rebaudioside T, rebaudioside T1, rebaudioside U, rebaudioside U2, rebaudioside V, rebaudioside W, rebaudioside W2, rebaudioside W3, rebaudioside Y, rebaudioside Z1, rebaudioside Z2, dulcoside A, dulcoside C, stevioside D, stevioside E, stevioside E2, stevioside F, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof.

[0208] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be used in combination with synthetic high intensity sweeteners such as sucralose, potassium acesulfame, aspartame, alitame, saccharin, neohesperidin dihydrochalcone, cyclamate, neotame, dulcin, suosan advantame, salts thereof, and combinations thereof.

[0209] Moreover, highly purified target steviol glycoside (s) particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in combination with natural sweetener suppressors such as gymnemic acid, hodulcin, ziziphin, lactisole, and others. Steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be combined with various umami taste enhancers. Steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be mixed with umami tasting and sweet amino acids such as glutamate, aspartic acid, glycine, alanine, threonine, proline, serine, glutamate, lysine, tryptophan and combinations thereof.

[0210] Highly purified target steviol glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in combination with one or more additive selected from the group consisting of carbohydrates, polyols, amino acids and their corresponding salts, poly-amino acids and their corresponding salts, sugar acids and their corresponding salts, nucleotides, organic acids, inorganic acids, organic salts including organic acid salts and organic base salts, inorganic salts, bitter compounds, flavorants and flavoring ingredients, astringent compounds, proteins or protein hydrolysates, surfactants, emulsifiers, flavonoids, alcohols, polymers and combinations thereof.

[0211] Highly purified target steviol glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be combined with polyols or sugar alcohols. The term "polyol" refers to a molecule that contains more than one hydroxyl group. A polyol may be a diol, triol, or a tetraol which contain 2, 3, and 4 hydroxyl groups, respectively. A polyol also may contain more than four hydroxyl groups, such as a pentaol, hexaol, heptaol, or the like, which contain 5, 6, or 7 hydroxyl groups, respectively. Additionally, a polyol also may be a sugar alcohol, polyhydric alcohol, or polyalcohol which is a reduced form of carbohydrate, wherein the carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. Examples of polyols include, but are not limited to, erythritol, maltitol, mannitol, sorbitol, lactitol, xylitol, inositol, isomalt, propylene glycol, glycerol, threitol, galactitol, hydrogenated isomaltulose, reduced isomalto-oligosaccharides, reduced xylo-oligosaccharides, reduced gentio-oligosaccharides, reduced maltose syrup, reduced glucose syrup, hydrogenated starch hydrolyzates, polyglycitols and sugar alcohols or any other carbohydrates capable of being reduced which do not adversely affect the taste of the sweetener composition.

[0212] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/orrebaudioside AM may be combined with reduced calorie sweeteners such as, for example, D-tagatose, L-sugars, L-sorbose, L-arabinose and combinations thereof.

[0213] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be combined with various carbohydrates. The term "carbohydrate" generally refers to aldehyde or ketone compounds substituted with multiple hydroxyl groups, of the general formula $(CH_2O)_n$, wherein n is 3-30, as well as their oligomers and polymers. The carbohydrates of the present invention can, in addition, be substituted or deoxygenated at one or more positions. Carbohydrates, as used herein, encompass unmodified carbohydrates, carbohydrate derivatives, substituted carbohydrates, and modified carbohydrates. As used herein, the phrases "carbohydrate derivatives", "substituted carbohydrate", and "modified carbohydrates" are synonymous. Modified carbohydrate means any carbohydrate wherein at least one atom has been added, removed, or substituted, or combinations thereof. Thus, carbohydrate derivatives or substituted carbohydrates include substituted and unsubstituted monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The carbohydrate derivatives or substituted carbohydrates optionally can be deoxygenated at any corresponding C-position, and/or substituted with one or more moieties such as hydrogen, halogen, haloalkyl, carboxyl, acyl, acyloxy, amino, amido, carboxyl derivatives, alkylamino, dialkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfo, mercapto, imino, sulfonyl, sulfenyl, sulfinyl, sulfamoyl, carboalkoxy, carboxamido, phosphonyl, phosphinyl, phosphoryl, phosphino, thioester, thioether, oximino, hydrazino, carbamyl, phospho, phosphonato, or any other viable functional group provided the carbohydrate derivative or substituted carbohydrate functions to improve the sweet taste of the sweetener composition.

[0214] Examples of carbohydrates which may be used in accordance with this invention include, but are not limited to, psicose, turanose, allose, tagatose, trehalose, galactose, rhamnose, various cyclodextrins, cyclic oligosaccharides, various types of maltodextrins, dextran, sucrose, glucose, ribulose, fructose, threose, arabinose, xylose, lyxose, allose, altrose, mannose, idose, lactose, maltose, invert sugar, isotrehalose, neotrehalose, isomaltulose, erythrose, deoxyribose, gulose, idose, talose, erythrulose, xylulose, psicose, turanose, cellobiose, amylopectin, glucosamine, mannosamine, fucose, glucuronic acid, gluconic acid, gluconolactone, abequose, galactosamine, beet oligosaccharides, isomalto-oligosaccharides (isomaltose, isomaltotriose, panose and the like), xylo-oligosaccharides (xylotriose, xylobiose and the like), xylo-terminated oligosaccharides, gentio-oligosaccharides (gentiobiose, gentiotriose, gentiotetraose and the like), sorbose, nigero-oligosaccharides, palatinose oligosaccharides, fructooligosaccharides (kestose, nystose and the like), maltotetraol, maltotriol, malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and the like), starch, inulin, inulo-oligosaccharides, lactulose, melibiose, raffinose, ribose, isomerized liquid sugars such as high fructose corn syrups, coupling sugars, and soybean oligosaccharides. Additionally, the carbohydrates as used herein may be in either the D- or L-configuration.

[0215] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be used in combination with various physiologically active substances or functional ingredients. Functional ingredients generally are classified into categories such as carotenoids, dietary fiber, fatty acids, saponins, antioxidants, nutraceuticals, flavonoids, isothiocyanates, phenols, plant sterols and stanols (phytosterols and phytostanols); polyols; prebiotics, probiotics; phytoestrogens; soy protein; sulfides/thiols; amino acids; proteins; vitamins; and minerals. Functional ingredients also may be classified based on their health benefits, such as cardiovascular, cholesterol-reducing, and anti-inflammatory. Exemplary functional ingredients are provided in WO2013/096420, the contents of which is hereby incorporated by reference.

[0216] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a high intensity sweetener to produce zero calorie, reduced calorie or diabetic beverages and food products with improved taste characteristics. It may also be used in drinks, foodstuffs, pharmaceuticals, and other products in which sugar cannot be used. In addition, highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA),

stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used as a sweetener not only for drinks, foodstuffs, and other products dedicated for human consumption, but also in animal feed and fodder with improved characteristics.

[0217] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a flavor modifier to produce zero calorie, reduced calorie or diabetic beverages and food products with modified flavor. When used as a flavor modifier, or a flavor with modifying properties (FMP), the highly purified target steviol glycoside is used in a consumable product below the detection level of the flavor modifier or FMP. The flavor modifier or FMP therefore does not impart a detectable taste or flavor of its own to the consumable product, but instead serves to modify the consumer's detection of tastes and/or flavors of other ingredients in the consumable product. One example of taste and flavor modification is sweetness enhancement, in which the flavor modifier or FMP itself does not contribute to the sweetness of the consumable product, but enhances the quality of the sweetness tasted by the consumer.

[0218] Examples of consumable products in which highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be used as a flavor modifier or flavor with modifying properties include, but are not limited to, alcoholic beverages such as vodka, wine, beer, liquor, and sake, etc.; natural juices; refreshing drinks; carbonated soft drinks; diet drinks; zero calorie drinks; reduced calorie drinks and foods; yogurt drinks; instant juices; instant coffee; powdered types of instant beverages; canned products; syrups; fermented soybean paste; soy sauce; vinegar; dressings; mayonnaise; ketchups; curry; soup; instant bouillon; powdered soy sauce; powdered vinegar; types of biscuits; rice biscuit; crackers; bread; chocolates; caramel; candy; chewing gum; jelly; pudding; preserved fruits and vegetables; fresh cream; jam; marmalade; flower paste; powdered milk; ice cream; sorbet; vegetables and fruits packed in bottles; canned and boiled beans; meat and foods boiled in sweetened sauce; agricultural vegetable food products; seafood; ham; sausage; fish ham; fish sausage; fish paste; deep fried fish products; dried seafood products; frozen food products; preserved seaweed; preserved meat; tobacco; medicinal products; and many others. In principle it can have unlimited applications.

[0219] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a foaming suppressor to produce zero calorie, reduced calorie or diabetic beverages and food products.

[0220] Examples of consumable products in which highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be used as a sweetening compound include, but are not limited to, alcoholic beverages such as vodka, wine, beer, liquor, and sake, etc.; natural juices; refreshing drinks; carbonated soft drinks; diet drinks; zero calorie drinks; reduced calorie drinks and foods; yogurt drinks; instant juices; instant coffee; powdered types of instant beverages; canned products; syrups; fermented soybean paste; soy sauce; vinegar; dressings; mayonnaise; ketchups; curry; soup; instant bouillon; powdered soy sauce; powdered vinegar; types of biscuits; rice biscuit; crackers; bread; chocolates; caramel; candy; chewing gum; jelly; pudding; preserved fruits and vegetables; fresh cream; jam; marmalade; flower paste; powdered milk; ice cream; sorbet; vegetables and fruits packed in bottles; canned and boiled beans; meat and foods boiled in sweetened sauce; agricultural vegetable food products; seafood; ham; sausage; fish ham; fish sausage; fish paste; deep fried fish products; dried seafood products; frozen food products; preserved seaweed; preserved meat; tobacco; medicinal products; and many others. In principle it can have unlimited applications.

[0221] During the manufacturing of products such as foodstuffs, drinks, pharmaceuticals, cosmetics, tabletop products, and chewing gum, the conventional methods such as mixing, kneading, dissolution, pickling, permeation, percolation, sprinkling, atomizing, infusing and other methods may be used.

[0222] Moreover, the highly purified target steviol glycoside(s) steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained in this invention may be used in dry or liquid forms.

[0223] The highly purified target steviol glycoside can be added before or after heat treatment of food products. The amount of the highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM depends on the purpose of usage. As discussed above, it can be added alone or in combination with other compounds.

[0224] The present invention is also directed to sweetness enhancement in beverages using steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM. Accordingly, the present invention provides a beverage comprising a sweetener and steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM as a sweetness enhancer, wherein steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM is present in a concentration at or below their respective sweetness recognition thresholds.

[0225] As used herein, the term "sweetness enhancer" refers to a compound capable of enhancing or intensifying the perception of sweet taste in a composition, such as a beverage. The term "sweetness enhancer" is synonymous

with the terms "sweet taste potentiator," "sweetness potentiator," "sweetness amplifier," and "sweetness intensifier."

[0226] The term "sweetness recognition threshold concentration," as generally used herein, is the lowest known concentration of a sweet compound that is perceivable by the human sense of taste, typically around 1.0% sucrose equivalence (1.0% SE). Generally, the sweetness enhancers may enhance or potentiate the sweet taste of sweeteners without providing any noticeable sweet taste by themselves when present at or below the sweetness recognition threshold concentration of a given sweetness enhancer; however, the sweetness enhancers may themselves provide sweet taste at concentrations above their sweetness recognition threshold concentration. The sweetness recognition threshold concentration is specific for a particular enhancer and can vary based on the beverage matrix. The sweetness recognition threshold concentration can be easily determined by taste testing increasing concentrations of a given enhancer until greater than 1.0% sucrose equivalence in a given beverage matrix is detected. The concentration that provides about 1.0% sucrose equivalence is considered the sweetness recognition threshold.

[0227] In some embodiments, sweetener is present in the beverage in an amount from about 0.5% to about 12% by weight, such as, for example, about 1.0% by weight, about 1.5% by weight, about 2.0% by weight, about 2.5% by weight, about 3.0% by weight, about 3.5% by weight, about 4.0% by weight, about 4.5% by weight, about 5.0% by weight, about 5.5% by weight, about 6.0% by weight, about 6.5% by weight, about 7.0% by weight, about 7.5% by weight, about 8.0% by weight, about 8.5% by weight, about 9.0% by weight, about 9.5% by weight, about 10.0% by weight, about 10.5% by weight, about 11.0% by weight, about 11.5% by weight or about 12.0% by weight.

[0228] In a particular embodiment, the sweetener is present in the beverage in an amount from about 0.5% of about 10%, such as for example, from about 2% to about 8%, from about 3% to about 7% or from about 4% to about 6% by weight. In a particular embodiment, the sweetener is present in the beverage in an amount from about 0.5% to about 8% by weight. In another particular embodiment, the sweetener is present in the beverage in an amount from about 2% to about 8% by weight.

[0229] In one embodiment, the sweetener is a traditional caloric sweetener. Suitable sweeteners include, but are not limited to, sucrose, fructose, glucose, high fructose corn syrup and high fructose starch syrup.

[0230] In another embodiment, the sweetener is erythritol.

[0231] In still another embodiment, the sweetener is a rare sugar. Suitable rare sugars include, but are not limited to, D-allose, D-psicose, D-ribose, D-tagatose, L-glucose, L-fucose, L-arabinose, D-turanose, D-leucrose and combinations thereof.

[0232] It is contemplated that a sweetener can be used alone, or in combination with other sweeteners.

[0233] In one embodiment, the rare sugar is D-allose. In a more particular embodiment, D-allose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0234] In another embodiment, the rare sugar is D-psicose. In a more particular embodiment, D-psicose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0235] In still another embodiment, the rare sugar is D-ribose. In a more particular embodiment, D-ribose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0236] In yet another embodiment, the rare sugar is D-tagatose. In a more particular embodiment, D-tagatose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0237] In a further embodiment, the rare sugar is L-glucose. In a more particular embodiment, L-glucose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0238] In one embodiment, the rare sugar is L-fucose. In a more particular embodiment, L-fucose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0239] In another embodiment, the rare sugar is L-arabinose. In a more particular embodiment, L-arabinose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0240] In yet another embodiment, the rare sugar is D-turanose. In a more particular embodiment, D-turanose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0241] In yet another embodiment, the rare sugar is D-leucrose. In a more particular embodiment, D-leucrose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.

[0242] The addition of the sweetness enhancer at a concentration at or below its sweetness recognition threshold increases the detected sucrose equivalence of the beverage comprising the sweetener and the sweetness enhancer compared to a corresponding beverage in the absence of the sweetness enhancer. Moreover, sweetness can be increased by an amount more than the detectable sweetness of a solution containing the same concentration of the at least one sweetness enhancer in the absence of any sweetener.

[0243] Accordingly, the present invention also provides a method for enhancing the sweetness of a beverage comprising a sweetener comprising providing a beverage comprising a sweetener and adding a sweetness enhancer selected from steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM ora combination thereof, wherein steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM are present in a concentration at or below their sweetness recognition thresholds.

[0244] Addition of steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM in a concentration at or below the sweetness recognition threshold to a beverage containing a sweetener may increase the detected sucrose equivalence from about 1.0% to about 5.0%, such as, for example, about 1.0%,

about 1.5%, about 2.0%, about 2.5%, about 3.0%, about 3.5%, about 4.0%, about 4.5% or about 5.0%.

[0245] The following examples illustrate preferred embodiments of the invention for the preparation of highly purified target steviol glycoside(s), particularly steviol-monoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM. It will be understood that the invention is not limited to the materials, proportions, conditions and procedures set forth in the examples, which are only illustrative.

EXAMPLES

Example 1

Protein Sequences of Engineered Enzymes Used in the Biocatalytic Process

SEQ ID 1:
>SuSy_At, variant PM1-54-2-E05 (engineered sucrose synthase; source of WT gene: *Arabidopsis thaliana*)
MANAERMITRVHSQRERLNETLVSERNEVLALLSRVEAKGKGILQQNQII

AEFEALPEQTRKKLEGGPFFDLLKSTQEAIVLPPWVALAVRPRPGVWEYL

RVNLHALVVEELQPAEFLHFKEELVDGVKNGNFTLELDFEPFNASIPRPT

LHKYIGNGVDFLNRHLSAKLFHDKESLLPLLDFLRLHSHQGKNLMLSEKI

QNLNTLQHTLRKAEEYLAELKSETLYEEFEAKFEEIGLERGWGDNAERVL

DMIRLLLDLLEAPDPSTLETFLGRVPMVFNVVILSPHGYFAQDNVLGYPD

TGGQVVYILDQVRALEIEMLQRIKQQGLNIKPRILILTRLLPDAVGTTCG

ERLERVYDSEYCDILRVPFRTEKGIVRKWISRFEVWPYLETYTEDAAVEL

SKELNGKPDLIIGNYSDGNLVASLLAHKLGVTQCTIAHALEKTKYPDSDI

YWKKLDDKYHFSCQFTADIFAMNHTDFIITSTFQEIAGSKETVGQYESHT

AFTLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPYTEEKRRLTKFHSEI

EELLYSDVENDEHLCVLKDKKKPILFTMARLDRVKNLSGLVEWYGKNTRL

RELVNLVVVGGDRRKESKDNEEKAEMKKMYDLIEEYKLNGQFRWISSQMD

RVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCKGGPA

EIIVHGKSGFHIDPYHGDQAADLLADFFTKCKEDPSHWDEISKGGLQRIE

EKYTWQIYSQRLLTLTGVYGFWKHVSNLDRLEHRRYLEMFYALKYRPLAQ

AVPLAQDD

SEQ ID 2:
>UGTSI2 variant 0234 (engineered glucosyltransferase; source of WT gene: *Solanum lycopersicum*)
MATNLRVLMFPWLAYGHISPFLNIAKQLADRGFLIYLCSTRINLESIIKK

IPEKYADSIHLIELQLPELPELPPHYHTTNGLPPHLNPTLHKALKMSKPN

FSRILQNLKPDLLIYDVLQPWAEHVANEQGIPAGKLLVSCAAVFSYFFSF

RKNPGVEFPFPAIHLPEVEKVKIREILAKEPEEGGRLDEGNKQMMLMCTS

RTIEAKYIDYCTELCNWKVVPVGPPFQDLITNDADNKELIDWLGTKPENS

TVFVSFGSEYFLSKEDMEEIAFALEASNVNFIWVVRFPKGEERNLEDALP

-continued

EGFLERIGERGRVLDKFAPQPRILNHPSTGGFISHCGWNSVMESIDFGVP

IIAMPIHNDQPINAKLMVELGVAVEIVRDDDGKIHRGEIAEALKSVVTGE

TGEILRAKVREISKNLKSIRDEEMDAVAEELIQLCRNSNKSK

SEQ ID 3:
>UGT76G1 variant 0042 (engineered glucosyltransferase; source of WT gene: *Stevia rebaudiana*)
MENKTETTVRRRRRIILFPVPFQGHINPILQLANVLYSKGFAITILHTNF

NKPKTSNYPHFTFRFILDNDPQDERISNLPTHGPLAGMRIPIINEHGADE

LRRELELLMLASEEDEEVSCLITDALWYFAQDVADSLNLRRLVLMTSSLF

NFHAHVSLPQFDELGYLDPDDKTRLEEQASGFPMLKVKDIKSAYSNWQIG

KEILGKMIKQTKASSGVIWNSFKELEESELETVIREIPAPSFLIPLPKHL

TASSSSLLDHDRTVFEWLDQQAPSSVLYVSFGSTSEVDEKDFLEIARGLV

DSGQSFLWVVRPGFVKGSTWVEPLPDGFLGERGKIVKWVPQQEVLAHPAI

GAFWTHSGWNSTLESVCEGVPMIFSSFGGDQPLNARYMSDVLRVGVYLEN

GWERGEVVNAIRRVMVDEEGEYIRQNARVLKQKADVSLMKGGSSYESLES

LVSYISSL

Example 2

Expression and Formulation of SuSy_at Variant of SEQ ID 1

[0246] The gene coding for the SuSy_At variant of SEQ ID 1 (EXAM PLE 1) was cloned into the expression vector pL E1A 17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.

[0247] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.2 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.

[0248] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (OD_{600})) with cell lysis buffer (100 mM Tris-HCI pH 7.0; 2 mM $MgCl_2$, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 μm filter and diluted 50:50 with distilled water, resulting in an enzymatic active preparation.

[0249] For enzymatic active preparations of SuSy_At, activity in Units is defined as follows: 1 mU of SuSy_At turns over 1 nmol of sucrose into fructose in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 400 mM sucrose at t_0, 3 mM $MgCl_2$, and 15 mM uridine diphosphate (UDP).

Example 3

Expression and Formulation of UGTSI2 Variant of SEQ ID 2

[0250] The gene coding for the UGTS12 variant of SEQ ID 2 (EXAM PLE 1) was cloned into the expression vector

pLE1A17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.

[0251] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.1 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.

[0252] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (0 D_{600})) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 μm filter and diluted 50:50 with 1 M sucrose solution, resulting in an enzymatic active preparation.

[0253] For enzymatic active preparations of UGTS12, activity in Units is defined as follows: 1 mU of UGTSI2 turns over 1 nmol of rebaudiosideA (RebA) into rebaudioside D (Reb D) in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at to, 500 mM sucrose, 3 mM $MgCl_2$, 0.25 mM uridine diphosphate (UDP) and 3 U/mL of SuSy_At.

Example 4

Expression and Formulation of UGT 76G1 Variant of SEQ ID 3

[0254] The gene coding for the UGT76G1 variant of SEQ ID 3 (EXAMPLE 1) was cloned into the expression vector pLE1A17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.

[0255] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.1 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.

[0256] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (OD_{600})) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 μm filter and diluted 50:50 with 1 M sucrose solution, resulting in an enzymatic active preparation.

[0257] For enzymatic active preparations of UGT76G1, activity in Units is defined as follows: 1 mU of UGT76G1 turns over 1 nmol of rebaudioside D (Reb D) into rebaudioside M (Reb M) in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at to, 500 mM sucrose, 3 mM $MgCl_2$, 0.25 mM uridine diphosphate (UDP) and 3 U/mL of SuSy_At.

Example 5

Synthesis of Rebaudioside AM from Stevioside in a One-Pot Reaction, Adding UGTS12, SuSy_At and UGT76G1 at the Same Time

[0258] Rebaudioside AM (reb AM) was synthesized directly from stevioside in a one-pot reaction (FIG. **3**), utilizing the three enzymes (see EXAMPLES 1, 2, 3 and 4): UGTS12 (variant of SEQ ID 2), SuSy_At-(variant of SEQ ID 1) and UGT76G1 (variant of SEQ ID 3). The final reaction solution contained 105 U/L UGTS12, 405 U/L SuSy_At, 3 U/L UGT76G1, 5 mM stevioside, 0.25 mM uridine diphosphate (UDP), 1 M sucrose, 4 mM $MgCl_2$ and potassium phosphate buffer (pH 6.6). First, 207 mL of distilled water were mixed with 0.24 g $MgCl_2 \cdot 6H$ 20, 103 g sucrose, 9.9 mL of 1.5 M potassium phosphate buffer (pH 6.6) and 15 g stevioside. After dissolving the components, the temperature was adjusted to 45° C. and UGTS12, SuSy_At, UGT76G1 and 39 mg UDP were added. The reaction mixture was incubated at 45° C. shaker for 24 hrs. Additional 39 mg UDP was added at 8 hrs and 18 hours. The content of reb AM, reb E, stevioside, reb M, reb B, steviolbioside and reb/at several time points was analyzed by HPLC.

[0259] For analysis, biotransformation samples were inactivated by adjusting the reaction mixture to pH 5.5 using 17% H_3PO_4 and then boiled for 10 minutes. Resulting samples were filtered, the filtrates were diluted 10 times and used as samples for HPLC analysis. HPLC assay was carried out on Agilent H P 1200 HPLC system, comprised of a pump, a column thermostat, an auto sampler, a UV detector capable of background correction and a data acquisition system. Analytes were separated using Agilent Poroshell 120 SB—C18, 4.6 mm×150 mm, 2.7 μm at 40° C. The mobile phase consisted of two premixes:

[0260] premix 1 containing 75% 10 mM phosphate buffer (pH 2.6) and 25% acetonitrile, and

[0261] premix 2 containing 68% 10 mM phosphate buffer (pH 2.6) and 32% acetonitrile.

[0262] Elution gradient started with premix 1, changed to premix 2 to 50% at 12.5 minute, changed to premix 2 to 100% at 13 minutes. Total run time was 45 minutes. The column temperature was maintained at 40° C. The injection volume was 5 μL. Rebaudioside species were detected by U V at 210 nm.

[0263] Table 3 shows for each time point the conversion of stevioside into identified rebaudioside species (area percentage). The chromatograms of stevioside and the reaction mixture at 24 hours are shown in FIG. **5** and FIG. **6**, respectively. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

TABLE 3

Biotransformation of stevioside to reb AM

Time,	% conversion from stevioside						
hrs	Reb E	Reb AM	Reb M	Reb I	Stevioside	Reb B	Steviolbioside
0	0	0	0	0	100	0	0
6	1.9	35.9	1.3	1.7	58.7	0.0	0.4
18	0.9	96.7	1.3	0.6	0.0	0.0	0.4
24	0.3	96.4	2.1	0.7	0.0	0.2	0.4

Example 6

Synthesis of Rebaudioside AM from Rebaudioside E in a One-Pot Reaction, SuSy_at and UGT76G1 at the Same Time

[0264] Rebaudioside AM (reb AM) was synthesized directly from rebaudioside E (reb E) in a one-pot reaction (FIG. **4**), utilizing the two enzymes (see EXAMPLES 1, 2 and 4): SuSy_At-(variant of SEQ ID 1) and UGT76G1 (variant of SEQ ID 3). The final reaction solution contained 405 U/L SuSy_At, 3 U/L UGT76G1, 5 mM reb E, 0.25 mM uridine diphosphate (UDP), 1 M sucrose, 4 mM $MgCl_2$. $6H_2O$ and potassium phosphate buffer (pH 6.6). First, 37 mL of distilled water were mixed with 40.3 mg $MgCl_2$, 17.12 g sucrose, 1.65 mL of 1.5 M potassium phosphate buffer (pH 6.6) and 5.04 g reb E. After dissolving the components, the temperature was adjusted to 45° C. and SuSy_At, UGT76G1 and 6.5 mg UDP were added. The reaction mixture was incubated at 45° C. shaker for 24 hrs. Additional 6.5 mg UDP was added at 8 hrs and 18 hours. The content of reb AM, reb E, stevioside, reb A, reb M, reb B, and steviolbioside at several time points was analyzed by HPLC.

[0265] For analysis, biotransformation samples were inactivated by adjusting the reaction mixture to pH 5.5 using 17% H_3PO_4 and then boiled for 10 minutes. Resulting samples were filtered, the filtrates were diluted 10 times and used as samples for HPLC analysis. HPLC assay was carried out on Agilent H P 1200 HPLC system, comprised of a pump, a column thermostat, an auto sampler, a UV detector capable of background correction and a data acquisition system. Analytes were separated using Agilent Poroshell 120 SB—C18, 4.6 mm×150 mm, 2.7 μm at 40° C. The mobile phase consisted of two premixes:

[0266] premix 1 containing 75% 10 mM phosphate buffer (pH 2.6) and 25% acetonitrile, and

[0267] premix 2 containing 68% 10 mM phosphate buffer (pH 2.6) and 32% acetonitrile.

[0268] Elution gradient started with premix 1, changed to premix 2 to 50% at 12.5 minute, changed to premix 2 to 100% at 13 minutes. Total run time was 45 minutes. The column temperature was maintained at 40° C. The injection volume was 5 μL. Rebaudioside species were detected by U V at 210 nm.

[0269] Table 4 shows for each time point the conversion of reb E into identified rebaudioside species (area percentage). The chromatograms of reb E and the reaction mixture at 24 hours are shown in FIG. **7** and FIG. **8**, respectively. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

TABLE 4

Biotransformation of reb E to reb AM

	% conversion from Reb E						
Time, hrs	Reb E	Reb AM	Reb M	Reb A	Stevioside	Reb B	Steviol-bioside
0	99.46	0	0	0.54	0	0	0
4	40.75	57.92	0	0.59	0	0.73	0
7	24.79	73.92	0	0.58	0	0.71	0
24	4.38	94.33	0	0.59	0	0.70	0

Example 7

Purification of Rebaudioside AM

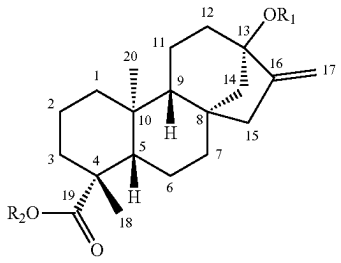
[0270] The reaction mixture of EXAM PLE 5, after 24 hrs, was inactivated by adjusting the pH to pH 5.5 with H_3PO_4 and then boiled for 10 minutes. After boiling the reaction mixture was filtered and diluted with RO water to 5% solids content. The diluted solution was passed through 1 L column packed with YWD03 macroporous adsorption resin (Cangzhou Y uanwei, China). Adsorbed steviol glycosides were eluted with 5 L 70% ethanol. The obtained eluate was evaporated until dryness to yield 16 g of dry powder which was dissolved in 80 mL of 70% methanol. The solution was crystallized at 20° C. for 3 days. The crystals were separated by filtration and dried in vacuum oven at 80° C. for 18 hours to yield 10.4 g of pure reb AM crystals with 95.92% purity, determined by HPLC assay. The chromatogram of reb AM is shown in FIG. **9**. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

Example 8

Structure Elucidation of Rebaudioside AM

[0271] NMR experiments were performed on a Bruker 500 M Hz spectrometer, with the sample dissolved in pyridine-d5. A long with signals from the sample, signals from pyridine-d5 at δ_C 123.5, 135.5, 149.9 ppm and δ_H 7.19, 7.55, 8.71 ppm were observed.

[0272] ^{1}H-NM R-spectrum of rebaudioside AM in pyridine-d_5 reveal the excellent quality of the sample (see FIG. **10**). The H SQC (see FIG. **11**) shows the presence of an exo-methylene group in the sugar region with a long-range coupling to C-15, observable in the H,H-COSY (FIG. **12**). Other deep-fielded signals of the quaternary carbons (C-13, C-16 and C-19) are detected by the HMBC (FIG. **13**). Correlation of the signals in the HSQC, HMBC and H,H-COSY reveal the presence of steviol glycoside with the following aglycone structure:



[0273] Correlation of HSQC and HMBC signals reveal five anomeric signals. The coupling constant of the anomeric protons of about 8 Hz and the broad signals of their sugar linkage allows the identification of these five sugars as β-D-glucopyranosides.

[0274] The observation of the anomeric protons in combination with HSQC and HMBC reveal the sugar linkage and the correlation to the aglycone. The assignment of the sugar sequence was confirmed by using the combination of HSQC-TOCSY (FIG. **14**) and HSQC.

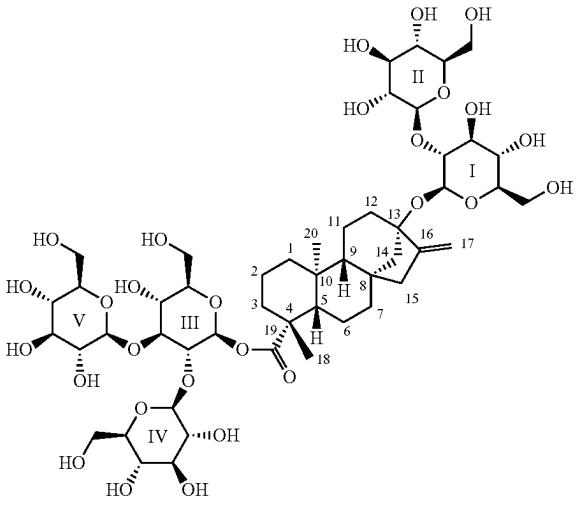
[0275] The NMR experiments above were applied to assign the chemical shifts of the protons and carbons, main coupling constants and main HMBC correlations (see Table 5).

TABLE 5

Chemical shifts of rebaudioside AM

Position	δ_C [ppm]	δ_H [ppm]	J [Hz]	HMBC (H → C)
Aglycone moiety				
1	39.9 t	0.68 m		
		1.64 m		
2	19.4 t	1.39 m		
		2.08 m		
3	37.4 t	1.05 m		
		2.80 m		
4	44.2 s	—		
5	57.3 d	0.95 m		
6	21.7 t	1.90 m		
		2.12 m		
7	41.0 t	1.26 m		
		1.38 m		
8	41.9 s	—		
9	53.3 d	0.85 m		
10	39.2 s	—		
11	20.1 t	1.59 m		
		1.61 m		
12	36.9 t	1.65 m		
		1.92 m		
13	85.9 s	—		
14	43.8 t	1.78 d	11.0	
		2.52 d	11.0	
15	47.4 t	2.00 d	16.0	7, 8, 9, 14
		2.06 d	16.0	
16	154.6 s	—		
17	104.3 t	5.03 br s		13, 15, 16
		5.71 br s		
18	28.5 q	1.40 s		3, 4, 5, 19
19	175.2 s	—		
20	16.2 q	1.06 s		1, 5, 9, 10
Sugar moiety				
Sugar I: β-D-Glucopyranoside				
1^{i}	97.5 d	5.13 d	7.7	13
2^{i}	84.0 d	4.14 m		
3^{i}	77.6 d	4.20 m		
4^{i}	71.3 d	4.19 m		
5^{i}	77.6 d	3.70 m		
6^{i}	62.0 t	4.23 m		
		4.32 m		
Sugar II: β-D-Glucopyranoside				
1^{ii}	106.3 d	5.26 d	8.0	2^{i}
2^{ii}	76.8 d	4.13 m		
3^{ii}	77.3 d	4.21 m		
4^{ii}	71.6 d	4.18 m		
5^{ii}	77.9 d	3.91 m		
6^{ii}	62.4 t	4.29 m		
		4.41 m		
Sugar III: β-D-Glucopyranoside				
1^{iii}	92.9 d	6.20 d	8.1	19
2^{iii}	77.0 d	4.46 m		
3^{iii}	88.1 d	4.24 m		
4^{iii}	69.0 d	4.12 m		
5^{iii}	78.4 d	3.82 m		
6^{iii}	61.3 t	4.20 m		
		4.33 m		
Sugar IV: β-D-Glucopyranoside				
1^{iv}	103.4 d	5.73 d	7.7	2^{iii}
2^{iv}	75.4 d	3.98 m		
3^{iv}	78.1 d	4.09 m		
4^{iv}	72.6 d	4.08 m		
5^{iv}	77.4 d	3.92 m		
6^{iv}	62.9 t	4.32 m		
		4.51 m		
Sugar V: β-D-Glucopyranoside				
1^{v}	104.4 d	5.29 d	8.1	3^{iii}
2^{v}	75.1 d	4.00 m		
3^{v}	78.2 d	4.24 m		
4^{v}	71.4 d	4.27 m		
5^{v}	78.2 d	3.99 m		
6^{v}	61.9 t	4.27 m		
		4.48 m		

[0276] Correlation of all NMR data indicates rebaudioside AM having five β-D-glucopyranoses attached to a steviol aglycone, as depicted with the following chemical structure:



[0277] The chemical formula of rebaudioside AM is $C_{50}H_{83}OO_{28}$, which corresponds to a calculated monoisotopic molecular mass of 1128.5. For LCMS analysis, rebaudioside AM was dissolved in methanol and analyzed using Shimadzu Nexera 2020 UFLC LCMS instrument on a Cortecs UPLC C18 1.6 μm, 50×2.1 mm column. The observed LCMS (negative ESI mode) result of 1127.3 (see FIG. **15***a* and FIG. **15***b* respectively) is consistent with rebaudioside AM and corresponds to the ion $(M-H)^-$.

SEQUENCE LISTING

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STFQEIAGSK ETVGQYESHT AFTLPGLYRV VHGIDVFDPK FNIVSPGADM SIYFPYTEEK  540
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DWLGTKPENS TVFVSFGSEY FLSKEDMEEI AFALEASNVN FIWVVRFPKG EERNLEDALP  300
EGFLERIGER GRVLDKFAPQ PRILNHPSTG GFISHCGWNS VMESIDFGVP IIAMPIHNDQ  360
PINAKLMVEL GVAVEIVRDD DGKIHRGEIA EALKSVVTGE TGEILRAKVR EISKNLKSIR  420
DEEMDAVAEE LIQLCRNSNK SK                                           442

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LITDALWYFA QDVADSLNLR RLVLMTSSLF NFHAHVSLPQ FDELGYLDPD DKTRLEEQAS  180
GFPMLKVKDI KSAYSNWQIG KEILGKMIKQ TKASSGVIWN SFKELEESEL ETVIREIPAP  240
SFLIPLPKHL TASSSSLLDH DRTVFEWLDQ QAPSSVLYVS FGSTSEVDEK DFLEIARGLV  300
DSGQSFLWVV RPGFVKGSTW VEPLPDGFLG ERGKIVKWVP QQEVLAHPAI GAFWTHSGWN  360
STLESVCEGV PMIFSSFGGD QPLNARYMSD VLRVGVYLEN GWERGEVVNA IRRVMVDEEG  420
EYIRQNARVL KQKADVSLMK GGSSYESLES LVSYISSL                          458
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1. (canceled)

2. A method for producing rebaudioside AM, comprising the steps of:

a. providing a starting composition comprising an organic compound with at least one carbon atom wherein the starting composition is selected from the group consisting of steviol, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, other steviol glycosides, and combinations thereof;

b. providing a biocatalyst selected from the group consisting of an enzyme preparation, a cell or a microorganism; said biocatalyst comprising at least one enzyme capable of converting the starting composition to rebaudioside AM;

c. contacting the biocatalyst with a medium containing the starting composition to produce a medium comprising rebaudioside AM.

3. The method of claim 2 further comprising the step of:

d. separating the rebaudioside AM from the medium to provide a highly purified rebaudioside AM composition.

4. (canceled)

5. The method of claim 2, wherein the microorganism is selected from the group consisting of *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., and *Yarrowia* sp.

6. The method of claim 2, wherein the enzyme is selected from the group consisting of: a steviol biosynthesis enzyme, a UDP glucosyltransferase, a UDP glucose recycling enzyme, a mevalonate (MVA) pathway enzyme, a 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzyme, geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase,

kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase, UGT74G1, UGT85C2, UGT91D2, EUGT11, UGTS12, UGT76G1, or mutant variant thereof having >85% amino-acid sequence identity, >86% amino-acid sequence identity, >87% amino-acid sequence identity, >88% amino-acid sequence identity, >89% amino-acid sequence identity, >90% amino-acid sequence identity, >91% amino-acid sequence identity, >92% amino-acid sequence identity, >93% amino-acid sequence identity, >94% amino-acid sequence identity, >95% amino-acid sequence identity, >96% amino-acid sequence identity, >97% amino-acid sequence identity, >98% amino-acid sequence identity, >99% amino-acid sequence identity; and combinations thereof.

7. The method of claim **3**, wherein the rebaudioside AM content in highly purified rebaudioside AM composition is greater than about 95% by weight on a dry basis.

8-14. (canceled)

15. The method of claim **3**, wherein the rebaudioside AM content in highly purified rebaudioside AM composition is greater than about 80% by weight on a dry basis.

* * * * *